Chapter 14

Proximity Labeling for the Identification of Coronavirus–Host Protein Interactions

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Abstract

Biotin-based proximity labeling circumvents major pitfalls of classical biochemical approaches to identify protein–protein interactions. It consists of enzyme-catalyzed biotin tags ubiquitously apposed on proteins located in close proximity of the labeling enzyme, followed by affinity purification and identification of biotinylated proteins by mass spectrometry. Here we outline the methods by which the molecular microenvironment of the coronavirus replicase/transcriptase complex (RTC), i.e., proteins located within a close perimeter of the RTC, can be determined by different proximity labeling approaches using BirA<sub>R118G</sub> (BioID), TurboID, and APEX2. These factors represent a molecular signature of coronavirus RTCs and likely contribute to the viral life cycle, thereby constituting attractive targets for the development of antiviral intervention strategies.

Key words Coronaviruses, Replication transcription complex RTC, Proximity labeling, Biotin ligase, Ascorbate peroxidase APEX2, BioID, TurboID, Affinity purification, Mass spectrometry, Proteomics, Replicase microenvironment

1 Introduction

Coronaviruses cause a wide range of diseases in animals and humans. In recent years, particular attention has been drawn by severe acute respiratory syndrome coronavirus (SARS-CoV), MiddleEast respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which can cause severe and lethal respiratory disease in humans [1–5]. Moreover, in the veterinary field, coronaviruses such as, feline infectious peritonitis virus, porcine epidemic diarrhea virus, or the avian infectious bronchitis virus, severely affect companion animals and livestock [6–8].

Viral pathogenicity is determined by the virus tissue and cell tropism and the host (inflammatory) responses to the infection, as well as viral countermeasures evading the host immune defense system.
On the cellular level, the expression of up to 15–16 non-structural proteins carrying various enzymatic functions result in the establishment of viral replication and transcription complexes (RTC) and the generation of membranous structures that host the RTC in the infected host cell cytosol [9, 10]. This stage is considered a major determinant of pathogenicity and infection outcome. Indeed, host proteins may be recruited to the RTC to promote viral replication [11, 12] or may display antiviral functions to restrict virus replication [13]. Knowledge on the composition of viral and host proteins located at the RTC is therefore of crucial importance to understand critical virus–host interactions taking place at the site of viral RNA synthesis.

Proximity labeling approaches have been implemented in an increasing number of investigations during the past years [14]. The strong enthusiasm of the scientific community is best exemplified by the constant improvement and adaptation of proximity labeling enzymes to a wide range of applications [15].

The hallmark of enzyme-catalyzed proximity labeling is the promiscuous and covalent biotin labeling of proteins located within a close perimeter (few nanometers). As such, the labeling is not dependent on the protein–protein interaction affinity. The high affinity between biotinylated residues and streptavidin, however, allows stringent and efficient affinity purification using streptavidin-coated beads. Eventually, affinity-purified proteins are sensitively identified by mass spectrometry. Additionally, proximity labeling is also suited to capture transient interactions, as the labeling time can vary from 1 min to several hours depending on the enzyme [15–18]. Of note, the labeling by most enzymes is not toxic and can be performed in live cells.

In this context, BirA_{R118G} (BioID) was the first proximity labeling enzyme successfully employed in different assays [16]. BirA_{R118G} is a promiscuous *E. coli* biotin ligase that uses free biotin in labeling reactions that require several hours of biotin incubation to obtain sufficient amounts of biotinylated proteins for further analysis. During viral infections, this feature can be advantageous when recording protein–protein interactions occurring at any stage during the entire course of infection in an unbiased screening approach [19].

The slow labeling kinetics of BioID were first improved by the development of BioID2 [18]. Recently, Branon et al. used directed evolution of BioID to engineer TurboID and miniTurbo, which contained few key amino acid substitutions conferring similar labeling capacities within 10 min instead of the 15–18 h required by BioID [15]. This significant improvement allows retaining the intrinsic advantages of proximity labeling enzymes while narrowing the labeling window. When applied to virus infections, TurboID enables to distinguish protein–protein interactions relevant at defined stages of infection.
Another popular proximity labeling enzyme is the soy bean-derived ascorbate peroxidase APEX2 [17, 20]. APEX2 catalyzes biotin-phenol substrates into biotin-phenoxyl radicals that react and tag neighboring proteins. This reaction is triggered by the addition of hydrogen peroxide to cells for 1 min. APEX2 can thereby be used to provide a “snapshot” of factors surrounding the protein of interest to which it is fused.

Alternatively, APEX2 catalyzes the polymerization of 3,3-diaminobenzidine (DAB) resulting in the deposition of insoluble DAB polymers at the site of production [21]. Given that DAB precipitates generate high contrast upon heavy-metal staining, this feature allows detecting, in a specific manner, the localization of an APEX2 fusion protein by electron microscopy.

Lastly, APEX2 has been demonstrated to label closely associated RNAs [22]. Upon purification of biotinylated RNAs, sequencing reveals the RNA population associated with a particular protein complex.

Here, we describe the use of BioID, TurboID, and APEX2 in the context of viral infections (Fig. 1). More specifically, by incorporating these proximity labeling enzymes into the RTC of a prototype coronavirus, these strategies enable the identification of critical host factors comprised within the coronavirus RTC microenvironment. The proximity labeling procedures described here are largely adapted from detailed protocols available for BioID [23],

![Fig. 1 Overview of proximity labeling assays using MHV-BiRAr118G-nsp2, MHV-TurboID-nsp2, and MHV-APEX2-nsp2](image-url)
TurboID [15], and APEX2 [17, 21]. Further procedures such as mass spectrometry of affinity purified proteins, electron microscopy, or data analysis are not covered in this chapter.

2 Materials

2.1 General Reagents and Equipment

1. 4% neutral buffered formalin.
2. Antibodies/reagents (see Table 1).
3. Branson Sonifier 250.
4. Cell culture plates, 6-well and 24-well.
5. Cell scrapers.
6. Confocal buffer (CB): phosphate-buffered saline (PBS) supplemented with 50 mM NH₄Cl, 0.1% (w/v) saponin and 2% (w/v) BSA. Filter-sterilize and store 50 ml aliquots at −20 °C.
7. Coomassie Brilliant Blue G250.
8. Coverslips #1.5H glass, 12 mm diameter, 0.170 mm thick.
9. Dimethyl sulfoxide (DMSO).
10. Western Blotting Transfer System, e.g., eBlot L1 Wet Transfer (Genscript).
11. Glass slides.
12. Hard setting IFA mounting medium containing DAPI.
13. L929 cells.

Table 1
Antibodies and reagents used in this protocol

| Antibodies/reagents                          | Supplier                        | Reference | Dilution |
|---------------------------------------------|---------------------------------|-----------|----------|
| Anti-dsRNA J2 (mouse monoclonal IgG2a, kappa chain) | English and Scientific Consulting |           | 1:200    |
| Anti-myc (mouse monoclonal)                 | Cell signaling                  |           | 1:8000   |
| Anti-MHV nsp2/3 (rabbit polyclonal)         | Gift from S. Baker              | [24]      | 1:200    |
| Anti-MHV nsp8 (rabbit polyclonal)           | Gift from S. Baker              | [25]      | 1:400    |
| Donkey anti-mouse 488                       | Jackson ImmunoResearch          |           | 1:400    |
| Donkey anti-rabbit 594                      | Jackson ImmunoResearch          |           | 1:400    |
| Donkey anti-rabbit 647                      | Jackson ImmunoResearch          |           | 1:400    |
| AlexaFluor 594-conjugated streptavidin      | ThermoFisher Scientific         |           | 1:300    |
| HRP-conjugated streptavidin                 | Dako                            |           | 1:2500   |
| Anti-V5                                     | Abcam                           |           | 1:1000   |

Antibodies stated here have been used successfully by us, other antibodies may also be suitable after validation.
14. Laemmli buffer (5×): 125 mM Tris–HCl pH 6.8, 20% (v/v) glycerol/glycerin (v/v), 5% (v/v) SDS, 10% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue.

15. Magnetic tube holder, e.g., DynaMag-2.

16. MEM+/+: Gibco minimum essential media (MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 IU/ml penicillin.

17. M-PER mammalian protein extraction reagent.

18. Nitrocellulose blotting membrane, supported 0.45 μm NC.

19. Phosphate-buffered saline (PBS).

20. PBS supplemented with 0.5% (v/v) Tween 20.

21. 1 M potassium chloride (KCl).

22. Protein-free blocking buffer, e.g., AdvanBlock-PF (Advansta).

23. 2% (w/v) sodium dodecyl sulfate (SDS).

24. SDS–PAGE equipment, e.g., Mini PROTEAN Tetra Cell (Biorad).

25. 0.1 M sodium carbonate (Na₂CO₃).

26. Streptavidin-coated magnetic beads, e.g., Dynabeads MyOne Streptavidin C1 or Pierce Streptavidin Magnetic Beads.

27. 50 mM Tris–HCl pH 7.4.

28. 10 mM Tris–HCl pH 8.0.

29. 20% (v/v) Triton X-100.

30. 2 M Urea, 10 mM Tris–HCl pH 8.0.

31. Widefield fluorescence microscope or a laser scanning confocal microscope.

2.2 BioID and TurboID Reagents

1. 100 mM Biotin stock in DMSO, sterile filtered, store aliquots at −20 °C.

2. BioID lysis buffer: 50 mM Tris–HCl pH 7.4, 500 mM NaCl, 0.2% (w/v) SDS, 1 mM DTT, and 1× protease inhibitor cocktail. Prepare fresh before use.

3. BioID wash buffer 2: 0.1% (w/v) deoxycholic acid, 1% (v/v) Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES pH 7.5. Prepare fresh before use.

4. BioID wash buffer 3: 0.5% (w/v) deoxycholic acid, 0.5% NP40, 1 mM EDTA, 250 mM LiCl, 10 mM Tris–HCl pH 7.4. Prepare fresh before use.

5. RIPA buffer: 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% SDS (v/v), 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1× protease inhibitor cocktail. Store 10–20 ml aliquots at −20 °C.
2.3 APEX Reagents

1. APEX lysis buffer: 50 mM Tris–HCl pH 7.4, 500 mM NaCl, 0.2% SDS, 1× protease inhibitor cocktail, 10 mM sodium ascorbate, 5 mM Trolox, 10 mM sodium azide. Prepare immediately before use.

2. APEX elution buffer: 75 mM Tris–HCl (pH 6.8), 6% 2-mercaptoethanol, 12% glycerol, 3% SDS, 0.06% bromophenol blue, 2 mM biotin, and 20 mM DTT.

3. APEX wash 1: 2% SDS in H₂O.

4. APEX wash 2: 2 M Urea, 10 mM Tris–HCl pH 7.4.

5. APEX wash 3: 0.1% (w/v) deoxycholate, 1% (v/v) Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES pH 7.5.

6. APEX wash 4: 0.5% (w/v) deoxycholate, 0.5% (vol/v) NP-40, 1 mM EDTA, 250 mM LiCl, 10 mM Tris–HCl pH 7.4.

7. APEX wash 5: 50 mM Tris–HCl pH 7.4.

8. Biotin-phenol (1000×): 500 mM biotin-phenol in DMSO. Sonicate to dissolve. Filter-sterilize the solution and store as 50 μl aliquots at −80 °C for several months.

9. H₂O₂ solution, 100 mM in PBS (100×). Prepare immediately before use (see Note 1).

10. Quencher solution, in PBS: 10 mM sodium ascorbate, 5 mM Trolox, and 10 mM sodium azide in PBS. Prepare immediately before use.

11. Sodium ascorbate (100×): 1 M sodium ascorbate in H₂O. Prepare fresh before mixing the Quencher solution.

12. Sodium azide (100×): 1 M sodium azide in H₂O. Aliquots can be stored at −20 °C for several months.

13. Trolox (100×): 500 mM Trolox in DMSO. Sonicate to dissolve if necessary. Prepare fresh before mixing the Quencher solution.

2.4 APEX Electron Microscopy Reagents

1. DAB stock (10×): Dissolve 50 mg 3,3′-diaminobenzidine (DAB) in 10 ml 0.1 M HCl. If DAB remains insoluble after prolonged vortexing at room temperature, pellet at 12,000 × g for 5 min and aliquot (10× 1 ml) the supernatant. Flash freeze aliquots in liquid nitrogen and store at −80 °C.

2. EM fixative: 2% (v/v) glutaraldehyde in 100 mM sodium cacodylate, pH 7.4, supplemented with 2 mM calcium chloride. Prepare fresh before use.

3. Sodium cacodylate buffer: 100 mM sodium cacodylate, pH 7.4, supplemented with 2 mM calcium chloride. Store at 4 °C.

4. 20 mM glycine in sodium cacodylate buffer.

5. 0.5 mg/ml DAB, 10 mM H₂O₂ in sodium cacodylate buffer.
3 Methods

3.1 Generation of Recombinant MHV, General Considerations Prior to Commencing Proximity Labeling

1. Cloning strategies and generation of recombinant viruses (Fig. 2) are adapted from Freeman et al. [26]. Coding sequences for BirA<sub>R118G</sub>, TurboID and APEX2 are derived from Addgene cat. no. 74223, 116904, and 72480, respectively, and cloned into a pGPT-1 vector as described previously [19]. Recombinant MHV viruses are generated using an established vaccinia virus-based reverse genetic system [27, 28]. N-terminally tagged proximity labeling enzymes are inserted in the MHV genome within ORF1a. Note the preserved polyprotein cleavage sites ensuring the release of the nsp2 fusion protein (Fig. 2, black arrows). Additionally, a flexible (SGG)<sub>3</sub> linker is placed between proximity labeling enzymes and nsp2 to provide structural flexibility and avoid potential steric hindrance of the fusion protein when embedded in the MHV RTC (see Note 2).

2. Generate and aliquot a virus working stock with sufficient titer to perform the planned experiments (if possible do not exceed 2–3 virus passages). Assess virus titers by plaque titration assays.

3. Ensure genetic integrity of every working stock and verify sequence identity by RT-PCR sequencing of the modified genomic region. Analytical agarose gel electrophoresis of RT-PCR products indicates potential truncations in the region of interest, or mixed populations in the virus stock (see Note 3).

4. Assess whether recombinant viruses have similar growth phenotype as compared to the parental strain. For this, perform growth curves and assess virus titers in the supernatant of infected cells.

Fig. 2 Schematic overview of recombinant MHV-BirA<sub>R118G</sub>-nsp2, MHV-TurboID-nsp2, and MHV-APEX2-nsp2. ORF1a nsp1, nsp2, and nsp3 are represented by gray boxes; BirA<sub>R118G</sub> (BioID), TurboID, and APEX2 by a dark gray box; and molecular V5 and myc tag by a black box. The amino acid and nucleotide sequence at the junction between nsp1 and the tagged proximity labeling enzyme are highlighted. The junction between the proximity labeling enzyme and nsp2, in which a flexible linker is incorporated is highlighted as well. Black arrows indicate proteolytic cleavage sites.
5. Establish that a functional fusion protein is expressed at the desired subcellular location by performing immunofluorescence microscopy and colocalization analyses and by detecting biotinylated proteins by both immunofluorescence microscopy and western blot analysis (see Subheadings 3.6 and 3.7).

### 3.2 Labeling Procedure Using MHV-BirAR118G-nsp2

1. Seed L929 cells \((8 \times 10^4 \text{ cells/ml})\) in 6-well cell culture plates or 24-well cell culture plates, using 2 ml/well or 0.5 ml/well, respectively. L929 cells are cultured in MEM+/-+. Incubate at 37 °C, 5% CO₂, for 16–18 h.

2. Infect L929 cells with MHV-BirAR118G-nsp2 (MOI = 1). Use 2 ml/well for 6-well plates and 0.5 ml/well for 24-well plates. MHV-BirAR118G-nsp2 is diluted in MEM+/-+ supplemented with 67 μM biotin.

3. Optional: At 1 h post infection (h.p.i.), wash the cells 2–3 times with PBS and incubate cells in fresh MEM+/-+ supplemented with 67 μM biotin (see Note 4).

4. At 15 h.p.i., wash the cells three times with PBS to remove free biotin contained in the cell culture medium.

5. Proceed by fixating the cell for immunofluorescence microscopy analysis or prepare lysates for western blot analysis as described in Subheadings 3.6 and 3.7.

### 3.3 Labeling Procedure Using MHV-TurboID-nsp2

1. Seed L929 cells \((8 \times 10^4 \text{ cells/ml})\) in 6-well cell culture plates or 24-well cell culture plates, using 2 ml/well or 0.5 ml/well, respectively. L929 cells are cultured in MEM+/-+. Incubate at 37 °C, 5% CO₂, for 16–18 h.

2. Infect L929 cells with MHV-TurboID-nsp2 (MOI = 1). Use 2 ml/well for 6-well plates and 0.5 ml/well for 24-well plates. MHV-TurboID-nsp2 is diluted in MEM+/-+.

3. At 1 h.p.i., wash the cells 2–3 times with PBS and incubate cells in fresh MEM+/-+.

4. At desired times post infection, add 500 μM biotin to the cell culture supernatant and incubate further. Labeling times can vary between 10 min up to several hours.

5. After labeling, wash the cells three times with PBS to remove free biotin contained in the cell culture medium.

6. Proceed by fixating the cell for immunofluorescence microscopy analysis or prepare lysates for western blot analysis as described in Subheadings 3.6 and 3.7.

### 3.4 Labeling Procedure Using MHV-APEX2-nsp2

1. Seed L929 cells \((8 \times 10^4 \text{ cells/ml})\) in 6-well cell culture plates or 24-well cell culture plates, using 2 ml/well or 0.5 ml/well, respectively. L929 cells are cultured in MEM+/-+. Incubate at 37 °C, 5% CO₂, for 16–18 h.
2. Infect L929 cells with MHV-APEX-nsp2 (MOI = 4). Mock controls receive fresh MEM+/+. Use 2 ml/well for 6-well plates and 0.5 ml/well for 24-well plates. MHV-APEX-nsp2 is diluted in MEM+/+.

3. After 1 h remove the inoculum, wash the cells three times with PBS and add fresh MEM+/+.

4. 30 min before the desired time point, exchange the medium with MEM+/+ supplemented with 500 μM biotin-phenol (BP).

5. At the desired time-point, add appropriate volume of 100× H₂O₂ solution to the culture medium to obtain a 1× H₂O₂ solution. Shake gently to mix.

6. Incubate for exactly 1 min at RT.

7. Quickly aspirate the labeling solution and wash the cells immediately three times with Quencher solution (see Note 5).

8. Proceed by fixating the cell for immunofluorescence microscopy analysis or prepare lysates for western blot analysis as described in Subheadings 3.6 and 3.7.

### 3.5 Controls

1. Appropriate control samples are of crucial importance and should be included in every experiment.

2. Parental recombinant viruses that do not express the proximity-labeling enzyme are used as negative control.

3. Alternatively, incubating cells in medium supplemented with DMSO instead of biotin (during MHV-BirA₁₁₈G-nsp2 or MHV-TurboID-nsp2 infections) will result in “background” biotinylation.

4. During MHV-APEX2-nsp2 infections, the omission of biotin-phenol or the H₂O₂ should be used as negative controls. In case of BP omission, supplement the medium with DMSO instead.

5. Mock infections followed by the labeling procedure can serve as additional negative controls.

### 3.6 Immunofluorescence Microscopy Analysis (IFA) of Biotinylated Proteins

1. Perform infections for IFA in 24-well cell culture plates.

2. Prior to seeding cells, place sterile glass coverslips in 24-well plates.

3. Perform infections and labeling procedures as indicated in Subheadings 3.2, 3.3, or 3.4.

4. Fix cells with 4% (v/v) formalin for 20 min at room temperature. Use 1 ml per well.

5. Wash cells three times with PBS.
6. Permeabilize, block unspecific antibody binding, and quench PFA by incubating the coverslips in confocal buffer (CB; 1 ml) for 1 h at room temperature. Gentle shaking is recommended.

7. Incubate coverslips, cells facing down, on a 30–50 μl drop of primary antibodies diluted in CB to label antigens of interest (see Note 6). 60 to 120 min incubation at room temperature is recommended. Antibodies used in our laboratory are listed in Table 1.

8. Place coverslips into a 12-well plate and wash coverslips three times with 1 ml CB for 5 min.

9. Incubate coverslips, cells facing down, on a 30–50 μl drop of secondary antibodies diluted in CB for 60 min at room temperature (see Note 6). Protect from light. Add fluorophore-conjugated streptavidin during this step to label biotinylated proteins.

10. Place coverslips into 12-well plate and wash coverslips twice with 1 ml CB for 5 min, wash once with 1 ml PBS, and once, briefly, with 1 ml dH2O.

11. Mount coverslip on glass slide using DAPI-containing mounting medium.

12. Cure by incubating glass slide overnight at room temperature. Protect from light.

13. Image cells using a widefield fluorescence microscope or a laser scanning confocal microscope. Use identical imaging setting for all samples and controls of same experiment. Further deconvolve 3D images if necessary.

14. For display purposes, adjust brightness and contrast to appropriate controls during post-acquisition processing of immunofluorescence microscopy images.

3.7 Western Blot Analysis of Biotinylated Proteins (Analytical Scale)

1. Perform infections and labeling procedures for analytical western blots in six-well cell culture plates as indicated in Subheadings 3.2, 3.3, or 3.4.

2. Prepare lysates using M-PER mammalian protein extraction reagent, RIPA, or other lysis buffers (see Subheadings 3.9–3.11) containing protease inhibitors. Typically, 100–200 μl/well lysis buffer is used, and cells are scraped off using an inverted pipette tip.

3. Collect lysate in 1.5 ml tube, centrifuge at ~14,000 × g for 5–10 min to pellet the cell debris and transfer supernatant to new tube. Add Laemmli buffer and boil for 5–10 min.

4. To assess biotinylated proteins in affinity-purified fractions, see Subheadings 3.9–3.11.

5. Separate proteins on a 10% (w/v) SDS–polyacrylamide gel.
6. Electroblot onto a nitrocellulose membrane.
7. Incubate nitrocellulose membrane in protein-free blocking buffer for 60 min at room temperature (see Note 7).
8. Incubate nitrocellulose membrane with horseradish peroxidase (HRP)-conjugated streptavidin diluted in protein-free blocking buffer. Incubate at 4 °C overnight.
9. Wash the nitrocellulose membrane three times with PBS supplemented with 0.5% (v/v) Tween 20 and once with PBS.
10. Visualize biotinylated proteins using an enhanced chemiluminescence (ECL) HRP substrate and a chemiluminescence CCD detector system.

3.8 Preparation of Cells Infected with MHV-APEX2-nsp2 for Electron Microscopy

1. Seed L929 cells (8–12 × 10⁴ cells/ml) in 24-well cell culture plates, using 0.5 ml/well. L929 cells are cultured in MEM+/. Incubate at 37 °C, 5% CO₂, for 16–18 h. Cells should be 90–100% confluent at the time of fixation.
2. Infect cells with MHV-APEX2-nsp2 at MOI = 3. MHV-APEX2-nsp2 is diluted in MEM+/(see Note 8).
3. At 1 h.p.i., wash the cells 2–3 times with PBS and incubate cells in fresh MEM+/. 
4. At desired time post infection, wash cells once with prewarmed (37 °C) PBS.
5. Fix cells using prewarmed (37 °C) EM fixative.
6. Place on ice for 60 min.
7. The following incubations are performed on ice in ice-cold buffers. Wash cells three times with sodium cacodylate buffer.
8. Quench with 20 mM glycine in sodium cacodylate buffer for 5 min.
9. Wash three times with sodium cacodylate buffer.
10. Stain cells in sodium cacodylate buffer containing 0.5 mg/ml DAB and 10 mM H₂O₂ for approximately 20 min until DAB precipitates are visible by light microscopy (see Notes 9 and 10). For this, use an inverted cell culture microscope and compare to a side-by-side control condition.
11. Wash three times with cacodylate buffer to stop the staining reaction.
12. Refer to electron microscopy facility’s guidelines for sample submission.

3.9 Sample Preparation for Mass Spectrometry, Infections with MHV-BirAR118G-nps2

1. Seed L929 cells in 4 × 150 cm² tissue culture flasks, 1 × 10⁷ cells per flask. Cells are cultured in MEM+/. Incubate at 37 °C, 5% CO₂, for 16–18 h.
2. Infect L929 cells with MHV-BirAR118G-nps2 (MOI = 1) in 25 ml MEM +/- supplemented with 67 μM biotin. Do not wash the cells after infection (see Note 11).
3. At 15 h.p.i., wash cells three times with a large excess of PBS (50 ml per tissue culture flask) *(see Note 12).*

4. Lyse the cells by adding 1 ml ice-cold BioID lysis buffer per flask and detach the cells using a cell scraper.

5. Keep the cells on ice for the rest of the procedure.

6. Pool the lysates of the four flasks into one 50 ml conical tube.

7. Add 400 μl 20% Triton X-100 to each tube.

8. Sonicate cells with two rounds of 20 pulses using a Branson Sonifier 250 (at 30% constant, 30% power). Put cells on ice during the sonication rounds.

9. Add 3.6 ml 50 mM Tris–HCl pH 7.4 to each tube.

10. Sonicate cells for one additional round of 20 pulses.

11. Transfer lysates to 4 × 2 ml tubes and centrifuge 10 min at 18,000 × g, 4 °C.

12. Wash streptavidin-coated magnetic beads (800 μl beads per condition) with 1 ml lysis buffer diluted 1:1 with 50 mM Tris–HCl pH 7.4. Place the cells on a magnetic tube holder and remove the wash solution.

13. Repeat wash with 1 ml of lysis buffer diluted 1:1 with 50 mM Tris–HCl pH 7.4, and distribute the solution to four 2 ml tubes. Remove wash solution using a magnetic tube holder.

14. Take an aliquot of 100 μl from the lysate (total 8 ml) before incubation with the magnetic beads as control and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.

15. Distribute the remaining lysate to the four 2 ml tubes containing the washed streptavidin-coated beads and incubate on a rotator at 15 rotations per minute at 4 °C overnight.

16. Place the samples on a magnetic tube holder and collect the “flowthrough” (non-bound protein lysate). Combine the beads into one tube.

17. Take an aliquot of 100 μl from the flowthrough and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.

18. Wash the beads as described below. After each washing step, place the tube on the magnetic tube holder and remove the washing solution.

19. Wash beads twice with 1.5 ml 2% (w/v) SDS by incubating beads for 5–8 min on a rotator.

20. Wash once with 1.5 ml BioID washing buffer 2 by incubating beads for 5–8 min on a rotator.
21. Wash once with 1.5 ml BioID washing buffer 3 by incubating beads for 5–8 min on a rotator.

22. Wash once with 1 ml 50 mM Tris–HCl pH 7.4.

23. Elute proteins from beads by the addition of 100 μl 1× Laemmli SDS-sample buffer supplemented with 0.5 mM biotin and heating at 95 °C for 10 min in a heating block while shaking at 700 rpm.

24. Keep 10% of eluate for western blot analysis.

25. Refer to the mass spectrometry facility’s guidelines for sample submission. Samples are typically separated 1 cm into an SDS-polyacrylamide gel, stained with Coomassie, and extracted from the gel for mass spectrometry analysis (see Note 13).

3.10 Sample Preparation for Mass Spectrometry, Infections with MHV-Turbo-nsp2

1. Seed L929 cells in 150 cm² tissue culture dishes, 1 × 10^7 cells per dish. Cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO₂, for 16–18 h.

2. Infect L929 cells with MHV-Turbo-nsp2 (MOI = 1) in 25 ml MEM +/+.

3. At 1 h.p.i., wash cells twice with PBS and incubate cells in 25 ml fresh MEM +/+ (see Note 14).

4. At a chosen time post infection, add biotin to a final concentration of 500 μM.

5. Incubate cells at 37 °C, 5% CO₂ for 10 min up to multiple hours.

6. At chosen time after biotin addition, wash the cells five times with 25 ml ice-cold PBS (see Note 12).

7. Lyse the cells in 1.5 ml RIPA buffer containing protease inhibitors. After addition of RIPA buffer, incubate for 1–2 min before using a 1 ml pipette to detach the cells from the surface of the dish.

8. Transfer the lysate to 2 ml tubes and spin at 12,000 × g, 4 °C.

9. Using a magnetic tube holder, wash streptavidin-coated magnetic beads twice with 1 ml RIPA buffer. Use 350 μl beads per condition (see Note 15).

10. Take a 100 μl aliquot of the lysate before incubation with the magnetic beads as control and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.

11. Add the lysate to the streptavidin-coated beads and incubate on a rotator at 4 °C overnight.

12. Place the samples on a magnetic tube holder and collect the “flowthrough” (non-bound protein lysate).
13. Take an aliquot of 100 μl from the flowthrough and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.

14. Wash the beads as described below. After each washing step, place the tube on the magnetic tube holder and remove the washing solution.

15. Wash the beads twice with 1 ml RIPA buffer for 2–5 min.

16. Wash the beads with 1 ml 1 M KCl for 2–5 min.

17. Wash the beads with 1 ml 0.1 M Na₂CO₃. Homogenize the beads with a 1 ml pipette and continue immediately with next washing step.

18. Wash the beads with 1 ml 2 M urea, 10 mM Tris–HCl (pH 8.0). Homogenize the beads with a 1 ml pipette and continue immediately with next washing step.

19. Wash the beads with 1 ml 10 mM Tris–HCl (pH 8.0).

20. Elute proteins from beads by the addition of 100 μl 1× Laemmli SDS-sample buffer supplemented with 0.5 mM biotin and heating at 95 °C for 10 min in a heating block while shaking at 700 rpm.

21. Place the beads on the magnetic tube holder and collect eluates in fresh 1.5 ml tubes.

22. Refer to the mass spectrometry facility’s guidelines for sample submission. Samples are typically separated 1 cm into an SDS–polyacrylamide gel, stained with Coomassie and extracted from the gel for mass spectrometry analysis (see Note 13).

3.11 Sample Preparation of Cells Infected with MHV-APEX2-nsp2 for Mass Spectrometry

1. Seed 2 × 10⁶ L929 cells in a 10 cm² dish. Cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO₂ for 16–18 h.

2. Infect the cells with MHV-APEX2-nsp2 or MHV-WT at MOI = 4 (see Note 16).

3. After 1 h remove the inoculum and wash the cells three times with PBS. Add fresh MEM+/+ (see Note 12).

4. Thirty minutes before the desired time point, exchange the medium with 10 ml MEM+/+ supplemented with 500 μM biotin-phenol (BP). Add fresh medium to APEX-negative controls.

5. At the desired time-point, add 100 μl H₂O₂ solution (100×) directly to the dish containing the 10 ml MEM+/+ supplemented with BP. Shake gently to mix.

6. Incubate for exactly 1 min at RT.

7. Quickly aspirate the labeling solution and wash the cells immediately three times with 10 ml of Quencher solution (see Note 17).
8. Remove the remaining Quencher solution and add 1 ml of APEX lysis buffer directly onto the cells. Use a cell scraper to detach all cells off the dish (see Note 18).

9. Collect the lysate and transfer it into a 50 ml tube and add 100 μl 20% Triton X-100.

10. Sonicate cells with two rounds of 30 pulses using a Branson Sonifier 250 (at 30% constant, 30% power). Put cells on ice during the sonication rounds.

11. Add 900 μl of 50 mM Tris–HCl pH 7.4 to each sample.

12. Sonicate samples one more time for 30 pulses.

13. Transfer samples into a 2 ml tube and centrifuge at 16,000 \( \times g \) at 4 °C for 10 min.

14. Transfer the supernatant into a fresh 2 ml tube. This is considered the Lysate (see Note 19).

15. Prepare 100 μl streptavidin-coated magnetic beads per sample in a 2 ml tube.

16. Add 1 ml of APEX lysis buffer to the beads and place the tube on a rotator at 15 rotations per minute for 8 min.

17. Place the tube on a magnetic tube holder for 1 min and subsequently remove all liquid from the tube without touching the beads on the wall.

18. Repeat the washing step again for a total of two washes with APEX lysis buffer.

19. Take an aliquot of 100 μl from the lysate and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.

20. Add the remaining lysate to the beads and incubate the samples on a rotator at 15 rotations per minute at 4 °C overnight.

21. Place the samples on a magnetic tube holder and collect the “flowthrough” (non-bound protein lysate).

22. Take an aliquot of 100 μl from the flowthrough and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.

23. Subject the beads to a series of washing steps, described below. Each step consists of 1 ml of the respective washing buffer followed by 8 min on the rotator and 1 min on magnetic tube holder. Always remove the wash buffers before the next washing step.

24. Wash the beads twice with APEX wash 1.

25. Wash the beads once with APEX wash 2.

26. Wash the beads twice with APEX wash 3.

27. Wash the beads twice with APEX wash 4.
28. Wash the beads once with APEX wash 5.
29. Remove the remaining wash buffer with a 20 μl pipette tip.
30. Add 50 μl of APEX elution buffer to the beads and elute biotinylated proteins by boiling each sample at 95 °C for 10 min while shaking at 700 rpm.
31. Vortex the beads briefly and cool the samples on ice. Quickly spin the samples to bring down condensation.
32. Place the beads on the magnetic tube holder and collect eluates in fresh 1.5 ml tubes.
33. Use 10 μl eluate for western blot analysis and 35 μl for mass spectrometry (see Note 20).
34. Refer to the mass spectrometry facility’s guidelines for sample submission. Samples are typically separated 1 cm into a SDS-polyacrylamide gel, stained with Coomassie and extracted from the gel for mass spectrometry analysis (see Note 13).

4 Notes

1. Keep H₂O₂ stock in small aliquots, cold and protected from light at all times.
2. The use of longer linkers can extend the biotinylation range [18].
3. One unique band, which is migrating at the expected molecular weight, is expected.
4. A washing step at 1 h.p.i. ensures that the cells are found in a similar stage of infection at any given time point. Also see Note 11.
5. Be careful not to detach too many cells during the washing. Remove the remaining liquid from the washes.
6. Alternatively, coverslips can be left in 24-well plate. In this case, dilute antibodies in a sufficient volume to cover coverslips.
7. Milk is to be avoided as it contains biotin, which competitively inhibits biotinylated protein recognition by HRP-coupled streptavidin [29].
8. Increase MOI if necessary in order to obtain 70–90% of infected cells.
9. Prepare 10 ml using a 10× DAB stock. Prepare fresh before use and discard unused buffer.
10. Add H₂O₂ to the buffer as a last step and just before use. Also see Note 1.
11. Do not wash the cells after infection. This will allow the cells to be infected in a non-synchronized fashion and enable to record
cells during different stages of the viral life cycle at the moment of lysis. Also see Note 4.

12. Repeated washing is important to completely remove free biotin, which might interfere with binding of proteins to streptavidin-coated beads.

13. Alternatively, proteins are not eluted from the beads and are submitted to on-bead tryptic digestion for mass spectrometry analysis.

14. If precise synchronization of the virus infection is desired, infect the cells at 4 °C and subsequently incubate them 60–90 min at 4 °C. This allows the virus particles to attach to the receptor but prevents entry. Afterwards place the cells in the incubator at 37 °C. Add 25 mM HEPES to the infection medium to buffer the cells.

15. The use of streptavidin-coated magnetic beads from Pierce are recommended when using the washing conditions described in Subheading 3.10. The use of MyOne Streptavidin C1 beads has proven to result in clumping and strong adherence to the walls of tubes, thus rendering washing and elution steps difficult. Lysis and affinity purification of biotinylated proteins in MHV-TurboID-nsp2-infected cells can alternatively be performed using the conditions described in Subheading 3.9. Nevertheless, most recent publications favor the use of buffers described in Subheading 3.10.

16. Prepare one extra 10 cm dish, which can be used to count the cells and calculate the MOI for the others.

17. Be careful not to detach too many cells during the washing. Remove the remaining liquid from the washes.

18. If you need to process multiple samples, store the falcon tubes containing the lysates on ice in the meantime.

19. The lysate can also be frozen at −80 °C until further processing.

20. Eluates can be immediately processed or frozen at −80 °C for storage.

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