Chapter 9

APEX2-Mediated Proximity Protein Labeling in *Dictyostelium*

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Abstract

Largely due to its simplicity, while being more like human cells compared to other experimental models, *Dictyostelium* continues to be of great use to discover basic molecular mechanisms and signaling pathways underlying evolutionarily conserved biological processes. However, the identification of new protein interactions implicated in signaling pathways can be particularly challenging in *Dictyostelium* due to its extremely fast signaling kinetics coupled with the dynamic nature of signaling protein interactions. Recently, the proximity labeling method using engineered ascorbic acid peroxidase 2 (APEX2) in mammalian cells was shown to allow the detection of weak and/or transient protein interactions and also to obtain spatial and temporal resolution. Here, we describe a protocol for successfully using the APEX2-proximity labeling method in *Dictyostelium*. Coupled with the identification of the labeled proteins by mass spectrometry, this method expands *Dictyostelium*'s proteomics toolbox and should be widely useful for identifying interacting partners involved in a variety of biological processes in *Dictyostelium*.

Key words *Dictyostelium discoideum*, Cellular signaling, Protein-protein interaction, Proximity labeling, Engineered ascorbic peroxidase, APEX, Biotinylation

1 Introduction

The recent development of protein proximity labeling methods allows for capturing and identifying transient and/or weak interactions. In these methods, the protein of interest (POI) is fused to an enzyme that catalyzes the covalent linking of the small molecule biotin to proteins that come in its vicinity (within estimated radius of ~20 nm) [1]. The biotinylated proteins can then be purified and identified by mass spectrometry (MS). The proximity-dependent biotin identification (BioID) method, and the improved TurboID, makes use of a promiscuous mutant of the *E. coli* biotin ligase BirA that, in the presence of ATP and biotin, generates an activated biotinoyl-5'-AMP molecule, which then reacts with primary amines of exposed lysine residues on neighboring proteins,

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resulting in their covalent biotin modification [2–4]. The BioID method was successfully adapted for use in *Dictyostelium* [5], but better temporal resolution is needed to capture dynamic protein interactions such as those involved in chemotactic signaling pathways. The more recently developed protein proximity labeling method that makes use of ascorbate peroxidase (APEX), and the improved APEX2, offers this possibility [6, 7].

1.1 APEX2 Proximity Labeling

APEX2 is a 27 kDA engineered monomeric ascorbate peroxidase from pea/soybean [6, 7]. In the presence of hydrogen peroxide (H_2O_2), APEX2 catalyzes the oxidation of biotin-phenol (BP) to a short-lived biotin-phenoxyl radical, which then reacts with tyrosine residues, and to a lesser extent with tryptophan and cysteine residues, on proteins proximal to APEX2, resulting in their covalent biotinylation [6–8]. Since BP alone does not trigger biotinylation and H_2O_2 readily penetrates cells, the rapid addition of H_2O_2 to cells previously incubated with BP is used to trigger APEX2-mediated biotinylation within seconds, with optimal labeling reached after ~1 min, and the reaction can be equally rapidly quenched using excess ascorbate and reducing agents. More recently, the development of an improved APEX2 enzyme with higher stability and catalytic efficiency really makes this method ideal for mapping interactomes in live cells when used in combination with appropriate spatial references and data analyses [9–11]. Indeed, because APEX2 labeling is so efficient, APEX2-fused POIs were shown to label not only proteins in the POI's interaction network but also neighboring proteins that diffuse through the reactive biotin cloud and/or reside in the same compartment (bystander proteins), and spatial references coupled with quantitative proteomics are then necessary to distinguish those from the POI's interactome [11]. Using the APEX2 protocol published by Hung et al. [10], and cAR1 as a prototypical POI, we have adapted the APEX2 method for use in *Dictyostelium*.

1.2 APEX2 Proximity Labeling for Proteomics Applications in Dictyostelium

Since APEX2 is the same size as GFP (27 kDa), its fusion to proteins that retain functionality when fused to GFP is expected to generate equally functional proteins. However, a recent study reported that fusing APEX2 to some proteins may result in reduced expression due to the presence of a cysteine on APEX2 (C32), and a C32S mutant was shown to fix this issue [12]. If expression issues are observed, it is then recommended to try the APEX2-C32S to generate POI-APEX2 fusion proteins. For proof of concept, we adapted the APEX2 proximity protein labeling method to *Dictyostelium* using a cAR1-APEX2 construct expressed in *carA* null cells. In troubleshooting experiments, we discovered that BP is not taken up by *Dictyostelium* without first permeabilizing the cells, which had also been reported to be the case in *S. cerevisiae* [13]. Further, as previously described, there are three endogenously biotinylated proteins in *Dictyostelium*, which can be observed by Western blot using streptavidin-HRP: methylcrotonyl-CoA carboxylase alpha

(77 kDa) and propionyl-CoA carboxylase alpha (80 kDa), which seem to migrate together (strong band around 75–80 kDa), and acetyl-CoA carboxylase (257 kDa) [5]. Only when cells were first permeabilized did we observe significant biotinylation of other proteins. We then performed a large-scale labeling reaction with cAR1-APEX2-expressing cells and purified the biotinylated proteins using streptavidin beads. Finally, to identify the biotinylated proteins, we performed in-gel trypsin digestion followed by liquid chromatography and tandem MS. Although we did not have a PM reference to properly distinguish bystanders from the cAR1 interactome, the cAR1-APEX2 proximity labeling allowed identification of many known cAR1-interacting proteins (e.g., $G\alpha_2$, Gβ, Gγ, and arrestin-like proteins) as well as cell cortex proteins and transport compartment-specific proteins (e.g., actin, myosin, clathrin, secretory complex proteins), consistent with similar APEX2 experiments performed with GPCRs in mammalian cells [11]. Therefore, this experiment serves as proof of principle demonstrating the successful adaptation of the APEX2 proximity labeling method for proteomics applications in *Dictyostelium*. Here, we describe this protocol, including the purification of biotin-labeled proteins and sample preparation for subsequent MS analysis (Fig. 1), which we originally published in the Journal of Biological Methods [14].

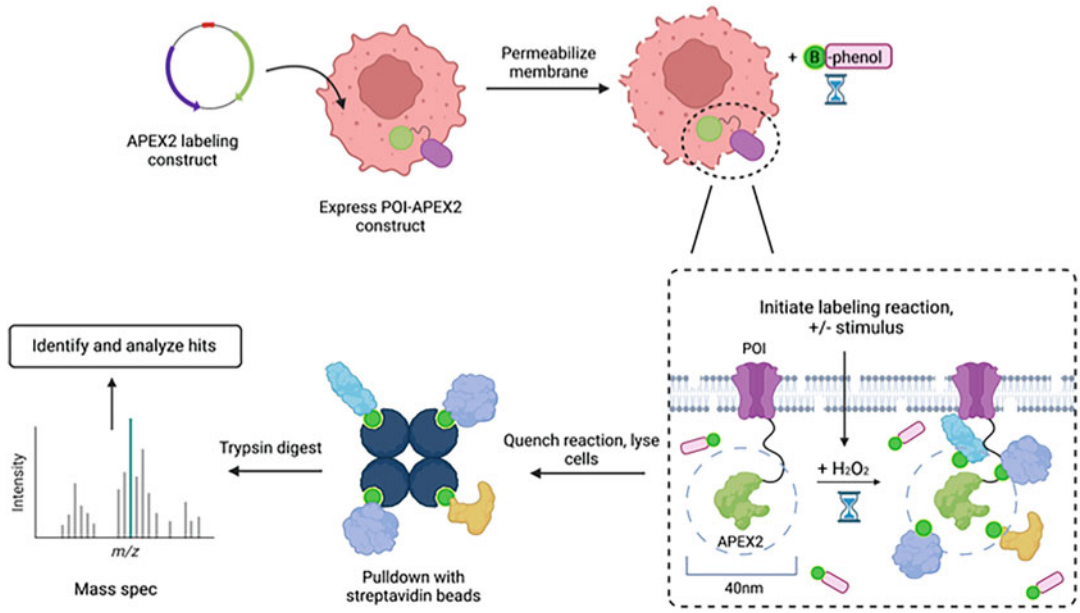

Fig. 1 Overview of the APEX2 protocol for proteomics applications in *Dictyostelium*. APEX2 constructs are prepared by fusing APEX2 in-frame to a POI, or reference control (not shown), and expressed in *Dictyostelium* cells. Cells are first permeabilized with digitonin followed by incubation with biotin-phenol (B-phenol). The reaction is started by adding H_2O_2, with or without stimulus, which will induce labeling of proteins within ~20 nm radius of APEX2. The reaction is then stopped by quenching at the desired time. Biotinylated proteins are then purified with streptavidin beads, eluted, and digested with trypsin prior LC/MS/MS analysis. Created with BioRender.com

2 Materials

Prepare all solutions with ultrapure deionized water (dH_2O) and sterilize the ones that are used with cells.

2.1 Preparing APEX2 Fusion Constructs

1. Any *Dictyostelium* expression vector (e.g., extrachromosomal pDM304): available through the Dicty Stock Center [15].
2. cDNA or gene of the POI and of control protein to be used.
3. APEX2 cDNA: Addgene ID# 72480, depositor: A. Ting, Stanford.

2.2 Validating the APEX2 Constructs

1. SDS-PAGE and Western blotting apparatus and necessary solutions.
2. Antibody recognizing the POI and control protein or the epitope tags used.
3. Fluorescence microscope.

2.3 Cell Permeabilization

1. 12 mM Na/K phosphate buffer: 2.5 mM Na_2HPO_4, 9.5 mM KH_2PO_4, and pH 6.1. Autoclave to sterilize and store at 22 °C (*see* **Note 1**).
2. 0.2 mM digitonin: Dissolve in 12 mM Na/K phosphate buffer, sonicate or heat to 95 °C to dissolve completely, and then cool on ice. Prepare immediately before use.
3. Gyratory shaker with rack for tubes.

2.4 APEX2 Labeling

1. 500 mM BP: Dissolve in DMSO. It may need to be sonicated to completely dissolve. Store as small aliquots at −80 °C. Thaw on ice before use and warm further if BP has precipitated.
2. 30 mM cAMP: Dissolve in 12 mM Na/K phosphate buffer. Store aliquots at −20 °C. Dilute in 12 mM Na/K phosphate buffer to make all working solutions.
3. 100 mM H_2O_2: Dilute the 30% (w/w) H_2O_2 reagent (~10 M H_2O_2 in water) in Na/K phosphate buffer. Prepare immediately before use.
4. 1 M sodium azide (NaN_3): Dissolve in dH_2O. Aliquot and stored at −20 °C.
5. 1 M sodium L-ascorbate: Dissolve in dH_2O. Prepare immediately before use.
6. 0.5 M Trolox: Dissolve in DMSO and sonicate to completely dissolve. Prepare immediately before use.
7. Quenching solution: 10 mM NaN_3, 10 mM sodium ascorbate, and 5 mM Trolox in Na/K phosphate buffer. Keep on ice. Prepare immediately before use.

8. RIPA lysis buffer: 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 10 mM NaN_3, 10 mM sodium ascorbate, 5 mM Trolox, 1 mM PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin, in dH_2O.

2.5 Purifying Biotin-Labeled Proteins

1. Streptavidin agarose beads (e.g., 6% slurry from Gold Biotechnology). Alternatively, streptavidin magnetic beads can also be used.
2. 1 M KCl: Dissolve in dH_2O. Store at room temperature.
3. 0.1 M Na_2CO_3: Dissolve in dH_2O. Store at room temperature.
4. 2 M urea: Dissolve in 10 mM Tris-HCl pH 8.0. Make the day it is to be used.
5. 100 mM biotin: Dissolve in DMSO. Aliquot and store at −20 °C.
6. 1 M DTT stock: Dissolve in dH_2O. Aliquot and store at −20 °C.

2.6 Analyzing Biotin Labeling by Western Blot

1. 6X Protein sample buffer: 0.375 mM Tris-HCl pH 6.8, 30% glycerol, 6% SDS, 600 mM DTT, and 6% bromophenol blue, in dH_2O. Aliquot and store at −20 °C.
2. Streptavidin-horseradish peroxidase (HRP).
3. Tris-buffer saline with Tween-20 (TBST): Dilute 0.1% (v/v) Tween-20 in TBS (50 mM Tris-HCl, 150 mM NaCl in dH_2O, pH 7.5).

2.7 Preparing Samples for LC-MS/MS

1. 50 mM and 100 mM ammonium bicarbonate buffers: Dissolve ammonium bicarbonate in dH_2O; pH should be ~7.8.
2. Destaining solution: 50% (v/v) methanol and 50 mM ammonium bicarbonate. Mix 1 volume methanol with 1 volume 100 mM ammonium bicarbonate buffer.
3. Reducing solution: Dissolve DTT to 25 mM (3.8 mg/mL) in 50 mM ammonium bicarbonate buffer.
4. Alkylating solution: Dissolve iodoacetamide to 55 mM (10.2 mg/mL) in 50 mM ammonium bicarbonate buffer.
5. Trypsin digestion solution: Dissolve 20 μg/mL mass spectrometry-grade Trypsin/Lysine-C mix in 50 mM ammonium bicarbonate buffer containing 0.1% ProteaseMAX™ surfactant Trypsin Enhancer.
6. Peptide extraction solution: 0.2% (v/v) formic acid, 45% acetonitrile (v/v), and 5% (v/v) isopropanol, in dH2O.

3 Methods

The protocol described is for identifying protein interactions involved in cAMP-induced signaling mechanisms, and for this, using ~5.5 h developed cells is recommended to have cells highly responsive to cAMP. Otherwise, no special preparation of the cells is necessary besides normal cell culturing.

3.1 Preparing APEX2 Fusion Constructs

1. Clone the cDNA or gene of the POI in-frame with the APEX2 cDNA in a *Dictyostelium* expression vector of choice. If possible, two APEX2-POI constructs should be made, one with APEX2 fused in N-terminus and one fused at the C-terminus to test both conformations.
2. Ideally, an APEX2 labelling control protein that localizes to the same subcellar compartment as the POI should also be prepared, which will help distinguish between specific interactions and bystander proteins (*see* **Note 2**).
3. An epitope tag or GFP can also be added to the APEX2 constructs to facilitate their analysis by Western blotting and imaging.

3.2 Validating the APEX2 Constructs

1. Culture and transform cells according to standard protocols (*see* **Note 3**).
2. Verify the integrity of APEX2 fusion proteins by Western blot of whole cell lysates using an appropriate antibody (*see* **Note 4**).
3. Verify the functionality of the APEX2-fused POI using appropriate activity assay and/or phenotypic rescue of null strains. This will vary with the POI under study.
4. Verify the localization of the APEX2-fused POI and APEX2 control by either immunofluorescence or fluorescence microscopy if using GFP (*see* **Note 5**).
5. Verify APEX2 functionality in a small-scale labelling assay after cell permeabilization, described in **steps 3.3** and **3.4**.

3.3 Cell Permeabilization

1. Transfer 5.5 h developed cells (10^8 for small-scale and 10^9 for large-scale assays) to 50 mL conical tubes, pellet by centrifugation at 500 × g for 3 min, and wash twice with cold 12 mM Na/K phosphate buffer.
2. Resuspend the cells to 2×10^7 cells/mL in ice-cold 12 mM Na/K phosphate buffer. Transfer cells to an ice-cold Erlenmeyer flask.
3. Add 1 volume of ice-cold 0.2 mM digitonin to the cells (0.1 mM final). Incubate at 4 °C or on ice with shaking for 5 min.

4. Transfer the cells to cold 50 mL conical tubes and pellet by centrifugation at 500 × g for 3 min, at 4 °C.
5. Gently wash the cells twice with ice-cold 12 mM Na/K phosphate buffer with moderate up and down pipetting.
6. Resuspend the cells to 10^8 cells/mL in 12 mM Na/K phosphate buffer at room temperature.

3.4 Small-Scale APEX2 Labeling

1. Perform **step 3.3** using APEX2-POI-expressing cells, APEX2 control-expressing cells, and wild-type cells as negative control. For each strain or condition to be tested, resuspend 10^8 5.5 h developed cells in 1 mL 12 mM Na/K phosphate buffer in a 50 mL conical tube (*see* **Note 6**).
2. Add 2 μL of 500 mM BP (1 mM final) and incubate for 30 min at 22 °C with shaking at ~140 rpm.
3. Start the labeling reaction by adding 20 μL of 100 mM H_2O_2 (2 mM final). Mix by swirling or pipetting up and down and incubate for 30 sec.
4. Stop the reaction by adding 1 mL ice-cold quenching solution and mix well by pipetting up and down. Transfer to 2 mL tubes.
5. Pellet the cells by centrifugation at 500 × g for 3 min, and quickly wash twice with 1 mL ice-cold quenching solution.
6. Resuspend the cells in 0.5 mL RIPA lysis buffer; vortex and incubate on ice for 2 min.
7. Analyze the samples by Western blot as described in Subheading 3.7. If the labeling is satisfactory, proceed with the large-scale labeling for subsequent MS/MS analysis.

3.5 Large-Scale APEX2 Labeling

1. For each cell strain, resuspend 10^9 5.5 h developed cells in 10 mL 12 mM Na/K phosphate buffer in a 50 mL conical tube.
2. Add 20 μL of 500 mM BP (1 mM final) and incubate for 30 min at 22 °C with shaking at ~140 rpm.
3. Start the labeling reaction and stimulate with cAMP at the same time by simultaneously adding 0.2 mL of 50 μM cAMP solution (1 μM final) and 0.2 mL of 100 mM H_2O_2 (2 mM final). Mix and incubate for 30 sec (*see* **Note 7**).
4. Stop the reaction by adding 40 mL ice-cold quenching solution and mix well.
5. Pellet the cells by centrifugation at 500 × g for 3 min, and quickly wash the cells twice with 40 mL ice-cold quenching solution (*see* **Note 8**).
6. Resuspend the cells in 5 mL RIPA lysis buffer; vortex and incubate at 4 °C with agitation for 4 hrs (*see* **Note 9**).

7. Vortex and transfer to tubes appropriate for high-speed centrifugation, and clarify the cell lysates by centrifugation at 15,000 × g for 10 min at 4 °C.
8. Transfer the clarified lysates to 15 mL conical tubes and keep on ice.
9. Take 10 μL aliquots for total biotin-labeled protein analysis by Western blot as described in Subheading 3.7, mix with 2 μL 6X protein sample buffer, and store at −20 °C until needed.

3.6 Purifying Biotin-Labeled Proteins

1. Quantify the lysates' protein content using a reducing agent compatible assay (e.g., Pierce™ 660 nm Protein Assay Reagent) (*see* **Note 10**).
2. Add 60 μL of streptavidin agarose beads (6% slurry) to the lysates for every 360 μg of protein in lysates, and incubate at 4 °C with agitation (e.g., on rotator or gyratory rocker) for at least 1 h and up to 16 h (*see* **Note 11**).
3. Pellet the beads by centrifugation at 1000 × g for 1 min and transfer the supernatants to different tubes. These are the "flowthrough" samples containing unbound proteins and are used to evaluate purification efficiency. Take 10 μL aliquots and mix with 2 μL 6X protein sample buffer for analysis by Western blot and store at −20 °C until needed.
4. Wash the beads with 10 mL of the following: twice with RIPA lysis buffer, once with 1 M KCl, once with 0.1 M Na_2CO_3, once with 2 M Urea in Tris-HCl pH 8.0, and then twice with RIPA lysis buffer. All buffers and samples should be kept on ice.
5. Elute the purified proteins by adding 300 μL of 3X protein sample buffer (prepared from 6X) supplemented with 2 mM biotin and 50 mM DTT; mix and incubate at 95 °C for 10 min. Vortex, cool on ice, and centrifuge briefly to bring down condensation.
6. Mix and then pellet the beads by centrifugation at 1000 × g for 1 min, transfer eluates to new tubes, and keep on ice or store at −20 °C until needed.

3.7 Analyzing Biotin Labeling by Western Blot

1. Thaw the clarified lysates and flowthroughs, and eluate samples containing sample buffer at room temperature, and then centrifuge briefly before incubating at 95 °C for 10 min.
2. Centrifuge the heated samples, and then vortex to ensure sample homogeneity before loading and separating on 8% SDS-PAGE gel.
3. Transfer the proteins to a nitrocellulose membrane and block in 3% (w/v) BSA in TBST at 4 °C for 16 hrs.

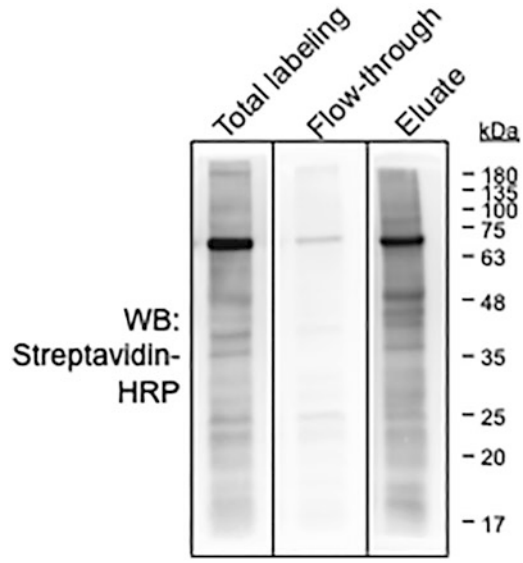

Fig. 2 APEX2 labeling in *Dictyostelium* cells using cAR1 as prototypical POI. The POI-APEX2-mediated protein labeling and efficiency of labeled protein purification should be verified by Western blot prior MS/MS. cAR1-APEX2-mediated labeling is shown as an example

4. Wash the membrane once in TBST and incubate with 0.3 μg/mL streptavidin-HRP in 3% BSA in TBST at room temperature for 1 h.
5. Wash the membrane in TBST for 30 min, followed by four washes of 5 min each.
6. Develop using a standard chemiluminescence reagent and reveal by autoradiography or using a digital imaging system. The clarified lysates and eluates should reveal robust biotinylation, whereas the flowthrough should have minimal biotinylated proteins (Fig. 2).

3.8 Preparing Samples for LC-MS/MS

There are multiple methods to accomplish this. Here, we describe an in-gel trypsin digestion method to prepare the samples for subsequent analysis by mass spectrometry in a core facility.

1. Load the samples on a 12.5% SDS-PAGE gel and perform electrophoresis only until the sample front is at ~1 cm in the gel.
2. Cut gel bands containing the proteins, including migration front with bromophenol blue, using sterile razor blades, and place in 1.7 mL tubes.

3. Incubate the gel bands with destaining solution on a vortex mixer at low speed for 1 h Repeat as necessary to remove all staining, changing the solution by pelleting the gel bands by brief centrifugation in a mini centrifuge and pipetting the supernatant out (*see* **Note 12**).
4. Incubate the gel bands with the reducing solution at 60 °C for 45 min. Pellet the gel bands and discard supernatant.
5. Incubate the gel bands with the alkylating solution in the dark at room temperature for 30 min. Pellet the gel bands and discard supernatant.
6. Wash the gel bands three times with 50 mM ammonium bicarbonate buffer on a vortex mixer at low speed, for 30 min each wash.
7. Transfer the gel bands to a clean glass plate (previously cleaned with methanol or isopropanol), and cut them into small pieces with a sterile razor blade.
8. Place the gel pieces in a 1.7 mL tube and dehydrate by incubating in acetonitrile for 10 min, vortexing a few times. Gel pieces will become white and stick together.
9. Pellet the gel pieces by brief centrifugation in a mini centrifuge, discard acetonitrile, and dry in a Speed Vacuum Concentrator (~30 min).
10. Rehydrate the gel pieces in trypsin digestion solution, enough to cover the gel pieces, for 15 min at room temperature. Vortex to mix.
11. Digest the proteins in the trypsin digestion solution at 37 °C for 16 h.
12. Add 10% formic acid to the in-gel protein digest mixture and vortex to mix. Verify the pH with pH test paper, which should be between 2 and 3. If needed, adjust pH with formic acid.
13. Pellet the gel pieces by brief centrifugation in a mini centrifuge, transfer the supernatant to a new tube, cool on ice, and centrifuge briefly to bring down condensation.
14. Extract the peptides from the gel pieces by adding peptide extraction solution, enough to cover the gel pieces, and sonicate 10 min. Pellet the gel pieces by brief centrifugation in a mini centrifuge. Transfer the supernatant, combining with that from **step 12**. Repeat the extraction procedure a second time.
15. Clarify the peptide solution by centrifugation at 16,000 × g at room temperature for 10 min. Transfer the supernatant to a new tube.
16. Reduce to desired sample volume using a Speed Vacuum Concentrator.

17. The peptides are then separated by standard liquid chromatography followed by tandem mass spectrometry.
18. To identify the proteins, mass spectra are searched against the SwissProt *Dictyostelium discoideum* protein database and an additional common contaminant database (e.g., trypsin, keratins).

4 Notes

1. An alternative buffer that can be used with *Dictyostelium* is the Development Buffer (DB; 5 mM Na_2HPO_4, 5 mM KH_2PO_4; 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 6.5).
2. The APEX2-mediated labeling of proteins is extremely robust and will lead to the labeling of all proteins that come near the POI, independently of whether they directly interact with it or not [11]. Therefore, ideally, a protein or engineered construct that localizes to the same subcellular compartments as the POI, but that does not interact with it, should be used as an APEX2 control to identify the "bystander" proteins, and distinguish them from true POI-interacting proteins. If stimulus-induced interactions are studied, analyzing the labeling obtained in unstimulated cells can help identify bystander proteins, but depending on the subcellular compartment where the POI localizes, this may not be sufficient.
3. If possible, using null strains of the POI under study is recommended. Using the APEX2-POI corresponding null strain will avoid competition of the POI with its endogenous counterpart for binding signaling proteins. Alternatively, APEX2 can be fused to the endogenous POI using CRISPR [16].
4. APEX2 is 27 kDa and thus the fusion proteins are expected to migrate proportionally slower when analyzed by Western blot. Verify that the APEX2 fusion proteins migrate at their expected molecular weight and that there are no other specific protein species, which would indicate proteolysis. Note that we have not had great success with anti-APEX antibodies and thus recommend using antibodies directed against the POI or tag used.
5. It is important to confirm that the APEX2-POI protein localizes to its expected cellular location, to ensure the significance of the identified interactome. It is also important that the APEX2 control localizes to the same cellular location as the APEX2-POI to identify the location-specific resident proteins that will be labelled by APEX2 even though they do not functionally interact with the POI (bystander proteins).

6. When performing the small-scale protocol for verifying the functionality of APEX2 in the fusion constructs, it is recommended to test conditions with and without BP and H_2O_2 treatments, as well as using cells that do not express APEX2, to distinguish APEX2-mediated labeling from endogenously biotinylated proteins [5].
7. To capture protein interactions involved in early signaling events that are triggered rapidly upon stimulation by a ligand such as cAMP, it is best to add H_2O_2 at the same time as the stimulus. This is achieved using a multichannel pipette.
8. Quenching of the labeling reactions must be done as fast and as thoroughly as possible to ensure best time resolution if comparing different time points after stimulation is desired.
9. Alternative lysis methods may be used, such as mechanical lysis by passing cells through filters, which prevents release of proteases from lysosomes. However, we find that with adequate protease inhibitors and keeping samples on ice and/or 4 °C at all times and performing all the subsequent purification steps in the cold room, proteolysis is minimized.
10. A 1:2 dilution of the samples is usually necessary before performing the protein quantification assay to obtain samples that will be within the linear range of the assay.
11. Use a pipette tip for which you have cut the end with a razor blade to make the opening wider and mix the bead solution well to ensure homogeneity right before pipetting the amount desired. If proteolysis is an issue during a longer incubation, a 1 h incubation can be used and is sufficient to pull down most of the biotinylated proteins.
12. The amount of solution used with the gel bands is minimal, just to cover them, except for the washing steps where 250 µL is suggested, or more depending on the gel band size.

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