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PROTOCOL

Proximity Protein Labeling In *Dictyostelium* With Engineered Ascorbic Acid Peroxidase 2

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Abbreviations used: APEX2, engineering ascorbic acid peroxidase 2; BioID, proximity-dependent biotin identification; BP, biotin-phenol; BSA, bovine serum albumin; cAMP, adenosine-3,5-cyclic; cAR1, cAMP receptor 1 protein; carA, cAR1-encoding gene; cDNA, complementary DNA; CoA, coenzyme A; CRISPR, clustered regularly interspaced short palindromic repeats; ddH2O, ultrapure deionized water; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; *E. coli, Escherichia coli*; Gα2, heterotrimeric G protein subunit alpha 2; Gβ, heterotrimeric G protein subunit beta; Gγ, heterotrimeric G protein subunit gamma; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; H_2O_2, hydrogen peroxide; HCl, hydrogen chloride; HRP, horseradish peroxidase; KCl, potassium chloride; KH_2PO_4, potassium phosphate monobasic; LC-MS/MS, liquid chromatography tandem MS; MS, mass spectrometry; Na_2CO_3, sodium carbonate; Na_2HPO_4, sodium phosphate dibasic; NaCl, sodium chloride; NaN_3, sodium azide; Na/K, sodium and potassium; PKB, protein kinas B; PKBR1, PKB-related 1; PM, plasma membrane; PMSF, phenylmethylsulfonyl fluoride; POI, protein of interest; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, tris-buffered saline; TBST, TBS-tween; TurboID, engineered Turbo biotin ligase for BioID

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ABSTRACT

To fully understand any cellular process, we not only need to identify the proteins implicated, but also how the protein network is structurally and spatially organized and changes over time. However, the dynamic nature of many protein interactions involved in cellular signaling pathways continues to be the bottleneck in mapping and studying protein networks. Fortunately, a recently developed proximity labeling method using engineered ascorbic acid peroxidase 2 (APEX2) in mammalian cells allows the identification of weak and/or transient protein interactions with spatial and temporal resolution. Here, we describe a protocol for successfully using the APEX2-proximity labeling method in *Dictyostelium*, using the cAMP receptor cAR1 as example. Coupled to 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*.

Keywords: Dictyostelium discoideum, Protein-protein interaction, Proximity labeling, Engineered ascorbic peroxidase, APEX, Biotinylation

BACKGROUND

Due to its simplicity and ease of use, *Dictyostelium* continues to be an ideal experimental model to study multiple cellular processes, including allorecognition, cytokinesis, chemotaxis, development, host-pathogen interactions, and quorum sensing, to name a few. Genetic screens have been and continue to be instrumental in these studies to identify key genes involved, but we also need to understand how the encoded proteins then interact with each other as part of networks that are both spatially and temporally regulated. This is particularly challenging when working with *Dictyostelium* because of its extremely fast signaling dynamics.

Currently, the best methods for capturing transient and/or weak protein interactions are based on proximity labeling, where a protein of interest (POI) is fused to an enzyme catalyzing the covalent addition of biotin to proteins that come in its proximity, followed by purification of the biotinylated proteins and their identification by mass spectrometry (MS) [1]. Two main proximity biotin labeling methods are currently used to investigate protein interactions in cells: proximity-dependent biotin identification (BioID) and engineered ascorbate peroxidase (APEX). BioID 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, resulting in their covalent biotin modification [2,3]. Subsequent improvements to the BioID method yielded BioID2 [4], and then TurboID, with an engineered biotin ligase with significantly increased catalytic efficiency that enables proximity

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labeling in cells in ~10 minutes after addition of biotin [5]. The BioID method was successfully adapted for use in *Dictyostelium* [6], but better temporal resolution is needed to capture dynamic protein interactions such as those involved in signaling pathways. APEX offers this possibility [7,8].

APEX is a 27 kDA engineered monomeric ascorbate peroxidase from pea/soybean [7,8]. In the presence of hydrogen peroxide (H_2O_2), APEX 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 APEX, resulting in their covalent biotinylation [7–9]. 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 APEX-mediated biotinylation within seconds, with optimal labeling reached after ~1 minute, 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 [10–12]. 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 to quantitative proteomics are then necessary to distinguish those from the POI's interactome [12]. Using the APEX2 protocol published by Hung *et al*. [11], and the cyclic AMP Receptor 1 (cAR1) as a prototypical POI, we have adapted the APEX2 method for use in *Dictyostelium*.

MATERIALS

Reagents

- ✓ Any *Dictyostelium* expression vector (*e.g.* extrachromosomal pDM304): available through the Dicty Stock Center [13].
- ✓ cDNA or gene of the POI and of control protein, if applicable, to be used.
- ✓ APEX2 cDNA (Addgene ID#72480, depositor: A. Ting, Stanford, CA).
- ✓ SDS-PAGE and western blotting reagents.
- ✓ Antibodies recognizing the POI and control protein, if applicable, or the epitope tags used.
- ✓ Streptavidin agarose beads (Cat. #S-105, Gold Biotechnology, St. Louis, MO).
- ✓ Streptavidin-horseradish peroxidase (HRP) (Cat. #18-152, MilliporeSigma, St. Louis, MO).
- ✓ Sodium phosphate dibasic (Na_2HPO_4) (Cat. #97061-586, VWR, Radnor, PA)
- ✓ Potassium phosphate monobasic (KH_2PO_4) (Cat. #97062-350, VWR)
- ✓ Digitonin (Cat. #D0540, Thermo Fisher Scientific, Waltham, MA)
- ✓ Biotin phenol (BP; biotin-tyramide) (Cat. #50-190-4983, Thermo Fisher Scientific)
- ✓ Dimethyl sulfoxide (DMSO) (Cat. #D4540, MilliporeSigma)
- ✓ Adenosine-3,5-cyclic (cAMP) (Cat. #102586-894, VWR)
- ✓ Hydrogen peroxide (H_2O_2) (Cat. # L14000-AP, Thermo Fischer Scientific)
- ✓ Sodium azide (NaN_3) (Cat. # 97064-646, VWR)
- ✓ Sodium L-ascorbate (Cat. #A0539, TCI Chemicals, Portland, OR)
- ✓ Trolox (Cat. #AC218940, Thermo Fisher Scientific)
- ✓ Tris-HCl (Cat. #T-095-1, Gold Biotechnology)
- ✓ Sodium chloride (NaCl) (Cat. #sc-203274C, Santa Cruz Biotechnology, Dallas, TX)
- ✓ Sodium dodecyl sulfate (SDS) (Cat. #L22010, Research Products International, Mount Prospect, IL)
- ✓ Sodium deoxycholate (Cat. # 30970, MilliporeSigma)
- ✓ Triton X-100 (Cat. # BP151, Thermo Fisher Scientific)
- ✓ Phenylmethylsulfonyl fluoride (PMSF) (Cat. # 52332, CalBioChem, San Diego, CA)
- ✓ Leupeptin (Cat. #501685873, Thermo Fisher Scientific)
- ✓ Aprotinin (Cat. #A-655, Gold Biotechnology)
- ✓ Potassium chloride (KCl) (Cat. #P9541, MilliporeSigma)
- ✓ Sodium carbonate (Na_2CO_3) (Cat. # BDH0284, VWR)
- ✓ Urea (Cat. # U-200-1, Gold Biotechnology)
- ✓ Biotin (Cat. #B-950, Gold Biotechnology)
- ✓ Dithiothreitol (DTT) (Cat. #DTT10, Gold Biotechnology)
- ✓ Glycerol (Cat. #MK509216, VWR)
- ✓ Bromophenol blue (Cat. # 97061-690, VWR)
- ✓ Pierce™ 660 nm Protein Assay Reagent (Cat. # 22660, Thermo Fisher Scientific)
- ✓ BSA (Cat. # A-420, Gold Biotechnology)
- ✓ Tween-20 (Cat. # J20605-AP, Thermo Fisher Scientific)
- ✓ SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Cat. #34580, Thermo Fisher Scientific)
- ✓ Ammonium bicarbonate (Cat. # 1066-33-7, Aeros Organies, Thermo Fisher Scientific)
- ✓ Methanol (Cat. # 67-56-1, Thermo Fisher Scientific)
- ✓ Iodoacetamide (Cat. # 144-48-9, MilliporeSigma)
- ✓ Trypsin/Lysine-C (Cat. # V5071, Promega, Madison, WI)
- ✓ ProteaseMAX™ (Cat. # V2071, Promega)
- ✓ Formic acid (Cat. # 85178, Thermo Fisher Scientific)
- ✓ Acetonitrile (Cat. # 75-05-8, Thermo Fisher Scientific)
- ✓ Isopropanol (Cat. # 67-63-0, Thermo Fisher Scientific)

Recipes

12 mM Na/K phosphate buffer

Prepare a 10X stock solution by dissolving 6.8 g Na_2HPO_4 and 26 g KH_2PO_4 in 2 L ultrapure deionized water (ddH_2O). Autoclave to sterilize and store at 22°C. To prepare 1X buffer, mix 400 mL of 10X with 3600 mL dH_2O, yielding a solution of 2.5 mM Na_2HPO_4 and 9.5 mM KH_2PO_4, pH ~6.1. Autoclave to sterilize and store at 22°C.

NOTE: An alternative buffer that can be used 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).

0.2 mM digitonin

Dissolve 1.25 mg digitonin in 5 mL (small scale protocol) or 12.5 mg in 50 mL (large scale protocol) of 12 mM Na/K phosphate buffer per sample. Sonicate or heat to 95°C to dissolve completely, then cool on ice. Prepare fresh.

500 mM BP

Dissolve 181.7 mg BP in 1 mL DMSO. Sonicate to completely dissolve. Store as 10 and 100 µL aliquots at −80°C. Thaw on ice before use and warm to room temperature if BP has precipitated. Do not refreeze, discard unused BP aliquot.

30 mM cAMP

Dissolve 395.04 mg cAMP in 40 mL 12 mM Na/K phosphate buffer. Store as 1 mL aliquots at −20°C. Dilute in 12 mM Na/K phosphate buffer to make the working solutions.

100 mM H_2O_2

Dilute 10 µL of the 30% (w/w; ~10 M) H_2O_2 reagent in 990 µL 12 mM Na/K phosphate buffer. Prepare fresh.

1 M sodium azide (NaN_3)

Dissolve 6.511 g NaN_3 in ddH_2O to 100 mL. Store as 5 mL aliquots at −20°C.

1 M sodium L-ascorbate

Dissolve 802.3 mg sodium L-ascorbate in 4.05 mL (small scale protocol) or 1.03 g in 5.2 mL (large scale protocol) ddH_2O per sample. Prepare fresh.

0.5 M Trolox

Dissolve 506.8 mg in 4.05 mL (small scale protocol) or 650.7 mg in 5.2 mL (large scale protocol) DMSO per sample and sonicate to completely dissolve. Prepare fresh.

Quenching solution

For the small-scale protocol: combine 50 µL of 1M NaN_3, 50 µL of 1M sodium L-ascorbate, 50 µL of 0.5 M Trolox, with 4.85 mL of 12 mM Na/K phosphate buffer per sample. For the large-scale protocol: combine 1.2 mL of 1M NaN_3, 1.2 mL of 1M sodium L-ascorbate, 1.2 mL of 0.5 M Trolox, with 116.4 mL of 12 mM Na/K phosphate buffer per sample (final concentrations: 10 mM NaN_3, 10 mM sodium ascorbate, 5 mM Trolox). Prepare fresh and keep on ice.

0.2 M PMSF

Dissolve 1.742 g PMSF in 5 mL ethanol. Store as 500 µL aliquots at −20°C.

5 mg/mL Leupeptin

Dissolve 5 mg leupeptin in 1 mL ddH_2O. Store as 50 µL aliquots at −20°C.

5 mg/mL Aprotinin

Dissolve 5 mg aprotinin in 1 mL ddH_2O. Store as 50 µL aliquots at −20°C.

RIPA lysis buffer

Dissolve 100 mg SDS, 500 mg sodium deoxycholate, 1 mL Triton X-100, 876.6 mg NaCl, 4 mL of 0.5 M Trolox, 4 mL of 1M NaN_3, 4 mL of 1M sodium L-ascorbate, 50 mL of 100 mM Tris-HCl pH 7.5, in ddH_2O to 100 mL (final concentrations: 0.1% w/v SDS, 0.5% w/v sodium deoxycholate, 1% v/v Triton X-100, 150 mM NaCl, 20 mM Trolox, 40 mM NaN_3, 40 mM sodium L-ascorbate, 50 mM Tris-HCl pH 7.5). Store at 4°C. Before use, prepare a working stock containing 1 mM PMSF, 5 µg/mL leupeptin, and 5 µg/mL aprotinin final, and keep on ice.

1 M KCl

Dissolve 7.46 g KCl in ddH_2O to 100 mL. Store at room temperature.

0.1 M Na_2CO_3

Dissolve 1.06 g Na_2CO_3 in ddH_2O to 100 mL. Store at room temperature.

2 M Urea

Dissolve 1.20 g urea in 10 mL of 10 mM Tris-HCl pH 8.0 per sample. Prepare fresh.

100 mM biotin

Dissolve 244.3 mg biotin in 10 mL DMSO. Store as 10 aliquots at −20°C.

1 M DTT stock

Dissolve 7.71 g DTT in 50 mL ddH_2O. Store as 1 mL aliquots at −20°C.

1 M Tris-HCl pH 6.8

Dissolve 30.2 g Tris-HCl in 250 mL ddH_2O. Autoclave and store at 4°C.

6X protein sample buffer

Mix 15 mL of 1M Tris-HCl pH 6.8 with 12 mL glycerol, then dissolve 2.4 g SDS, 4 g DTT, and 2.4 mg Bromophenol blue, heating to 37°C to completely dissolve, and add ddH_2O to 40 ml (final concentrations: 375 mM Tris-HCl pH 6.8, 30% v/v glycerol, 6% w/v SDS, 600 mM DTT, 6% w/v bromophenol blue). Store as 1 mL aliquots at −20°C.

3X biotin elution protein sample buffer

Mix 20 µL of 100 mM biotin (2 mM biotin final) with 500 µL 6X protein sample buffer and 480 µL ddH_2O. Prepare fresh.

Tris buffer saline with Tween-20 (TBST)

Dissolve 8.2 g NaCl, 0.22 g KCl, and 6 g Tris-HCl in ddH_2O, pH to 7.4 with HCl, then mix in 1 mL Tween-20 and bring to 1 L with ddH_2O (final concentrations: 0.1% v/v Tween-20, 50 mM Tris-HCl, 150 mM NaCl).

Blocking buffer

Dissolve 3 g BSA in 100 mL TBST (3% w/v final). Store at 4°C.

Ammonium bicarbonate buffers

To make the 50 mM buffer, dissolve 0.1976 g ammonium bicarbonate in 50 mL ddH_2O, and to make the 100 mM buffer, dissolve 0.3953 g ammonium bicarbonate in 50 mL ddH_2O. pH should be ~7.8. Prepare fresh.

De-staining solution

Mix 1 volume methanol with 1 volume 100 mM ammonium bicarbonate buffer, to yield 50% v/v methanol and 50 mM ammonium bicarbonate solution. Prepare fresh.

Reducing solution

Dissolve 3.8 mg DTT in 1 mL 50 mM ammonium bicarbonate buffer (25 mM final). Prepare fresh.

Alkylating solution

Dissolve 10.2 mg iodoacetamide in 1 mL of 50 mM ammonium bicarbonate buffer (55 mM final). Prepare fresh.

Trypsin digestion solution

Dissolve 2 µg mass spectrometry grade Trypsin/Lysine-C mix (20 ng/µL final) and dilute 1 µL 10% ProteaseMAX™ surfactant Trypsin Enhancer (0.1% final) in 99 µL of 50 mM ammonium bicarbonate buffer. Prepare fresh.

Peptide extraction solution

Mix 2 µL formic acid (0.2% v/v final), 449.1 µL acetonitrile (45% v/v final), and 49.9 µL isopropanol (5% v/v final) with 499 µL ddH_2O to 1 mL. Prepare fresh.

Equipment

- ✓ SDS-PAGE apparatus (Cat. # 1658002FC, Mini-PROTEAN® Tetra Vertical Electrophoresis system, Bio-Rad, Hercules, CA)
- ✓ Western blot apparatus (Cat. # 1703935, Mini Trans-Blot Module, Bio-Rad)
- ✓ Confocal imaging system (*e.g.* Marianas SDC, 3i)
- ✓ Benchtop centrifuge (Cat. # 75007221, SL8 Small Benchtop Centrifuge, Thermo Fisher Scientific)
- ✓ High-speed centrifuge (Cat. # 369001, Avanti J-E Centrifuge, Beckman-Coulter, Brea, CA)
- ✓ Microcentrifuge (Cat. # B31600, Microfige 20, Beckman-Coulter)
- ✓ Mini centrifuge (Cat. # 75004061, mySPIN 6, Thermo Fisher Scientific)
- ✓ Orbital shaker (Cat. #12620-940, VWR® Mini Shaker, VWR International)
- ✓ Platform rocker (Cat. # 07-202-200, Thermo Fisher Scientific)
- ✓ Vortex mixer (Cat. # 76549-928, VWR International)
- ✓ Tube rotator (Cat. # 88-861-049, Fisherbrand Multi-Purpose Tube Rotator, Thermo Fisher Scientific)
- ✓ Chemiluminescence imager (Cat. # IS1026, Sapphire Biomolecular Imager, Azure Biosystems, Dublin, CA)
- ✓ Speed vacuum concentrator (Cat. # SPD130DLX-115, Savant Speedvac, Thermo Fisher Scientific)
- ✓ Block heater (Cat. # D1100, Accublock digital dry bath, Labnet International, Edison, NJ)
- ✓ Bath sonicator (FS60D, FS6 Ultrasonic Cleaner, Thermo Fisher Scientific)
- ✓ Multichannel pipette (Cat. # FA10036, Gilson, Middleton, WI)
- ✓ Pipette controller (Cat. #4-000-101, Portable Pipet-Aid® XP, Drummond Scientific Company, Broomall, PA)
- ✓ Glass 125 ml Erlenmeyer flask (Cat. # 10536-912, VWR International)
- ✓ Glass plate (Cat. # GLP5X5-PK/12, Novatech international, Kingwood, TX)
- ✓ Polycarbonate tubes (Cat. # 31170150, Thermo Fisher Scientific)
- ✓ 50 ml conical tubes (Cat. # 89039-658, VWR International)
- ✓ 15 ml conical tubes (Cat. # 89039-666, VWR International)
- ✓ 5 ml tubes (Cat. # 1162A08, Thomas Scientific)
- ✓ 1.7 ml tubes (Cat. # 24-281, Genesee Scientific, El Cajon, CA)
- ✓ Razor blades (Cat. # CUT-40475, Economy Cutter Blades, Garvey, Blue Anchor, NJ)
- ✓ pH test strips (Cat. # 13-640-521, Thermo Fisher Scientific)

PROCEDURE

We describe a protocol for identifying protein interactions involved in cAMP-induced signaling mechanisms in *Dictyostelium* and, thus, the use of ~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 *Dictyostelium* cell culturing (for cell culture protocols see [14]).

1. Preparing APEX2 fusion constructs

1.1. 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.

NOTE: 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.

1.2. Ideally, an APEX2 labeling 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.

NOTE: 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 [12]. 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. Alternatively, if stimulus-induced interactions are studied, analyzing the labeling obtained in unstimulated cells can help identifying bystander proteins but, depending on the subcellular compartment where the POI localizes, this may not be sufficient.

1.3. An epitope tag or GFP can also be added to the APEX2 constructs to facilitate their analysis by western blot and imaging.

2. Validating the APEX2 constructs

2.1. Culture and transform cells according to standard protocols [14].

NOTE: If available, using null strains of the POI under study is recommended as the cellular background for APEX2-POI expression. This will prevent competition of the APEX2-POI with its endogenous counterpart for binding signaling proteins. Alternatively, APEX2 can be fused to the endogenous POI using CRISPR [15].

2.2. Verify the expression and integrity of APEX2 fusion proteins by western blot of whole cell lysates using a POI- or tag-specific antibody and standard SDS-PAGE and western blot protocols.

NOTE: 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 good results using commercial APEX2 antibodies for APEX2-POI western blot.

2.3. 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.

2.4. Verify the localization of the APEX2-fused POI and APEX2 control by confocal immunofluorescence or fluorescence imaging if using GFP.

NOTE: 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 labeled by APEX2 even though they do not functionally interact with the POI (bystander proteins).

2.5. Verify APEX2 functionality in a small-scale labeling assay after cell permeabilization, described in Sections 3 and 4 below.

3. Cell permeabilization

3.1. Transfer the developed cells (1×10^8 for small scale assay; 1×10^9 for large scale assay) to 50 mL conical tubes, pellet by centrifugation at 500 xg for 3 minutes at 4°C and wash twice with 40 mL cold 12 mM Na/K phosphate buffer, pelleting the cells by centrifugation at 500 xg for 3 minutes at 4°C after each wash.

3.2. Resuspend the cells to 2×10^7 cells/mL in ice-cold 12 mM Na/K phosphate buffer (5 mL for small scale assay; 50 mL for large scale assay). Transfer cells to an ice-cold 125 mL Erlenmeyer flask.

3.3. 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 gentle shaking on orbital shaker at ~100 rpm for 5 minutes.

3.4. Transfer the cells to cold 50 mL conical tubes and pellet by centrifugation at 500 xg for 3 minutes at 4°C.

3.5. Wash the cells twice with 40 mL ice-cold 12 mM Na/K phosphate buffer with gentle up and down pipetting, pelleting the cells by centrifugation at 500 xg for 3 minutes at 4°C after each wash.

3.6. Resuspend the cells to 1×10^8 cells/mL in 12 mM Na/K phosphate buffer (1 mL small scale assay; 10 mL large scale assay) at room temperature.

4. Small scale APEX2 labeling

4.1. Perform the cell permeabilization steps (Section 3 above) using APEX2-POI-expressing cells, APEX2-control-expressing cells, and wild-type cells as reference control. For each strain or condition to be tested, resuspend the 1×10^8 developed, permeabilized cells in 1 mL room temperature 12 mM Na/K phosphate buffer in a 50 mL conical tube.

NOTE: 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_2O2 treatments, as well as using cells that do not express APEX2, to distinguish APEX2-mediated labeling from endogenously biotinylated proteins [6].

4.2. Add 2 µL of 500 mM BP (1 mM final) and incubate for 30 minutes at 22°C with shaking on orbital shaker at ~140 rpm.

4.3. 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 30 seconds.

4.4. Stop the reaction by adding 1 mL ice-cold quenching solution and mix well by pipetting up and down. Transfer to 5 mL tubes.

4.5. Pellet the cells by centrifugation at 500 xg for 3 minutes at 4°C and quickly wash twice with 1 mL ice-cold quenching solution, pelleting the cells by centrifugation at 500 xg for 3 minutes at 4°C after each wash.

4.6. Resuspend the cells in 0.5 mL RIPA lysis buffer, vortex and incubate on ice for 2 minutes.

4.7. Analyze the samples by western blot as described in Section 7 below. If the labeling is satisfactory, proceed with the large-scale labeling for subsequent MS/MS analysis.

5. Large-scale APEX2 labeling

5.1. For each cell strain, resuspend 1×10^9 developed cells in 10 mL room temperature 12 mM Na/K phosphate buffer in a 50 mL conical tube.

5.2. Add 20 µL of 500 mM BP (1 mM final) and incubate for 30 minutes at 22°C with shaking at ~140 rpm.

5.3. Start the labeling reaction and stimulate with cAMP at the same time by simultaneously adding 200 µL of 50 µM cAMP solution (1 µM final) and 200 µL of 100 mM H_2O_2 (2 mM final). Mix and incubate 30 seconds.

NOTE: 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.

5.4. Stop the reaction by adding 40 mL ice-cold quenching solution and mix well by pipetting up and down.

5.5. Pellet the cells by centrifugation at 500 xg for 3 minutes at 4°C and quickly wash the cells twice with 40 mL ice-cold quenching solution, pelleting the cells by centrifugation at 500 xg for 3 minutes at 4°C after each wash.

NOTE: 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.

5.6. Resuspend the cells in 5 mL RIPA lysis buffer, vortex and incubate at 4°C or on ice with shaking on orbital shaker at ~140 rpm for 4 hours.

NOTE: 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 minimal.

5.7. Vortex and transfer to polycarbonate tubes and clarify the cell lysates by centrifugation at 15,000 xg for 10 minutes at 4°C in a high-speed centrifuge.

5.8. Transfer the clarified lysates to 15 mL conical tubes and keep on ice.

5.9. Take 10 µL aliquots for total biotin-labeled protein analysis by western blot as described in Section 7 below, mix with 2 µL 6X protein sample buffer in 1.7 mL tube and store at −20°C until needed.

6. Purifying biotin-labeled proteins

6.1. Quantify the lysates' protein content using a reducing agent compatible assay (*e.g.* Pierce™ 660 nm Protein Assay Reagent) according to the manufacturer's protocol.

NOTE: 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.

6.2. 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 (on rotator or rocker) for at least 1 hour, and up to 16 hours.

NOTE: 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 desired amount. 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.

6.3. Pellet the beads by centrifugation at 1,000 xg for 1 minute at 4°C and transfer the supernatants to new 1.7 mL 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.

6.4. Always keeping buffers and samples on ice, wash the beads as follows: twice with 10 mL RIPA lysis buffer, once with 10 mL 1 M KCl, once with 10 mL 0.1 M Na_2CO_3, once with 10 mL 2 M Urea in Tris-HCl pH 8.0, and twice with 10 mL RIPA lysis buffer, pelleting the beads by centrifugation at 1,000 xg for 1 minute at 4°C between washes.

6.5. Elute the purified proteins by adding 300 µL of 3X biotin elution protein sample buffer to the beads, mix and incubate at 95°C for 10 minutes. Vortex, cool on ice, and centrifuge briefly using a mini centrifuge to bring down condensation.

6.6. Mix and then pellet the beads by centrifugation at 1,000 xg for 1 minute at 4°C, transfer eluates to new 1.7 mL tubes and keep on ice or store at −20°C until needed.

7. Analyzing biotin labeling by western blot

7.1. Thaw the clarified lysates, flowthroughs, and eluate samples containing sample buffer to room temperature, then centrifuge briefly in a mini centrifuge before incubating at 95°C for 10 minutes.

7.2. Centrifuge the heated samples in a mini centrifuge, and then vortex to ensure sample homogeneity before loading and separating on 8% SDS-PAGE gel.

7.3. Transfer the proteins to a nitrocellulose membrane and incubate in blocking buffer at 4°C for 16 hours with rocking on platform rocker.

7.4. Wash the membrane once with 20 mL TBST and incubate with 0.3 µg/mL streptavidin-HRP in blocking buffer at room temperature for 1 hour with rocking on platform rocker.

7.5. Wash the membrane in 20 mL TBST for 30 minutes with rocking on platform rocker, followed by 4 similar washes of 5 minutes each.

7.6. Develop using chemiluminescence and reveal by autoradiography or using a digital imaging system.

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.

8.1. Load the entirety of each sample in one large well per sample on a 12.5% SDS-PAGE gel and perform electrophoresis only until the migration front is at ~1 cm in the gel.

8.2. Cut gel bands containing the proteins, including migration front with bromophenol blue, using sterile razor blades and transfer the bands to 1.7 mL tubes. The gel bands can then be kept at −20°C until used further.

8.3. Incubate the gel bands with de-staining solution, minimal amount just to cover them, on a vortex mixer at low speed for 1 hour. Repeat with fresh de-staining solution 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.

8.4. Incubate the gel bands with the reducing solution at 60°C for 45 minutes. Pellet the gel bands and discard supernatant.

8.5. Incubate the gel bands with the alkylating solution in the dark at room temperature for 30 minutes. Pellet the gel bands and discard supernatant.

8.6. Wash the gel bands 3 times with 250 µL of 50 mM ammonium bicarbonate buffer on a vortex mixer at low speed, for 30 minutes each wash.

8.7. 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.

8.8. Place the gel pieces in a 1.7 mL tube and dehydrate by incubating in acetonitrile, just enough to cover the gel pieces, for 10 minutes, vortexing a few times. Gel pieces will become white and stick together.

8.9. Pellet the gel pieces by brief centrifugation in a mini centrifuge, discard acetonitrile and dry in a Speed Vacuum Concentrator (~30 minutes).

8.10. Rehydrate the gel pieces in trypsin digestion solution, just enough to cover the gel pieces, for 15 minutes at room temperature. Vortex to mix.

8.11. Digest the proteins in the trypsin digestion solution at 37°C using block heater filled with water, for 16 hours.

8.12. Add 10% formic acid final to the in-gel protein digestion mixture and vortex to mix. Verify the pH with pH test strips, which should be between 2–3. If needed, adjust pH with formic acid.

8.13. Pellet the gel pieces by brief centrifugation in a mini centrifuge, transfer the supernatant to a new 1.7 mL tube, cool on ice and centrifuge briefly to bring down condensation.

8.14. Extract all peptides from the gel pieces by adding peptide extraction solution, just enough to cover the gel pieces, and sonicate 10 minutes. Pellet the gel pieces by brief centrifugation in a mini centrifuge. Transfer the supernatant, combining with that from Step 8.13. Repeat the extraction procedure a second time and combine all supernatants in a 1.7 mL tube.

8.15. Clarify the peptide solution by centrifugation at 16,000 xg in a microcentrifuge, at room temperature for 10 minutes. Transfer the supernatant to a new 1.7 mL tube.

8.16. Reduce to ~20 µL using a Speed Vacuum Concentrator.

8.17. The peptides are then separated by standard liquid chromatography followed by tandem mass spectrometry.

8.18. 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).

ANTICIPATED RESULTS

Herein, we describe a step-by-step protocol for the APEX2-mediated proximity labeling of proteins in *Dictyostelium* cells, fol-

lowed by the purification of biotin-labeled proteins and sample preparation for subsequent MS analysis. This protocol allows for the identification of transient protein-protein interactions such as those implicated in the cAMP signaling chemotaxis pathways.

We have optimized this protocol using the cAMP chemoattractant receptor cAR1 as proof of principle. We generated a cAR1-APEX2 construct by cloning the cAR1 cDNA without stop codon in the BglII site of the pDM304 vector, and then cloning APEX2 without its initiator methionine in the SpeI site, resulting in a fusion construct with a 5 amino acid linker. We introduced this plasmid in *carA* null (*carA*-) cells by transformation using electroporation, and we confirmed that cAR1-APEX2 is properly expressed as a fusion protein and is functional (**Fig. 1A, 1B**). We also attempted to express a plasma membrane (PM) reference that is used to identify bystander proteins in APEX2 experiments with G protein-coupled receptors (GPCRs) in mammalian cells [12], the Lyn PM targeting sequence, but it did not sufficiently localize to the PM in *Dictyostelium* (not shown). Furthermore, we determined that BP is not taken up by *Dictyostelium* without first permeabilizing the cells (**Fig. 1C**), which had also been reported to be the case in *S. cerevisiae* [16]. As previously reported, 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) [6]. Therefore, these proteins will be purified and detected along any other biotin-labelled proteins in the assay and can be used to determine purification efficiency but represent background biotinylation that does not depend on APEX2-mediated labeling. In support of this, we only observed significant biotinylation of other proteins when we treated cells with either sorbitol or digitonin to permeabilize the plasma membrane (**Fig. 1C**). However, we also determined that only the digitonin-treated cells remained responsive to the cAMP stimulus and, therefore, we suggest that only digitonin should be used to permeabilize cells to BP for the APEX2 assay in *Dictyostelium* (**Fig. 1D**). It is also important to confirm that digitonin-treated cells effectively respond to the stimulus, here cAMP, in the presence of BP and H_2O_2 (**Fig. 1E**). We assessed the cAMP-stimulated phosphorylation of PKB and PKBR1, but depending on the POI under study, other functional responses should be monitored to ensure proper conditions are used.

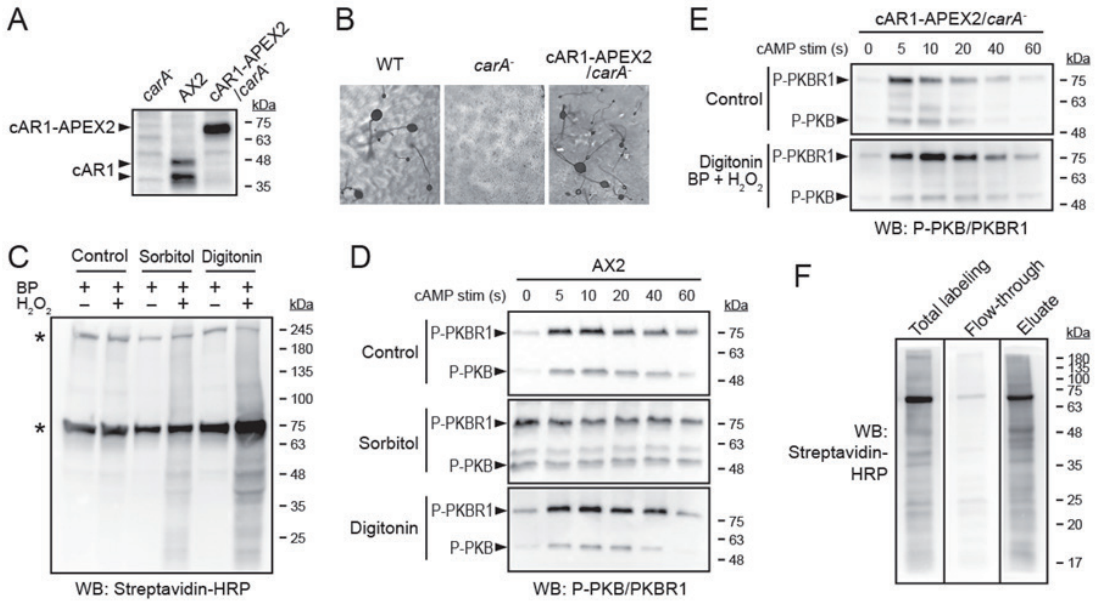

Figure 1 APEX2 labeling in *Dictyostelium* cells using cAR1 as prototypical POI. A. cAR1-APEX2 fusion construct (expected 71 kDa) expressed in *carA*- cells is detected by western blot of cell lysates using anti-cAR1 antibody as described previously [17]. Lysates of *carA*- and wild-type (AX2) cells are used as control. cAR1 is 44.3 kDa and APEX2 is 27 kDa. **B.** cAR1-APEX2 rescues the developmental defects of *carA*- cells. Pictures of cells plated on non-nutrient agar were taken at 48 h after onset of starvation. WT, AX2 cells. **C.** Cell permeabilization is necessary for BP to penetrate cells and APEX2-mediated labeling of proteins in cells in the presence of H_2O_2. In a small scale APEX2 labeling assay, cAR1-APEX2 cells were treated or not (control) with either 800 mM sorbitol or 0.1 mM digitonin for 5 minutes at 4°C prior incubation with BP and addition of H2O2. Biotinylated proteins were analyzed by western blot (WB) using streptavidin-HRP. **D.** Digitonin- and not sorbitol-treated cells remain responsive to cAMP. AX2 cells were treated or not (control) with sorbitol or digitonin prior stimulating with cAMP for the time indicated and lysing cells directly in sample buffer, followed by analyses of cAMP-induced PKB and PKBR1 phosphorylation by western blot, as previously described [17]. **E.** cAR1-APEX2 effectively transduces the cAMP signal in the presence of BP and H_2O_2. cAR1-APEX2-expressing carA- cells were pretreated with digitonin before BP incubation, and H_2O_2 was added to cells at the same time as cAMP. Cells were lysed and analyzed as described above. **F.** Verifying cAR1-APEX2-mediated protein labeling and labeled protein purification by western blot prior LC-MS/MS.
* The endogenous biotinylated proteins can be observed migrating at ~75 kDa and ~240 kDa.

We then performed a large-scale labeling reaction with cAR1-APEX2-expressing cells and purified the biotinylated proteins using streptavidin beads (**Fig. 1F**). Here, the clarified lysates and eluates should reveal robust biotinylation, whereas the flowthrough should have minimal biotinylated proteins. 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 a large cAR1 proximal proteome that includes 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), which is expected for membrane-localized GPCRs and consistent with similar APEX2 experiments performed in mammalian cells [12]. Even with the lack of a location-specific APEX2 control, if +/- stimulation conditions are used, the unstimulated condition can serve as a good control for identifying stimulus-induced interactions. Therefore, this experiment serves as proof-of-principle demonstrating the successful adaptation of the APEX2 proximity labeling method for proteomics applications in *Dictyostelium*.

TROUBLESHOOTING

Steps	Problems	Causes	Suggestions
2	Low APEX2 fusion protein expression levels	-Low transformation efficiency -Unstable fusion protein due to APEX2 cysteine residue 32 (C32) that can affect folding of some proteins [18]	-Generate stable, clonal cell lines -Use an APEX2-C32S mutant [18]
4	No or little biotin labeling	-Reagents not fresh -Reagents not fully dissolved in their solvent -Cell lysis	-It is important to prepare many of the reagents fresh, where indicated, the day of the experiment and even within ~2 hours. of utilization. -Make sure that digitonin and BP are completely dissolved by sonicating the solutions in a bath sonicator. -Be very careful and gentle when permeabilizing the cells with digitonin and washing them after the treatment, rough manipulation of the cells at these steps will lead to cell lysis.
6	Inefficient purification	-Left over BP in lysates, competing for binding the beads -Low binding of the biotin-labeled proteins to the streptavidin beads -The biotin-labeled proteins did not elute	-Increase the quenching solution volume and number of washes -Make sure that the pH of the RIPA buffer is ~7.5 and to use 60 µL or more of 6% streptavidin-agarose slurry for each 360 µg of lysate proteins. Also try longer incubations of lysates with the streptavidin beads. -Make sure that biotin in the 100 mM stock solution is completely dissolved after thawing an aliquot to prepare the 3X biotin elution sample buffer, and warm up the 6X protein sample buffer + ddH_2O to at least 37°C before adding the biotin to prevent precipitation. This will ensure adequate concentration of biotin and optimal elution.

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