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Identification of Lipid Droplet Proteomes by Proximity Labeling Proteomics Using APEX2

Kirill Bersuker and **James A. Olzmann**

Abstract

Lipid droplets (LDs) are ubiquitous lipid storage organelles composed of a neutral lipid core surrounded by a phospholipid monolayer that is decorated with integral and peripheral proteins. Accurate identification of LD proteins using biochemical fractionation methods has been challenging due to the presence of contaminant proteins from co-fractionating organelles. Here, we describe a method to identify high-confidence LD proteomes that employs an engineered ascorbate peroxidase (APEX2) to induce spatially and temporally restricted biotinylation of LD proteins. This proximity labeling method can be broadly applied to define the composition of the LD proteome in any cultured cell line and can be utilized to examine LD proteome dynamics.

Keywords

Proximity labeling; Biotinylation; Lipid droplet; APEX; APEX2; Proteome; Organelle

1 Introduction

Lipid droplets (LDs) are endoplasmic reticulum (ER)-derived organelles that regulate cellular lipid and energy homeostasis through the dynamic sequestration and release of fatty acids [1, 2, 3, 4]. LDs store fatty acids as neutral lipids (e.g., triacylglycerol and sterol esters) within the LD core, which is encircled by a phospholipid monolayer containing integral and peripheral proteins [5, 6]. The complement of LD-associated proteins, which is referred to as the LD proteome, regulates all known LD functions [5, 6]. Some well-characterized examples of LD proteins include triacylglycerol synthesis machinery (e.g., ACSL3, GPAT4, DGAT2), lipolytic enzymes (e.g., ATGL, HSL, MGL), and scaffold proteins that regulate LD stability (e.g., the perilipin family of proteins: PLIN1–5). LDs are evolutionarily conserved and are present in nearly all cell types. However, the levels, morphology, and protein composition of LDs vary between cell types.

To understand LD functions and mechanisms of regulation, previous studies analyzed the proteomes of LD-enriched buoyant fractions isolated by centrifugation of cell lysates in a sucrose gradient [7, 8]. While this technique was successfully used to identify *bona fide* LD-associated proteins, it has several limitations. For example, fragments of organelles such as ER and mitochondria co-fractionate with LDs, which increases the potential for misidentification of ER/mitochondrial proteins as LD proteins. In addition, mechanical disruption of cells prior to biochemical fractionation may result in aberrant association of non-LD proteins with LDs. In general, the presence of contaminating proteins is a major drawback when using biochemically purified organelles to investigate organelle proteomes.

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This technical limitation was recently overcome through the development of proximity-labeling systems that label the organelle proteome in vivo, often bypassing the need for pure biochemical fractions [9–11]. A method employing an engineered ascorbate peroxidase (APEX2) targeted to the organelle of interest has been widely used to define organelle and sub-organelle proteomes in cultured cells [10, 12–20] and in model organisms [21, 22]. In the presence of hydrogen peroxide (H_2O_2), APEX2 catalyzes the conversion of biotin-phenol to a phenoxyl-radical that covalently reacts with residues, primarily tyro-sine [23], found in proximal proteins (within a 20 nm radius) over a short time period (<1 min). These biotin adducts can be affinity purified with streptavidin-coated beads and identified by shotgun mass spectrometry. This method allows for comprehensive and accurate identification of organelle proteomes.

We recently adapted the APEX2 labeling approach to define the composition of LD proteomes in U2OS osteosarcoma and Huh7 hepatoma cell lines [18]. Our results yielded high-confidence LD proteomes that contained known and novel LD proteins, but lacked contaminants identified in previous LD proteomics studies [18]. Here, we provide a protocol for the use of APEX2 proximity biotinylation to define high-confidence LD proteomes in any cellular system. In this approach, APEX2 is targeted to LDs by generating stable cell lines that express APEX2 genetically fused to LD-resident proteins. Cells expressing soluble, cytosolic APEX2 are analyzed in parallel to control for any proteins that are nonspecifically labeled and/or isolated during LD purification. The cells expressing the APEX2 fusion proteins are then treated with reagents to induce biotinylation of LD proteins. Buoyant fractions enriched in biotinylated LD proteins are isolated by sucrose gradient centrifugation, biotinylated proteins are affinity purified using streptavidin-coated beads, and LD proteins are identified using mass spectrometry.

2 Materials

2.1 APEX2 Fusion Protein Expression

1. Appropriate cell lines: Flp-In T-REx U2OS and tetracycline-inducible Huh7 were used in our published study [18]. Other cultured cells could be employed (e.g., HeLa, HEK293, mouse embryonic fibroblasts) (*see* Note 1).

2. Growth medium: Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS).

3. Mammalian expression plasmids for APEX2-fusion proteins: Expression plasmids for soluble, cytosolic APEX2 and LD-targeted APEX2 are required. For example, we previously generated plasmids encoding cytosolic V5-tagged APEX2 (V5-APEX2) and LD-targeted V5-APEX2 consisting of V5-APEX2 fused to the C-terminus of PLIN2 (PLIN2-V5-APEX2) or mutant ATGL (ATGL(S47A)-V5-APEX2) (*see* Note 2). Constructs were cloned into pcDNA5/FRT/TO (Thermo Fisher Scientific), which allows for homologous recombination into a cell line containing an FRT homologous recombination site when co-transfected with POG44 Flp-Recombinase plasmid (Thermo Fisher Scientific).

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4. Transfection reagent: Fugene6 or X-tremeGENE HP.
5. Doxycycline: A 1 mg/mL stock solution is prepared in double-distilled water (ddH_2O) and stored at −20 °C in the dark.
6. Puromycin dihydrochloride: A 1 mg/mL stock solution is prepared in ddH_2O and stored at 4 °C.
7. Hygromycin B: Sold as a 50 mg/mL stock solution and stored at 4 °C.

2.2 Induction of LD Protein Biotinylation

1. Oleate: A 200 mM oleate stock solution is prepared by resuspension in 100% ethanol and stored at −20 °C.
2. Fatty acid-free bovine serum albumin (BSA): A 10% fatty acid-free BSA stock solution is prepared in phosphate-buffered saline (PBS), sterile filtered, and stored at −20 °C.
3. 10× Oleate-BSA complex: Sterile filtered 10% fatty acid-free BSA in PBS is diluted in DMEM to a 1% final concentration. Oleate is added from the 200 mM stock to a 2 mM final concentration. The mixture is vortexed vigorously and immediately added to cells in growth medium at a 1:10 v/v ratio to achieve a final concentration of 200 μM oleate.
4. Biotin-phenol (also known as biotin-tyramide): A 250 mM stock solution is prepared in DMSO and stored at −80 °C in aliquots.
5. Hemin: A 483 μM stock solution is freshly prepared in 10 mM NaOH and used immediately.
6. 30% Hydrogen peroxide (H_2O_2): Store at 4 °C. A 1 M H_2O_2 working solution is prepared by diluting the 30% solution with ddH_2O immediately prior to use.
7. Quenching buffer: 10 mM Sodium ascorbate and 5 mM Tro-lox are prepared by resuspension in PBS by vigorous stirring at medium heat, followed by sterile filtration. Prepare immediately prior to use.

2.3 Lysis and Fractionation

1. 10× Pierce Protease Inhibitor stock: Resuspend one tablet of Pierce Protease Inhibitor Mini Tablets, EDTA free, in 1 mL of ddH_2O by vigorously vortexing. 1× Pierce Protease Inhibitor is diluted from 10× stock using the indicated buffer.
2. HLM buffer: 20 mM Tris–HCl pH 7.4, 1 mM EDTA.
3. 7 mL Glass Dounce Homogenizer with tight and loose-fitting pestles.
4. 13.2 mL Ultra-Clear tubes.
5. Tube slicer.
6. SW41 Ti Swinging-Bucket Rotor.

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7. 60% Sucrose in HLM buffer: Dilute 60 g of sucrose in 50 mL of HLM. Stir vigorously to dissolve, add HLM buffer to a final volume of 100 mL, and sterile filter. Store at 4 °C.

8. 5% Sucrose in HLM buffer: Dilute 60% sucrose/HLM stock with HLM. Prepare immediately prior to use.

2.4 Affinity Purification, Digestion of Biotinylated Proteins, and Mass Spectrometry

1. Streptavidin-coated agarose. Store at 4 °C.

2. PBST: Add 1 mL of Tween-20 to 1 L of PBS for a final concentration of 0.1% Tween-20. Mix vigorously and store at room temperature (RT).

3. Ammonium bicarbonate: 50 mM and 100 mM solutions are freshly prepared in ddH_2O.

4. RapiGest SF solution: Prepare 0.02% RapiGest SF Surfactant (Waters) in 50 mM ammonium bicarbonate (w/v) and use immediately.

5. Trypsin protease, MS grade: Resuspend 20 μg trypsin in 40 μL of 50 mM ammonium bicarbonate for the on-bead protein digestion method. Resuspend 20 μg trypsin in a solution containing 40 μL of 5 mM ammonium bicarbonate and 5% acetonitrile for the in-gel protein digestion method.

6. Biotin elution buffer: Resuspend biotin to a 3 mM final concentration in a 2% sodium dodecyl sulfate (SDS) solution prepared in ddH_2O.

7. 5× Laemmli buffer: 60 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% *β*-mercaptoethanol, and 0.01% bromophenol blue.

8. Gel-fixing buffer: 50% Ethanol and 10% acetic acid (v/v).

9. Gel-washing buffer: 50% Methanol and 10% acetic acid (v/v).

10. 50 mM Ammonium bicarbonate containing 50% acetonitrile: Mix 100 mM ammonium bicarbonate and 100% acetonitrile in a 1:1 (v/v) ratio.

11. Gel extraction buffer: Dilute formic acid to a final concentration of 5% (v/v) in 100% acetonitrile.

12. 500 mM Hydrochloric acid (HCl) stock: Dilute concentrated HCl with ddH_2O and store at RT in an acid safety cabinet.

13. MaxQuant proteomic software package [24, 25]: www.biochem.mpg.de/5111795/maxquant.

2.5 Immunoblotting

1. BSA: Prepare at a 5% final concentration (w/v) in PBST and use immediately.

2. Primary antibodies: Rabbit polyclonal anti-Plin2 antibody (Abgent), rabbit polyclonal anti-α-tubulin antibody (Cell Signaling Technology, Inc.), rabbit polyclonal anti-calnexin antibody (Proteintech Group Inc.), and mouse

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monoclonal anti-V5 tag antibody (Invitrogen). Antibodies are diluted 1:1000 in PBST containing 5% BSA for immunoblotting and stored at −20 °C for reuse.

3. Secondary antibodies: Anti-rabbit IRDye800-conjugated secondary (LI-COR Biosciences) and anti-mouse Alexa Fluor 680-conjugated secondary (Invitrogen). Antibodies are diluted 1:25,000 in PBST containing 5% BSA.
4. IRDye800-conjugated streptavidin (LI-COR Biosciences): IRDye800-conjugated streptavidin is diluted 1:5000 in PBST containing 5% BSA and stored at 4 °C in the dark for reuse.

2.6 Immunofluorescence Microscopy

1. BODIPY™ 558/568 C_{12} (Thermo Fisher Scientific): A 1 mM stock is prepared by resuspension in 100% ethanol and stored at −20 °C in the dark.
2. Streptavidin-Alexa Fluor-488 conjugate: Stored in aliquots at −20 °C in the dark.
3. Primary antibodies: Mouse monoclonal anti-V5 tag antibody is stored at 4 °C.
4. Goat anti-mouse Alexa Fluor™ 488 secondary antibody: Stored in aliquots at −20 °C in the dark.
5. 4% Paraformaldehyde: Prepare by diluting 16% paraformaldehyde 1:4 (v/v) in PBS. Store at 4 °C.
6. 6. 0.1% Triton X-100: Prepare by diluting 100% Triton X-100 in PBS containing 1% BSA. Store at RT.
7. 0.01% Digitonin: Freshly prepared in PBS containing 1% BSA. Use immediately after preparation.
8. Blocking solution: Freshly prepared 1% BSA in PBS (w/v).
9. Fluoromount G: Store at RT.

3 Methods

3.1 Generation of FRT Flp-In Cell Lines Stably Expressing Inducible APEX2 Fusion Proteins

1. Plate a Flp-In cell line of choice (HEK293, U2OS, HeLa) containing a stably integrated FRT homologous recombination site in DMEM/10% FBS (*see* Note 1). Allow cells to reach 50% confluency. Include an additional plate that will be used as an untransfected control.
2. Transfect cells with V5-APEX2 plasmids using an appropriate transfection reagent (e.g., Fugene6, or X-tremeGENE HP) according to the manufacturer's instructions. For the transfection, use a DNA mix consisting of V5-APEX2:POG44 at a 1:9 (w/w) ratio. Incubate for 24 h (cell culture growth incubations are at 37 °C with 5% CO_2 unless otherwise noted).
3. Replace growth medium after 24 h and incubate for an additional 24 h.

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4. Split cells into growth medium containing 100–500 µg hygromycin B (concentration depends on the cell type and must be determined empirically). Incubate until all cells in the untransfected control plate have died, replacing the growth medium with fresh growth medium containing hygromycin B every 3 days.
5. Once all control cells have died and colonies have formed in the transfected plate, replace growth medium with new growth medium containing 1/5 the original amount of hygromycin B. Allow the cells to recover and grow to confluency.

3.2 Characterization of APEX2 Fusion Protein Expression and Biotinylation by Immunoblotting

1. Plate cells in a 6-well plate and incubate with doxycycline for 48 h to induce APEX2 fusion protein expression. For the initial characterization of new cell lines, test a range of doxycycline concentrations (e.g., 0–100 ng/mL).
2. Treat cells with 7 µM hemin 24 h prior to harvesting.
3. To induce biotinylation, treat cells with 500 µM biotin-phenol for 30 min and add 1 mM hydrogen peroxide for 1 min.
4. Quench the biotinylation reaction by rapidly aspirating the media and washing cells with 1 mL of quenching buffer. Repeat the wash with quenching buffer.
5. Wash cells with 1 mL PBS once.
6. Harvest cells by scraping down in 0.5 mL of fresh PBS and transfer to a 1.5 mL microcentrifuge tube. Pellet cells by centrifugation at 500 × *g* for 5 min at 4 °C.
7. Remove PBS and resuspend the cell pellet in 1% SDS to lyse. Sonicate lysates at 15% power for 10 s and heat at 95 °C for 5 min. Allow lysates to cool to RT.
8. Employing standard approaches, measure the protein concentration of lysate, separate equal amounts of protein by SDS-PAGE, and transfer proteins to nitrocellulose.
9. Immunoblot using anti-V5 tag antibody (1:1000) to detect the APEX2 fusion proteins and anti-tubulin antibody (1:1000) as a loading control. Fluor-compatible secondary antibody (1:25,000) and IRDye800 streptavidin (1:5000) are added for a final 30-min incubation to detect V5/tubulin and biotinylated proteins. All blotting reagents are prepared in PBST/5% BSA.

3.3 Characterization of APEX2 Fusion Protein Expression and Biotinylation by Fluorescence Microscopy

1. Place sterile, circular coverslips into a 12-well tissue culture plate.
2. Plate cells and incubate with doxycycline for 48 h.
3. 24 h prior to fixation, treat cells with 200 µM oleate-BSA, 7 µM hemin, and 1 µM BODIPY™ 558/568 C_{12}.

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4. To induce biotinylation, treat cells with 500 μM biotin-phenol for 30 min and add 1 mM hydrogen peroxide for 1 min.
5. Quench the biotinylation reaction by rapidly aspirating the media and washing cells with 1 mL of quenching buffer. Wash once more with 1 mL quenching buffer.
6. Wash cells once with 1 mL PBS.
7. Fix cells by incubating in 1 mL of 4% paraformaldehyde in PBS for 15 min at RT in the dark.
8. Wash cells three times with 1 mL PBS.
9. To permeabilize cells, add 1 mL of PBS containing 0.1% Triton and 1% BSA. Alternatively, to better preserve the integrity of LDs, permeabilize using PBS containing 0.01% digitonin and 1% BSA. Incubate for 5 min at RT in the dark.
10. Wash cells three times with PBS containing 1% BSA.
11. Block cells for an additional 30 min in 1 mL of blocking solution at RT in the dark.
12. Remove coverslips from the tissue culture plate with tweezers and place on a parafilm sheet.
13. Overlay each coverslip with 150 μL of anti-V5 primary antibody (1:500 in blocking solution) and incubate for 1 h at RT in the dark.
14. Wash coverslips three times with blocking solution.
15. Overlay each coverslip with 150 μL of goat anti-mouse Alexa Fluor secondary antibody (1:500 in blocking solution) to stain V5-APEX2. Alternatively, overlay with 150 μL of Streptavidin-Alexa Fluor™ 488 (1:500 in blocking solution) to stain biotinylated proteins. Incubate for 1 h at RT in the dark.
16. Wash three times with blocking solution.
17. To mount coverslips, apply 10 μL of Fluoromount G to a glass slide while avoiding bubbles, remove excess blocking solution by dabbing the edge of the coverslips against a paper towel, and gently apply each coverslip cell side down on the slide. Allow slides to dry overnight at RT in the dark.
18. Image slides using a wide-field fluorescence microscope equipped with a FITC filter to visualize V5-APEX2/streptavidin and a Texas Red filter to visualize BODIPY-stained LDs.

3.4 Biotinylation of LD Proteins for Proteomics

1. Seed eighteen 15 cm plates of U2OS cells stably expressing LD-targeted or cytosolic APEX2 (*see* Note 3). If induction of LD-targeted APEX2 with doxycycline is required, seed cells at a density that allows for 48 h of growth in the presence of doxycycline. Allow cells to reach a confluency of 30–50% after induction.

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2. To induce formation of LDs (*see* Note 1), treat cells with 200 μM oleate-BSA complex 24 h prior to initiating the biotinylation reaction. Concurrently, treat cells with 7 μM hemin, which functions as a cofactor for the APEX2-catalyzed reaction.
3. Prior to inducing biotinylation, pretreat the cells with 500 μM biotin-phenol for 30 min.
4. To induce APEX2-catalyzed biotinylation, add hydrogen peroxide from the 1 M hydrogen peroxide stock to the media to achieve a final concentration of 1 mM, mix by gently swirling or tilting the plate, and incubate at RT for 1 min (*see* Note 4).
5. Rapidly aspirate the media and quench the reaction by adding 10 mL of quenching buffer. Aspirate the quenching buffer and wash each plate once more with 10 mL of quenching buffer.
6. Wash cells with 10 mL PBS.
7. Gently scrape cells in 3–5 mL of PBS per plate and centrifuge at 500 × *g* for 10 min to pellet the cells. Aspirate the PBS and transfer cell pellets to ice.

3.5 Cell Lysis and LD Fractionation

1. Resuspend the cell pellet in 4 mL of cold HLM buffer containing 1× cOmplete EDTA-free Protease Inhibitor Cocktail (*see* Note 5). Mix gently by pipetting and incubate on ice for 10 min.
2. Transfer resuspended cell pellets to a pre-chilled Dounce homogenizer and Dounce using 10–80 strokes (*see* Note 5).
3. Centrifuge lysates at 1000 × *g* for 10 min at 4 °C to pellet nuclei and unbroken cells. Transfer the supernatant to a 13.2 mL Ultra-Clear ultracentrifuge tube.
4. Dilute the supernatant to a final concentration of 20% sucrose-HLM using a 60% sucrose-HLM stock solution and mix gently by pipetting.
5. Overlay the lysate in 20% sucrose-HLM with 3.5 mL HLM buffer containing 5% sucrose by pipetting (*see* Note 6). Check that the combined volumes of the cell lysate in 60% sucrose-HLM and the solutions added in **steps 5** and **6** do not exceed the 13.2 mL capacity of the ultracentrifuge tube.
6. Overlay the 5% sucrose-HLM solution with 3.5 mL HLM by pipetting.
7. Transfer the tubes to tube holders for a SW41 swinging-bucket rotor (*see* Note 7). To separate the LD-enriched buoyant fraction, centrifuge the samples at 15,000 × *g* for 30 min at 4 °C.
8. Use a Beckman-Coulter tube slicer to collect the floating buoyant fraction (white layer on the surface). Position the blade to slice the tube 0.5–1 mL below the top of the liquid level. After cutting the tube, pipette the buoyant fraction up and down several times to mix, and transfer to a 1.5 mL micro-centrifuge tube on ice (*see* Note 8).

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9. Place the bottom portion of the sliced tube in a tube holder. Collect 1 mL fractions with a micropipette by pipetting from the top. Resuspend the pellet fraction in 1 mL of HLM buffer.

10. Dilute all fractions with a 20% SDS solution to a final concentration of 1% SDS. Vortex briefly to mix.

11. Sonicate each fraction for 15–30 s at 15% power using a tip sonicator and incubate at 65 °C for 10 min. To solubilize LDs, incubate the buoyant fraction at 37 °C for 1 h, sonicating for 15–30 s at 15% power every 20 min, followed by a final incubation at 65 °C for 10 min.

12. To assess the purity of the buoyant fraction and determine if biotinylated proteins are present, run all fractions on SDS-PAGE and immunoblot with Streptavidin-Alexa Fluor™ 488 and primary antibodies against known LD, cytosolic, and ER membrane proteins (*see* Note 9).

3.6 Isolation of Biotinylated Proteins from Buoyant Fractions

1. Dilute the buoyant fraction 1:10 (v/v) with PBST to a final concentration of 0.1% SDS.

2. Prepare 0.2 mL of streptavidin-conjugated agarose by washing0.4 mL of the bead slurry with 1 mL PBST (*see* Note 10). Pellet beads by centrifugation for 30 s at 2000 × *g* and discard the supernatant. Wash the beads with PBST two additional times.

3. Add the diluted buoyant fraction to beads in a 15 mL conical tube. Incubate at RT for 4 h with constant mixing on a tube rotator.

4. Wash beads five times with 1 mL PBST followed by three washes with 1 mL PBS.

5. Proceed to preparation of biotinylated peptides using on-bead or in-gel trypsin digestion (*see* Note 11).

3.7 On-Bead Digestion of Biotinylated Proteins

1. Wash beads three times with 1 mL of freshly prepared 50 mM ammonium bicarbonate.

2. Resuspend beads in one bead volume of 0.02% RapiGest SF (w/v) in 50 mM ammonium bicarbonate. Mix using a cut 200 μL pipette tip to prevent mechanical disruption of the beads.

3. Incubate beads in RapiGest SF at 65 °C for 15 min. Allow the mixture to cool to RT.

4. Add 1 μg of mass spectrometry-grade trypsin resuspended in 50 mM ammonium bicarbonate and pipette to mix. Cover tube with parafilm to prevent evaporation and incubate for 16–24 h at 37 °C.

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5. Pellet the beads by centrifugation for 30 s at 2000 × *g* and transfer the supernatant containing peptides in RapiGest SF to a new tube with a gel-loading tip.
6. To precipitate the RapiGest SF, acidify the samples to a pH 2 with 500 mM HCl (*see* Note 12). Incubate for 45 min at RT.
7. To remove precipitated RapiGest SF, spin sample at 20,000 × *g* for 15 min at RT. Transfer the supernatant to a new tube, avoiding transfer of any pelleted material at the bottom. Perform this procedure again to remove any residual RapiGest SF.
8. Dry down peptides to a final volume of 15–20 μL in a vacuum centrifuge (*see* Note 13).

3.8 In-Gel Digestion of Biotinylated Proteins

1. Wash beads five times with 1 mL PBST and three times with 1 mL PBS.
2. Elute biotinylated proteins by mixing beads with 1 bead volume of biotin elution buffer (2% SDS + 3 mM biotin) using a cut 200 μL pipette tip. Incubate at RT for 15 min followed by an incubation at 95 °C for 15 min. Pellet the beads by centrifugation for 30 s at 2000 × *g* and transfer the supernatant containing the eluted proteins to a new tube.
3. Mix the eluted proteins with 5× Laemmli buffer 4:1 (v/v) and run the protein sample 2–3 cm into a polyacrylamide gel using SDS-PAGE.
4. Wash gel three times with ddH_2O using enough volume to cover the gel.
5. Add gel-fixing buffer and incubate gel at RT while gently shaking for 1 h.
6. Remove gel-fixing buffer and add gel-washing buffer to gel. Incubate at RT while gently shaking for 30 min.
7. Wash gel three times with ddH_2O.
8. Excise gel pieces containing biotinylated peptides and transfer to a 1.5 mL microcentrifuge tube (*see* Note 14).
9. Overlay the gel pieces with a solution (~300 μL) containing 50 mM ammonium bicarbonate and 50% acetonitrile. Incubate at RT for 40 min, briefly vortexing every 5–10 min, and remove the solution.
10. To dehydrate, overlay the gel pieces with 100% acetonitrile. Incubate at RT with occasional vortexing for 30–60 min or until the pieces turn white. Remove the acetonitrile solution.
11. To digest proteins, add 0.5 μg of mass spectrometry-grade trypsin in 5 mM ammonium bicarbonate containing 5% acetonitrile to the gel pieces, wrap the tube in parafilm, and incubate for 16–24 h at 37 °C.
12. Extract peptides by incubating gel pieces in 5% formic acid in acetonitrile for 15 min at 37 °C with constant shaking. Transfer supernatant to a new tube.

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13. Dry down peptides to a final volume of 15–20 μL in a vacuum centrifuge.

3.9 Analysis of Biotinylated Proteins by Mass Spectrometry

1. Run the maximum allowable amount (typically 1 μg) of digested peptides on a Thermo Scientific Q Exactive Orbitrap Mass spectrometer or equivalent (*see* Note 15).

2. We typically analyze our peptides at a proteomics core facility that employs the following setup: Digested peptides are desalted using a 100 × μm 25 mm Magic C18 100-Å 5U reverse-phase trap and then separated using a 75 μm × 150 mm Magic C18 200-Å 3U reverse-phase column. Peptides are eluted using a 300 nL/min flow rate and MS/MS spectra acquired using a top 15 method. Additional parameters include an isolation mass window of 1.6 *m/z* for precursor ion selection and normalized collection energy of 27% for fragmentation.

3. Analyze .RAW files in MaxQuant [24, 25] or an equivalent proteomics software, using the reviewed human protein database from UniProt. Set variable modifications to include protein N-terminal acetylation and methionine oxidation and set the false discovery rate to 1%.

4. Compare MS/MS counts or label free quantification scores of samples from cells expressing LD-targeted APEX2 with the samples from cells expressing the cytosolic APEX2 control (*see* Notes 16 and 17).

4 Notes

1. Since this protocol requires isolation of LDs, a cell model that either contains abundant LDs at basal state or forms abundant LDs after treatment with fatty acids (e.g., oleate) should be chosen. Cells with low levels of LDs can be used, but this may require culturing large numbers of cells to obtain sufficient amounts of LDs for proteomics. Therefore, LD content should first be investigated in the cell line of interest in order to select the appropriate model system. Use of cell lines in which expression of APEX2 can be induced (e.g., cells that express a tetracycline-inducible system) will limit potential undesirable consequences of constitutive APEX2 expression.

2. APEX2 was fused to PLIN2 and ATGL(S47A) because their localizations are restricted to LDs. Since ATGL is the rate-limiting enzyme in lipolysis, the S47A inactive mutant of ATGL was used to avoid excessive lipolytic degradation of LDs caused by ATGL expression. Overexpression of any LD-associated protein can lead to changes in LD function, and saturation of the LD surface by overexpressed proteins may cause changes in LD proteome composition and/or result in aberrant localization of LD-targeted APEX2 to other cellular compartments. Thus, the effects of overexpression on LDs should be investigated in the cell line of interest by Western blotting against known LD proteins to detect potential global changes in LD protein levels, and by immunofluorescence microscopy to identify changes in LD size or morphology. If possible, expression

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levels should be tuned to restrict localization of LD-targeted APEX2 to LDs by using an inducible expression system.

3. The number of plates is dependent on the capacity of the cell type to form LDs. Based on our experience, 10–20 confluent plates are required to isolate a sufficient amount of LDs from oleate-treated U2OS cells, while adipocyte lines may require five plates or fewer. If many plates are required, cells can be cultured in 245 mm × 245 mm square plates to facilitate incubator storage and cell collection.

4. The length of incubation in hydrogen peroxide must be determined empirically and depends on the expression level and proper localization of LD-targeted APEX2. Long incubation times may increase background labeling, while short incubation times may cause incomplete labeling of the LD proteome. If many plates are required, it is recommended to process a few plates at a time to ensure similar labeling times between plates.

5. Use a 1 mL Dounce homogenizer for sample volumes 2 mL to perform two separate homogenizations per sample. Otherwise, use a 7 mL Dounce homogenizer for larger volumes. We typically use 1 mL HLM per 5 × 15 cm plates of U2OS cells. Exceeding 4 mL HLM per sample will interfere with downstream centrifugation steps. For most cells (e.g., U2OS), homogenization should be performed using 10–80 fast strokes and a tight-fitting pestle. However, for cells such as adipocytes that contain large LDs, 10–15 strokes using a loose-fitting pestle is sufficient to lyse the cells and is also necessary to prevent mechanical disruption of LDs.

6. To overlay the sucrose gradient without disturbing the previous layer, it is best to use a pipette-aid set to the slowest speed, allowing the user to dispense the next solution drop by drop. A good strategy is to drip the solution against the wall of the tube, keeping the pipette in close proximity to the liquid surface. Extra care should be taken when pipetting the final HLM layer on top of the 5% sucrose-HLM layer. The volume of each layer can be varied but is limited by the total volume of the ultracentrifuge tube (13.2 mL).

7. The SW41 rotor, tube holders, and ultracentrifuge should be pre-chilled to 4 °C prior to centrifugation. After loading tubes into tube holders, balance the samples to within 0.1 g by adding the appropriate volume of HLM buffer. Exercise care not to disturb the samples when loading the rotor and after centrifugation.

8. A tube slicer is not absolutely required for this step, but it significantly simplifies the procedure. Pipetting the buoyant fraction is not recommended. We refer the reader to this source for an additional LD isolation technique that does not require a tube slicer [26]. Prior to processing samples, perform the slicing procedure on tubes filled with known volumes of HLM. Use these tubes to test the tube slicer setup for leaks and to determine how the slicing position should be adjusted to achieve the desired volume of the buoyant fraction in HLM. If tubes are filled to the top with HLM, the cutting step may cause buffer to spurt from

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the tube during cutting. These filled tubes should be covered with parafilm prior to cutting to collect any material ejected from the buoyant fraction.

9. IRDye800-conjugated streptavidin is used to detect biotinylated proteins in the buoyant fraction. Examples of abundant LD, cytosolic, and ER proteins that can be analyzed using immunoblotting are PLIN2, tubulin, and calnexin, respectively. It is expected that significant levels of biotinylated proteins will also be present in cytosolic and pellet fractions since APEX2 targeted to the LD surface has access to the cytosol and may be in proximity to ER. However, the majority of the proteins labeled in the cytosol and ER are separated from the buoyant fraction during sucrose gradient fractionation, greatly reducing background signal. Any background labeling that remains can be excluded by analyzing proteins in the buoyant fraction that are labeled by cytosolic APEX2.

10. The binding capacity of streptavidin-conjugated beads is large relative to the levels of biotinylated proteins in buoyant fractions. The volume of bead slurry should be minimized due to the potential generation of streptavidin peptides by trypsin during on-bead digestion, which can interfere with MS analysis. To determine the minimal bead amount required, perform affinity purifications using a test sample of biotinylated proteins and varying volumes of beads. Analyze binding efficiency by immunoblotting input, streptavidin-purified, and flow-through fractions with IRDye800-conjugated streptavidin.

11. Contamination of the biotinylated protein sample with streptavidin must be minimized for downstream mass spectrometry analysis. On-bead digestion is efficient, but may result in contamination of samples with streptavidin peptides. In-gel protein digestion avoids this issue but requires careful elution of streptavidin-bound proteins without disruption of the tetrameric streptavidin complex, which is only partially covalently conjugated to beads. In practice, on-bead digestion can be optimized by determining the minimal volume of beads required to bind biotinylated proteins, thus minimizing contamination of the samples by streptavidin-derived peptides (*see* Note 10). If detection of the biotinylated residue within peptides is desired, the in-gel method should be used because it includes an elution step. Otherwise, the biotinylated peptide may remain associated with bead-conjugated streptavidin.

12. Typically, 12–15 μL of 500 mM HCl is required to acidify every 100 μL of RapiGest. Add HCl in increments, mix well, and then test the pH of the solution by transferring ~0.2 μL to colorimetric pH strips.

13. Medium heat can be used to accelerate the drying procedure, but it is best to dry without heat under high vacuum.

14. If sample volume exceeds the well volume of the gel, samples can be run in multiple wells. Staining with Coomassie solution prior to excision is unnecessary, as the concentration of eluted peptides is very small. The excision boundaries can be set by cutting each lane from the top of the well to the bottom of the dye front.

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15. Excessive levels of streptavidin or trypsin peptides will show up as large peaks on the mass chromatogram that could complicate analysis. If this is the case, optimize the sample preparation to decrease the bead volume used in the streptavidin affinity purification or the amount of trypsin used during digestion. Alternatively, consider using the in-gel digestion method.

16. This protocol can be adapted to use quantitative proteomics methods, such as stable isotope labeling using amino acids in culture (SILAC) [18] or tandem mass tags (TMT). In practice, we have observed that the background labeling in buoyant fractions from cells expressing cytosolic APEX2 is relatively low when compared to LD-targeted APEX2, suggesting that semiquantitative methods using MS/MS counts are sufficient. However, high background labeling would warrant the use of quantitative methods to avoid identification of false-positive LD proteins. Common false positives labeled in the buoyant fraction that would likely indicate problems in the procedure include ER proteins such as BiP, grp94, Sec61, PDI, and calnexin.

17. Sample .RAW mass spectrometry files from APEX2 proximity labeling experiments can be found on the PRoteomics IDEntifications (PRIDE) database (accession #: PXD007695).

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