Video Article

Lipid Droplet Isolation for Quantitative Mass Spectrometry Analysis

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Abstract

Lipid droplets are vital to the replication of a variety of different pathogens, most prominently the Hepatitis C Virus (HCV), as the putative site of virion morphogenesis. Quantitative lipid droplet proteome analysis can be used to identify proteins that localize to or are displaced from lipid droplets under conditions such as virus infections. Here, we describe a protocol that has been successfully used to characterize the changes in the lipid droplet proteome following infection with HCV. We use Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) and thus label the complete proteome of one population of cells with "heavy" amino acids to quantitate the proteins by mass spectrometry. For lipid droplet isolation, the two cell populations (*i.e.* HCV-infected/"light" amino acids and uninfected control/"heavy" amino acids) are mixed 1:1 and lysed mechanically in hypotonic buffer. After removing the nuclei and cell debris by low speed centrifugation, lipid droplet-associated proteins are enriched by two subsequent ultracentrifugation steps followed by three washing steps in isotonic buffer. The purity of the lipid droplet fractions is analyzed by western blotting with antibodies recognizing different subcellular compartments. Lipid droplet-associated proteins are then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining. After tryptic digest, the peptides are quantified by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Using this method, we identified proteins recruited to lipid droplets upon HCV infection that might represent pro- or antiviral host factors. Our method can be applied to a variety of different cells and culture conditions, such as infection with pathogens, environmental stress, or drug treatment.

Video Link

The video component of this article can be found at https://www.jove.com/video/55585/

Introduction

Lipid droplets are highly dynamic cytoplasmic (and nuclear) cell organelles composed of a core of neutral lipids (triglycerides (TG) and cholesterol ester (CE)) enclosed by a monolayer of phospholipids with embedded proteins[1]. All cell types produce lipid droplets, but they vary in size, lipid composition, and protein decoration. Lipid droplets fulfill diverse functions, including serving as energy and membrane precursor reservoirs or as protein deposits. In addition, through the uptake of lipids, they protect cells from lipotoxicity, release lipids as signaling molecules, and are involved in protein degradation and endoplasmic reticulum (ER) stress responses[2]. As such, a host of proteins bind to lipid droplets and govern their generation, degradation, trafficking, and interaction with other organelles. Among them are the perilipin family of *bona fide* lipid droplet binding proteins (PLIN1-5)[3].

Lipid droplet biogenesis likely starts at the ER, where ER-resident enzymes catalyze the synthesis of neutral lipids that accumulate within the membrane bilayer, forming a lens of neutral lipids, a process that was recently visualized nicely in yeast[4]. Membrane bending and elevated phosphatidic acid and diacylglycerol levels are then thought to attract proteins involved in phospholipid biosynthesis, as the simultaneous synthesis of the core neutral lipids and the shielding phospholipids is required for lipid droplet generation[5]. Enzymes harboring transmembrane domains that reside at the ER catalyze this process. Expansion to large lipid droplets requires the activity of a different class of lipid-synthesizing enzymes that harbor an amphipathic helix and can thus travel from the ER to lipid droplets. The mobilization of lipids from lipid droplets occurs through the local activation of the triglyceride and diacylglycerol lipases adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) or by different autophagic pathways, such as macro- and microlipophagy or chaperone-mediated autophagy[6]. Lipid droplets interact with other cellular organelles, such as mitochondria (for beta-oxidation and lipid synthesis) and ER (for lipid synthesis and protein trafficking), but also with lysosomes, endosomes, and the vacuoles induced by intracellular bacteria[7]. Indeed bacteria, viruses, and even parasites target lipid droplets for replication and persistence, among them HCV[8].

HCV infection is one of the leading causes of liver-related morbidity and mortality worldwide, accounting for approximately 0.5 million deaths per year[9]. The true number of HCV infections is unknown, but recent estimates suggest that 130 - 150 million people are chronically infected. No vaccine exists, but the recently approved direct-acting antivirals dramatically increase therapeutic responses compared to the standard interferon-based therapy. However, worldwide, the treatment of patients will likely be restricted due to the extremely high costs of the new therapeutics. About half of all individuals chronically infected with HCV develop fatty liver disease (steatosis), a condition characterized by the

excessive accumulation of lipid droplets in hepatocytes. Intriguingly, lipid droplets also emerged as vital cellular organelles for HCV replication, putatively serving as viral assembly sites[10,11].

In HCV-infected cells, the viral protein core and NS5A localize to lipid droplets in a process that depends on triglyceride biosynthesis, as inhibitors of diacylglycerol acyltransferase-1 (DGAT1) impair trafficking to lipid droplets and subsequent HCV particle production[12,13,14,15]. In addition, mutations in the lipid droplet-binding domains of either core or NS5A suppress HCV assembly[16,17]. Core and NS5A then recruit all other viral proteins, as well as viral RNA replication complexes, to membranes closely associated with lipid droplets[16]. A concerted action of all viral proteins is required for the successful production of infectious viral progeny[10,11]. The structural proteins are part of the virions, and the nonstructural proteins promote the protein-protein interactions required for this process. Intriguingly, the *bona fide* lipid droplet-binding protein PLIN3/TIP47 is required for both HCV RNA replication and the release of virions[18,19,20]. Despite these recent advances, the mechanistic details, especially of virus-host interactions during the late stages of HCV replication, remain ill-defined, and the precise function of the lipid droplets is unknown.

Here, we describe a method to isolate lipid droplets for the quantitative mass spectrometry of associated proteins. Using this method, we found profound changes in the lipid droplet proteome during HCV infection and identified annexin A3 as a host protein that co-fractionates with lipid droplets and is required for efficient HCV maturation[21].

Protocol

1. Preparation of Media for Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)

NOTE: Here, the SILAC Protein Quantitation Kit - DMEM supplemented with 50 mg of $^{13}C_6$ L-Arginine-HCl was used for SILAC labeling. The dialyzed Fetal Calf Serum (FCS) is provided with the SILAC Protein Quantitation Kit.

1. Remove 50 mL from each bottle of DMEM medium and add 50 mL of dialyzed FCS.
2. Dissolve 50 mg of $^{13}C_6$ L-Lysine-2HCl and 50 mg of $^{13}C_6$ L-Arginine-HCl in 1 mL of medium. Mix thoroughly and add the amino acids to the DMEM + FCS medium.
3. Add 1x Pen/Strep and 1x L-glutamine substitute. Sterile-filter the medium using a 0.45 µm filter. Label the bottle as "heavy" SILAC medium.
4. To prepare the "light" medium, repeat steps 1.1 - 1.3 using 50 mg of L-Arginine-HCl and 50 mg of L-Lysine-2HCl. Label the bottle as "light" SILAC medium.

2. SILAC-labeling and Amino Acid Incorporation Control

1. Trypsinize cells (1x Trypsin-EDTA) and split 1 x 10^5 Huh7.5 cells into 2 wells of a 6-well culture plate containing 2 mL of medium, one well with the "heavy" SILAC medium and one with the "light" SILAC medium.
2. Culture the cells for at least 6 passages (split ratio: 1:6); after 6 passages, the incorporation of the "heavy" amino acids should be more than 95%.
3. Harvest 1 x 10^6 cells of the "heavy"- and "light"-labeled cell population to analyze the incorporation efficacy. Wash the cells in 1x PBS and pellet the cells by centrifugation for 5 min at 160 x g and 4 °C.
4. Resuspend the cell pellets in 150 µL of MS-buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, and 1 mM EDTA supplemented with 1x protease inhibitor cocktail) and incubate the cells on ice for 30 min. Lyse the cells by sonication at 4 °C.
   NOTE: The following are the sonication settings used here: timer, hold; output control, 8; duty cycle, 80%; and 2 x 30 pulses.
5. Remove the cell debris by centrifugation for 10 min at 11,000 x g and 4 °C. Transfer the supernatant to a new tube and determine the protein concentration with a detergent-compatible protein assay.
6. Mix 75 µg of protein with 6x sample buffer (375 mM Tris-HCl, pH 6.8, 25.8% glycerol, 123 mg/mL SDS, 600 µg/mL bromophenol blue, and 60 µL/mL β-mercaptoethanol), boil at 95 °C for 5 min, and separate the proteins by SDS-PAGE in SDS running buffer (3.02 g/L Tris base, 18.8 g/L glycine, and 1 g/L SDS) at 180 V for approximately 1 h or according to the manufacturers' instructions.
7. Transfer the gel into colloidal Coomassie staining solution. Excise the same protein band from each lane. Digest the proteins with trypsin and analyze the incorporation efficacy by MS analysis, as described[22].

3. Lipid Droplet Isolation of SILAC-labeled Huh7.5 Cells

1. Infect one population of cells (*i.e.* those labeled with "light" amino acids) with an HCV reporter virus by incubating with virus stocks (*e.g.,* $Jc1^{NS5AB\text{-}mKO2\text{-}BSD}$, MOI 1) for 4 h at 37 °C, as described[21].
   NOTE: $Jc1^{NS5AB\text{-}mKO2\text{-}BSD}$ is an HCV virus carrying a fluorescence reporter (monomeric Kusabira Orange 2, mKO2) to monitor infection rates followed by a Blasticidin Resistance Gene (BSD) between a duplicated NS5A-NS5B cleavage site, described previously[21,23]. Work with HCV requires BSL2+ (USA) or S3** (Germany) biosafety-level containment and practices. Instead of infection with HCV, cells can be infected with different pathogens.
2. Expand HCV-infected "light" cells and noninfected "heavy" cells. During the passaging of the cultures, fix an aliquot with 4% paraformaldehyde (PFA) in PBS and determine the HCV infection rates by flow cytometry of the fluorescent marker protein (*e.g.,* mKO2), as described[21]; the infection rates should be higher than 90% for lipid droplet isolation. NOTE: If using the $Jc1^{NS5AB\text{-}mKO2\text{-}BSD}$ HCV strain, add 10 µg/mL blasticidin S to the "light" medium to select for HCV-positive cells.
3. 1 d prior to lipid droplet isolation, wash the cells with PBS, trypsinize, resuspend in "light" and "heavy" medium, and count the cells using a Neubauer counting chamber. Seed 7 x 10^6 cells of each population in a 150 cm^2 cell culture dish. Prepare at least 5 dishes per "light" and "heavy" cell population. Culture the cells in 30 mL of medium/dish O/N.
4. Remove the medium and wash the cells in 1x PBS. Detach the cells in 1x PBS using a cell scraper.

5. Count both cell populations using a Neubauer counting chamber and pool equal cell numbers in a 50 mL centrifuge tube. Pellet the cells by centrifugation for 5 min at 160 x g and 4 °C.
6. Remove the PBS and resuspend the cell pellet in 1 mL of sucrose buffer (0.25 M sucrose, 1 mM EDTA, and 1 mM DTT, supplemented with protease inhibitor cocktail). Transfer the cell suspension to a tight-fitting Dounce homogenizer and lyse the cells with 200 strokes on ice.
NOTE: Confirm complete cell lysis using trypan blue staining.
7. Transfer the lysate into a 1.5 mL microfuge tube and spin down the nuclei and cell debris for 10 min at 1,000 x g and 4 °C.
8. After centrifugation, store an aliquot of 25 µL of the post-nuclear fraction (PNS) at -20 °C as an input control.
9. Place the rest of the PNS fraction at the bottom of centrifuge tube (11 x 60 mm) and overlay with lipid droplet wash buffer (~3 mL; 4 mL total) (50 mM potassium phosphate buffer pH 7.4, 100 mM potassium chloride, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride).
10. Centrifuge for 2 h at 100,000 x g and 4 °C. Harvest the floating lipid droplet fraction using a bent, blunt cannula from the top of the tube (approximately 250 - 500 µL, depending on the amount of lipid droplets). Place the lipid droplet fraction in a centrifuge tube (11 x 60 mm), overlay with lipid droplet wash buffer (~3.5 mL; 4 mL total), and repeat the centrifugation step.
11. Harvest the floating lipid droplet fraction using a bent, blunt cannula from the top of the tube and transfer the lipid droplets to a new 1.5 mL microfuge tube. Add 500 µL of lipid droplet wash buffer and spin for 20 min at 21,000 x g and 4 °C.
NOTE: Lipid droplets appear as a white band floating on top of the buffer.
12. Remove the subjacent wash buffer using a gel loading pipette tip and repeat this washing step three times.
13. After the final removal of the washing buffer using a gel loading pipette tip, mix 5 µL of lipid droplet fractions with 10 µL of NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1% NP-40, supplemented with protease inhibitor cocktail). Incubate the sample on ice for 1 h to inactivate the virus if working with infectious material. Determine the protein level with a detergent-compatible protein assay; at least 35 µg of protein is needed for MS-analysis.
14. Store the lipid droplet fractions at -20 °C.
15. Mix the corresponding volume of the lipid droplet fraction with 4x loading dye. Incubate on ice for 1 h to inactivate the virus if working with infectious material. Boil at 95 °C for 5 min.
16. Separate the proteins using SDS-PAGE according to the manufacturers' protocol. Transfer the gel into colloidal Coomassie staining solution. Excise all the protein bands from the gel. After tryptic in-gel digestion[24], evaporate the samples and dissolve them in 0.1% formic acid. Analyze by LC-MS/MS.
NOTE: Here, LC-MS/MS analyses were performed on a Quadrupole-Time-of-Flight mass spectrometer (Q-TOF) or on a Linear Trap Quadrupole (LTQ) orbitrap mass spectrometer. Both instruments were coupled with an ESI-source to a nano-UPLC system. Data analyses and LC-MS/MS analyses on the Q-TOF and on the orbitrap mass spectrometer were performed as described[21,22]. Take great care not to contaminate the sample with human keratin. Always use keratin-free pipette tips and tubes. Prepare buffers under the laminar flow hood with keratin-free chemicals. Before usage, clean all surfaces and devices with distilled water and ethanol. Always wear gloves and a lab coat.

4. Analysis of Lipid Droplet Purity

1. Dilute aliquots of lipid droplet fractions 1:2 and input fractions (see step 3.9) 1:10 with NP-40 lysis buffer. Incubate the samples on ice for 1 h to inactivate the virus if working with infectious material. Determine the protein level with a detergent-compatible protein assay.
2. Mix equal amounts of protein with 6x sample buffer and incubate the samples on ice for 1 h to inactivate the virus if working with infectious material. Proceed with SDS-PAGE and transfer the proteins onto a nitrocellulose membrane by tank blotting at 80 V for 90 min.
NOTE: After blocking the membrane in blocking buffer (5% nonfat dried milk powder in TBS-T (10 mM Tris-HCl pH 7.6, 150 mM NaCl, and 0.05% Tween20)), incubate with antibodies directed against lipid droplet markers (*e.g.,* PLIN1, PLIN2, or PLIN3) and markers of other subcellular compartments (*e.g.,* CALR, MnSOD, or tubulin), followed by secondary HRP-coupled antibodies. Use chemiluminescence for the detection of proteins.

Representative Results

Lipid droplets are vital to HCV infection as the putative sites of virion assembly, but the molecular mechanisms of morphogenesis and egress of virions are largely unknown. To identify novel host dependency factors involved in that process, we performed quantitative lipid droplet proteome analysis of HCV-infected cells[21] (**Figure 1A**). We established a protocol for purifying lipid droplets and routinely detected a strong enrichment of the lipid droplet-binding proteins PLIN2/ADRP and PLIN3/TIP47 and a depletion of markers of other cellular compartments, such as β-tubulin for microtubules, MnSOD for mitochondria, or calreticulin/calnexin for the ER (**Figure 1B, C**). We compiled a list of proteins that reliably cofractionate with lipid droplets in Huh7.5 cells (**Figure 1D**) and, using isotope labeling, identified proteins that are specifically recruited to or displaced from lipid droplets in HCV-infected cells (**Figure 1E**). Our results indicate that HCV disconnects lipid droplets from their regular metabolic function and/or regulation and identify putative host dependency and restriction factors.

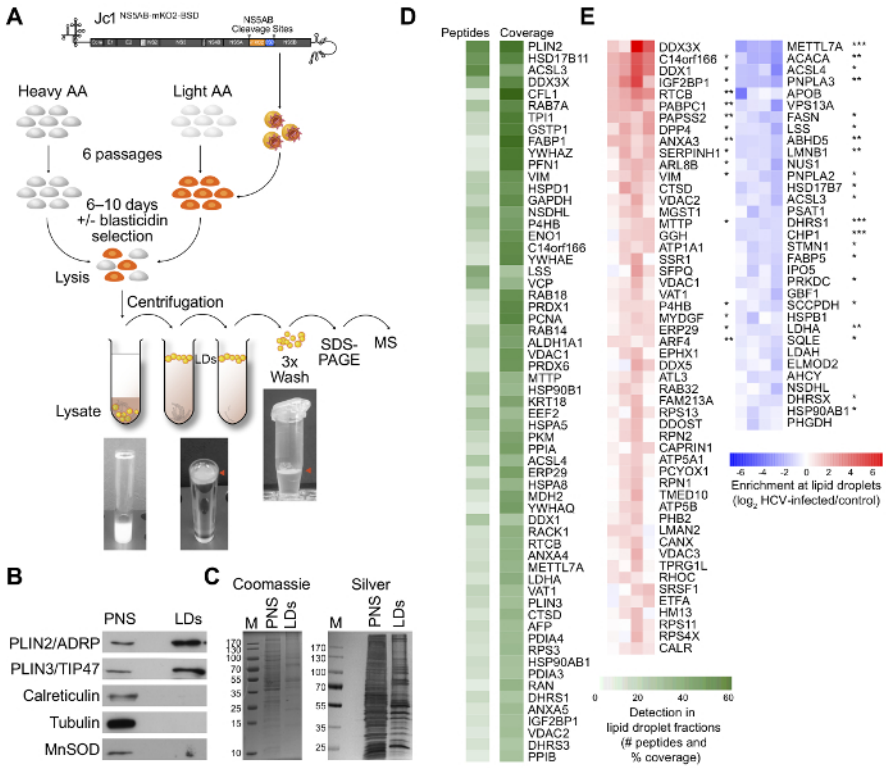

Figure 1: (A) Scheme of the Experiment. Naïve Huh7.5 cells were labeled with "heavy" amino acids or "light" amino acids. Cells carrying "light" amino acids were infected with an HCV reporter virus (Jc1$^{NS5AB\text{-}mKO2\text{-}BSD}$). "Light" and "heavy" amino acid-labeled populations were mixed, and lipid droplets were isolated by two subsequent ultracentrifugation and three washing steps, separated by SDS-PAGE, and analyzed by LC-ESI-MS/MS. Note the floating white lipid droplet fraction (red arrow) after ultracentrifugation and washing in microfuge tubes. **(B)** Western blot analysis of post-nuclear and lipid droplet fractions shows an enrichment of lipid droplet marker proteins in lipid droplet fractions and a depletion of markers of other cellular compartments. **(C)** Coomassie blue and silver staining of post-nuclear supernatant (PNS) and lipid droplet fractions separated by SDS-PAGE. Silver staining is presented for visualization only. **(D)** Heatmap of the number of peptides and percentage of protein coverage of proteins identified in lipid droplet fractions of Huh7.5 cells (cutoff: ≥5 peptides and ≥20% coverage). **(E)** Heatmap depicting enriched or depleted lipid droplet-associated proteins after infection with HCV (normalized to the median, 1.5-fold cutoff, *p < 0.05, **p < 0.01, ***p < 0.001). Modified from [21]. Please click here to view a larger version of this figure.

Discussion

Here, we describe a protocol to isolate lipid droplets for quantitative lipid droplet proteome analysis to compare the enrichment and depletion of proteins associated with lipid droplets under diverse culture conditions, such as viral infections. As an alternative method, the proteome analysis can be performed with label-free quantifications based on total peak intensities. This method has no dynamic range limitation and avoids metabolic problems. The advantage of the SILAC approach is that the samples are pooled prior lipid droplet isolation, and therefore the results are independent of errors in sample preparation, digest, and LC-MS/MS analysis. We highly recommend this approach for quantitative lipid droplet proteome analysis.

As the enrichment or depletion of lipid droplet-associated proteins observed in the MS analysis could reflect an induction or repression of protein expression, the analysis of expression levels by quantitative RT-PCR and (preferably) western blotting is advised. In addition, two methods can be used to verify the enrichment or depletion of specific proteins in lipid droplets: lipid droplet isolation followed by western blotting and immunofluorescence analysis with lipophilic dyes, like BODIPY or LD540, that stain lipid droplets as described[21].

Perform experiments with swapped labeling conditions to ensure that the labeling with "heavy" amino acids does not influence protein expression or lipid droplet localization and to identify protein contaminants from the "light" medium and environmental sources. For protein identification, the search should be performed with a false discovery rate (FDR) of 0.01 on both the peptide and protein level. To ensure high confidence results, we advise performing at least 3 - 4 independent experiments, with cells from different passages and different virus stock preparations. Depending on the magnitude of change, more independent experiments might be required.

For normalizing the quantitative MS data to correct for slightly different cell numbers or lipid droplets, center the detection ratios of the "light" over the "heavy" peptides, or vice versa, in swapped labeling conditions by dividing through the median of all identified proteins, as described[25]. If the amount of lipid droplets differs significantly between the samples, normalizing to lipid droplet marker proteins, such as PLIN2, might be advisable. When we normalize our MS data to PLIN2 levels, we find similar results as when we normalize to the median (analysis not shown). Of note, under the cell culture conditions we use, we do not detect significant lipid droplet accumulation upon HCV infection.

Lipid droplets are in close contact with other cellular organelles, most notably mitochondria and the ER. Therefore, proteins from these compartments can co-fractionate with lipid droplets during isolation. If such a "contaminant" protein is unaltered in abundance in response

to infection or treatment, it will not affect the comparative lipid droplet proteome analysis. It must be noted, however, that under some circumstances, the contact between lipid droplets and other organelles might be altered. For example, under lipolytic conditions, mitochondrial proteins are detected at higher frequencies in lipid droplet fractions from lipolytically stimulated 3T3-L1 adipocytes compared to basal conditions[26]. Stimulated *de novo* lipogenesis, on the other hand, might lead to enhanced association with the ER. These changes then reflect changes in organelle interaction induced by the various stimuli and might be interesting, even if not reflective of pure lipid droplet localization.

We used this protocol for an extensive quantitative lipid droplet proteome analysis of HCV-infected, versus uninfected control cells to reveal the perturbations caused by HCV infection and to identify regulators of HCV replication[21]. In the hepatoma cell line Huh7.5, we routinely identified up to 2,900 proteins within lipid droplet fractions, with ~300 proteins identified with multiple peptides in each experiment. Following infection with HCV, we observed the both recruitment and depletion of host proteins. Several proteins identified as highly enriched at lipid droplets of HCV-infected cells have been previously published as HCV host factors (*e.g.,* DEAD box proteins 1 and 3 (DDX1, DDX3) or insulin-like growth factor-II mRNA-binding protein 1 (IGF2BP1)), indicating the reliability of the SILAC approach[27,28,29]. In addition, recruited proteins are often annotated for RNA-binding proteins, highlighting the tight association of the viral RNA replication complexes with lipid droplet fractions. In contrast, proteins depleted from lipid droplets were mainly annotated for lipid metabolic processes, indicating that HCV perturbs the protein composition to disconnect the lipid droplets from their normal metabolic regulation and function.

The protocol we describe is amendable to different cell lines and culture conditions and could help in deciphering the function of lipid droplets in the life cycle of different pathogens that depend on lipid droplets for replication.

Disclosures

The authors have nothing to disclose.

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