CHAPTER

Analysis of Lipid Droplets in Hepatocytes

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Huajin Wang*, Ariel D. Quiroga†, and Richard Lehner‡

*Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut, USA

†Instituto de Fisiología Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

‡Group on Molecular and Cell Biology of Lipids, Departments of Pediatrics and Cell Biology, University of Alberta, Edmonton, Alberta, Canada

CHAPTER OUTLINE

Methods in Cell Biology, Volume 116 **ISSN 0091-679X**

http://dx.doi.org/10.1016/B978-0-12-408051-5.00007-3

Abstract

The liver plays an important role in triacylglycerol (TG) metabolism. It can store large amounts of TG in cytosolic lipid droplets (CLDs), or it can package TG into very-low density lipoproteins (VLDL) that are secreted from the cell. TG packaged into VLDL is derived from TG stored within the endoplasmic reticulum in lumenal lipid droplets (LLDs). Therefore, the liver contains at least three kinds of LDs that differ in their protein composition, subcellular localization, and function. Hepatic LDs undergo tremendous changes in their size and protein composition depending on the energetic (fasting/feeding) and pathological (viral infection, nonalcoholic fatty liver disease, etc.) states. It is crucial to develop methodologies that allow the isolation and analyses of the various hepatic LDs in order to gain insight into the differential metabolism of these important lipid storage/transport particles in health and disease. Here, we present detailed protocols for the isolation and analysis of CLDs and LLDs and for monitoring CLD dynamics.

INTRODUCTION AND RATIONALE

All organisms and cell types investigated so far can store triacylglycerols (TGs), sterol esters in lipid droplets (LDs). Until recently, LDs were largely considered to be inert organelles, functioning only as lipid-storing bodies. However, it is now clear that LDs are active and dynamic organelles that play multiple roles in lipid metabolism, signal transduction, protein storage, and lipid trafficking (Olofsson et al., 2009; Walther & Farese, 2012).

The liver plays a central role in lipid metabolism and storage. In addition to the critical role of this organ in TG synthesis, storage, and provision of substrates for β-oxidation and ketogenesis, the liver is a specialized organ that secretes TG into the blood in apolipoprotein B (apoB)-containing very-low density lipoprotein (VLDL) particles. Therefore, hepatocytes contain several types of LDs, each with a specific subcellular localization and distinct protein composition. In addition to cytosolic LDs (CLDs) that are present in most other cell types, hepatocytes contain at least two more types of LDs in the lumen of the endoplasmic reticulum (ER) where

VLDL is assembled: the lumenal apoB-free lipid droplets (LLDs) and the apoB-containing particles (VLDL and its precursors). LLDs have been proposed to provide TG source for the bulk lipidation of VLDL precursors. Because the hepatic CLDs, LLDs, and VLDL are important contributors to whole body energy homeostasis, it has become important to characterize these intracellular TG storage entities. The most challenging issue in characterization of CLDs, LLDs, and the nascent VLDL is a reliable method to separate these very distinct LDs. Added complication is the rapidly changing composition of LD-associated proteins with various metabolic states of the hepatocyte (feeding/fasting, stress induced by viral infection, etc.). It is therefore important to take into consideration these factors when studying hepatic LDs.

CLDs are composed of a lipid core, mainly TG, cholesterol esters (CE), and retinyl esters, surrounded by an amphipathic lipid monolayer (phospholipids and free cholesterol) decorated with LD-associated proteins including the PAT (Perilipin1, ADRP/Perilipin2, TIP47/Perilipin3) protein family (Bickel, Tansey, & Welte, 2009; Farese & Walther, 2009; Kuhnlein, 2012; Yang et al., 2012). Hepatic CLDs also contain numerous proteins that are also found on CLDs in adipocytes and other cell types, such as ER-resident proteins, Rab GTPases, and cytoskeleton components, indicating that LDs might share similar regulation in all cell types. To date, only a few studies have been performed investigating protein composition of hepatic CLDs. One of these studies was performed in rat liver following partial hepatectomy, where 50 proteins were identified, including perilipin 2, ER-resident proteins, lipid and vitamin metabolism enzymes, cytoskeletal components, cell signaling and cell activation regulation proteins, and a number of proteins that participate in diverse intracellular trafficking pathways and exocytosis (Turro et al., 2006). Another work performed in the HuH7 hepatoma cell line identified 17 proteins, including perilipin 2 and lipid and steroid metabolism enzymes as the most abundant proteins (Fujimoto et al., 2004). Except for the presence of PAT protein family, hydroxysteroid dehydrogenases, and Rab5 GTPase, no similarities in protein contents between the two LD compositions were observed, possibly reflecting the differences between human hepatoma cell line (HuH7) and the liver, and/or differences in the metabolic state of the cells. Lack of overlap between the proteomes published by different groups also to a certain degree reflects the various degree of contamination present in all subcellular organelles isolated by density centrifugation. Even though a large amount of contaminants are removed while LDs partition across layers of a density gradient, it is impossible to remove all, especially since most common contaminations arise from hydrophobic proteins nonspecifically interacting with LDs, as well as from abundantly expressed proteins. It is also likely that some LDs are in the continuum with the ER (the site of LD origin), thus a small amount of the ER proteome may copurify with LDs.

Similarly, studies investigating hepatic LLDs are limited, mainly because they have proved very challenging. Light microscopy is not suitable for observing LLDs since their size is within or below the range of lipoproteins (ranging from 7 to 200 nm) and is under the detection limit of conventional light microscopy. Even with the state-of-the-art super resolution microscopy techniques, it is difficult to

distinguish LLDs from presecretory VLDL or VLDL precursor particles. The only reported success in observing LLDs was by immunogold electron microscopy, where they were identified as VLDL-sized particles in the smooth ER lacking immunodetectable apoB (Alexander, Hamilton, & Havel, 1976). Subsequently, chylomicron-sized LLDs were detected in the ER of enterocytes lacking apoB expression, further supporting this observation (Hamilton, Wong, Cham, Nielsen, & Young, 1998). No further progress has been made to provide additional evidence for the presence of these LDs. We have developed a method to purify LLDs by subcellular fractionation and to biochemically characterize protein and lipid properties of the isolated LLDs, thus providing an approach to biochemically study the formation and metabolism of LLDs. The purification of LLDs will become a powerful tool to study events involved in the lipidation step of VLDL assembly. LLDs were found to be heterogeneous in size, possibly reflecting the various states of synthesis/turnover (Wang, Gilham, & Lehner, 2007). Whereas in mouse CLDs, TG accounts for up to 80% of total lipids, phospholipid for about 15%, and cholesterol with CE for the remaining 5%, LLDs contained on average a lower percentage of TG (60%) and a higher percentage of phospholipid (25%) (Wang et al., 2007); lipid ratios similar to those found in mature VLDL. Proteomic analysis of LLDs revealed the presence of microsomal TG transfer protein (MTP), protein disulfide isomerase (PDI), apolipoprotein E (apoE), and several other ER-resident proteins including two members of the carboxylesterase family, carboxylesterase 3/triacylglycerol hydrolase (Ces3/TGH, Ces1d) and carboxylesterase 1/esterase-x (Ces1/Es-x, Ces1g) (Wang et al., 2007).

Despite the important functions LDs serve in many cellular processes, our knowledge of the cell biology of LDs lacks behind that of other intracellular organelles. It is generally believed that LD biogenesis in eukaryotes initiates from the ER where TG biosynthesis takes place. However, little is known about the mechanism by which nascent LDs accrue additional TG and grow in size after nascent formation. This gap is in part due to the lack of good tools to visualize the flux of lipids. Traditional lipophilic dyes such as BODIPY 493/503, Nile Red, and LD540 are excellent tools to visualize the morphology of already formed CLDs by fluorescence microscopy; however, they do not allow tracking the dynamics of initial LD formation and cannot distinguish different pools of LDs at the different stages of biogenesis. Thus, a method is needed that distinguishes between preformed and newly synthesized CLDs. Additionally, CLD formation is a rapid process that occurs almost immediately (within 15 min) after OA addition (Wang et al., 2010). Thus, real-time cell imaging is necessary to capture this short window during CLD formation.

Many lipid analogues have been developed in the past decade, including a variety of fluorescent fatty acid analogues, thus enabling the tracking of initial lipid incorporation into CLDs with real-time microscopy. These analogues include, but are not limited to, NBD-conjugated fatty acids (Chattopadhyay, 1990), BODIPY-conjugated fatty acids (Pagano, Martin, Kang, & Haugland, 1991),

polyene-fatty acids (Kuerschner et al., 2005), etc. With these tools in hands, we are able to start answering important questions regarding the mechanism of CLDs biogenesis, such as the formation of nascent CLDs and addition of lipids to preformed CLDs.

This protocol covers the isolation of CLDs and LLDs from the liver of fasted C57BL/6J mouse maintained on chow diet. Analysis protocols related to the characterization of CLDs and LLDs and visualization of CLD dynamics will also be briefly discussed.

7.1 MATERIALS

7.1.1 Isolation of CLDs

Reagents:

1. Phosphate-buffered saline (PBS): NaCl 137 mM, KCl 2.7 mM, $Na_2HPO_4 \cdot 2H_2O$ 10 mM, KH_2PO_4 2.0 mM, pH 7.4
2. Hypotonic medium (HM): 20 mM Tris–HCl, pH 7.4, 1 mM EDTA. Prepare fresh, keep refrigerated
3. 60% sucrose-HM: HM supplemented with 60 g/100 ml sucrose. Prepare fresh, keep refrigerated for no more than 1 day
4. 5% sucrose-HM: HM supplemented with 5 g/100 ml sucrose. Prepare fresh, keep refrigerated for no more than 1 day

All hypotonic media contain protease and phosphatase inhibitors at their optimal concentrations.

5. Sodium dodecyl sulfate (SDS) 10% (w/v)

Other materials and equipment:

6. Plastic tubes
7. Eppendorf tubes (1.5 ml)
8. Low-speed refrigerated centrifuge with appropriate centrifuge tubes
9. Ultracentrifuge with swinging-bucket rotor
10. Thin-walled polycarbonate ultracentrifuge tubes
11. Reagents and equipment for SDS–PAGE and immunoblotting

7.1.2 Isolation of LLDs

Reagents:

1. Tris-buffered saline (TBS): 20 mM Tris–HCl, 150 mM NaCl
2. Homogenization buffer: 250 mM sucrose, 20 mM Tris–HCl, pH 7.4, 1 mM EDTA
3. Washing buffer: 20 mM Tris–HCl, 0.5 M NaCl (pH 7.4)
4. 1 mM Tris–HCl, pH 8.8
5. Glycerol
6. Protease inhibitor cocktail (Roche)

7. Goat anti-apoB antibody (Chemicon)
8. 4× SDS–PAGE sample buffer: 200 mM Tris–HCl, pH 6.8, 8% SDS, 40% glycerol (v/v), 40% β-mercaptoethanol (v/v), 0.4% Bromophenol Blue

Other materials and equipment:

9. Surgical tools for removing the mouse liver
10. Motor-driven Potter–Elvehjem tissue homogenizer with loose-fitting Teflon pestle (Wheaton), 15 ml capacity
11. Table-top refrigerated centrifuge with swinging-bucket rotor
12. Beckman ultracentrifuge with SW41Ti swinging-bucket rotor
13. 15 ml Falcon conical bottom tubes
14. Beckman 13.2 ml Ultra-Clear™ or thin-wall polyallomer ultracentrifuge tubes

Additional reagents and equipment for:

15. SDS–PAGE and immunoblotting, lipid extraction, thin-layer chromatography (TLC) plates (lipid analysis), and fast protein liquid chromatography (FPLC) and Superose 6 (gel filtration column for determining particle size).

7.1.3 Use of BODIPY fatty acids to visualize CLD dynamics

Reagents:

1. Collagen solution from calf skin (type I): 0.1% solution in 0.1 M acetic acid
2. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4
3. Bovine serum albumin (BSA): essentially fatty acid free (Sigma)
4. Hepatocyte culture media: Dulbecco's modified Eagle's medium (DMEM), 10% FBS (heat inactivated at 56 °C for 30 min), 100 U/ml penicillin, and 100 μg/ml streptomycin
5. 20× oleic acid complexed to BSA (OA/BSA): 7.5 mM OA, 10% BSA, dissolved in DMEM (see Section 2.3.2. for protocol)
6. Labeling medium A: DMEM, 1× OA/BSA, 6 μM BODIPY FL C_{12} or BODIPY 558/568 C_{12} (Molecular Probes)
7. Labeling medium B: QTB fatty acid uptake reagent (Molecular Devices) reconstituted in 10 ml DMEM containing 1× OA/BSA
8. BODIPY 493/503 dye: 1 mg/ml in DMSO (Molecular Probes)

Other materials and equipment:

9. Spinning-disk confocal microscope with 488 and 543 nm laser or similar
10. Environment chamber for live-cell imaging with temperature control and CO_2 supply
11. Stage adaptor and culture chamber for live-cell imaging
12. Volocity software for image capture and processing

7.2 METHODS

7.2.1 Isolation of CLDs from mouse liver

Before we start: all samples and solutions throughout the preparation of LDs should be kept on ice, and make sure that protease inhibitor cocktail is freshly added to all buffers used during the preparation.

7.2.1.1 Mice, diets, and feeding states

In order to have a successful (purer) CLD fractionation, the amount of lipid present in the liver should be considered before starting any LD isolation procedures. As a general rule, the larger amount of lipid in the tissue, the higher the chance of contamination of LDs with other organelles. Therefore, three key interrelated issues must be taken into account before isolating CLDs from mouse livers: (i) the diet, (ii) the fasting time, and (iii) the amount of TG present in the tissue. First, high-fat diets increase the amount of TG in the liver but also make the tissue much more brittle thereby increasing the likelihood of obtaining contaminated LD fractions. On the other hand, low-fat diets may result in livers with less TG, but CLDs become easier to isolate since the chances of contamination with other intracellular membranes are minimized. Second, and equally important, is the feeding state of the animals: it is known that fasting induces the accumulation of hepatic TG and therefore CLD number, size, and also protein composition vary depending on the feeding states of the animals. Fasting times longer than 24 h are not recommended for the mouse, since the animal enters into a starvation mode at such a prolong fasting period, and the proteome does not represent a normal physiological state. On the other hand, we determined that refeeding times (after a 24 h fast) should be no longer than 6 h if one is to study CLD composition in animals with defined nutritional states. This is as a mean of synchronicity: after a 24 h fast, animals are eager to eat, thus all mice feed at a fairly constant pace for at least 5 h. From 6 h on, mice start to eat less and at a different pace from each other, which has an impact on CLD size, number, and protein/lipid composition. Refeeding times shorter than 6 h might result in a significant overlap in CLD protein composition between fasting and refeeding states. Third, the amount of lipid present in the liver may be first evaluated taking into account the genetic background of the animal, since defined liver lipid phenotypes have been described for different strains of mice. For instance, $Ces1^{-/-}$ mice present with obesity and mild steatosis (Quiroga et al., 2012). Similarly, virus-induced knockdown of Atgl also results in hepatic steatosis (Ong, Mashek, Bu, Greenberg, & Mashek, 2011).

7.2.1.2 CLD fractionation

CLDs are isolated by methods developed by Brasaemle and Wolins (2006) with some modifications specific to the liver tissue (Table 7.1). Mice should be anesthetized by inhalation with isoflurane and exsanguinated by cardiac puncture. Quickly but carefully, livers are harvested, perfused with ice-cold PBS, and cleaned from any

Table 7.1 Lipid Droplet Isolation from Mouse Livers

1. Harvest livers quickly and carefully
2. Clean livers from any other tissues
3. Rinse livers thoroughly in ice-cold PBS
4. Homogenize livers by soft homogenization in HM on ice
5. Transfer homogenates to 15 ml tubes
6. Centrifuge at $500 \times g$ for 10 min at 4 °C
7. Spin the supernatant at $15{,}000 \times g$ for 10 min at 4 °C
8. Recover fat cakes into new clean tubes
9. Wash twice at $15{,}000 \times g$ for 10 min at 4 °C
10. Dilute fat cakes with 1/3 volume of ice-cold HM containing 60% sucrose (final concentration 20% sucrose)
11. Disperse aggregates of LDs by gently pipetting
12. Layer the diluted aggregates carefully at the bottom of ultracentrifuge tubes
13. Overlay these tubes with double the volume HM containing 5% sucrose
14. Carefully overlay the tubes with same volume of HM
15. Ultracentrifuge tubes at $28{,}000 \times g$ for 30 min
16. Recover LDs carefully (use a pipet or a slicer) into Eppendorf tubes
17. Analysis

(modified from Brasaemle & Wolins, 2006)

leftover adipose tissue. Individual livers are transferred to a beaker containing ice-cold PBS and are thoroughly rinsed. Then, livers are weighed and immediately subjected to soft homogenization on ice in HM (20% homogenate, w/v) by 10 gentle strokes with a motor-driven Potter–Elvehjem homogenizer set at a speed of 3. This step is crucial to preserve LD integrity and to prevent damage to intracellular organelles (such as the ER, which would otherwise leak lumenal contents including VLDL and LLDs that would contaminate CLD preparations). Homogenates are transferred to 15 ml tubes and centrifuged 10 min at $500 \times g$, 4 °C. The supernatant containing the floating fat layer (fat cake) is then spun at $15{,}000 \times g$ for 10 min to remove mitochondria and to further allow fat cake separation. Fat cakes are then recovered into new tubes and washed twice with ice-cold HM at $15{,}000 \times g$ for 10 min. Recovered fat cakes are then diluted with 1:3 (v/v) of ice-cold HM containing 60% sucrose to yield a final 20% sucrose-adjusted homogenate. Aggregates of LDs should be finely and thoroughly dispersed by gentle pipetting. It is highly recommended to use a pipet tip with a wide opening. Diluted CLD aggregates are now carefully layered at the bottom of ultracentrifuge tubes and overlayed with double the volume HM containing 5% sucrose. These tubes are now carefully overlayed with same volume of HM. Tubes should be ultracentrifuged at $28{,}000 \times g$ for 30 min. After ultracentrifugation, fat cakes are carefully recovered (using a pipet or a slicer) and kept in Eppendorf tubes for further analysis. This step could be repeated after further dilution of recovered fat cake with 60% sucrose in order to get a highly enriched (purified) CLD fraction.

After isolation, recovery and purity of LDs should be evaluated by SDS–PAGE followed by immunoblotting (see below how to solubilize CLD-associated proteins).

Known CLD proteins should be immunoblotted as controls using specific antibodies. For this purpose, perilipin 2 is an excellent marker for CLD recovery in the liver. In order to determine the purity of the preparation, cytosolic proteins and resident polytopic membrane proteins from other organelles such as the ER and mitochondria should be analyzed by immunoblotting with specific antibodies. Polytopic ER membrane proteins (such as phosphatidylethanolamine-*N*-methyltransferase) are good markers for the absence of ER contaminations from CLDs. Likewise, fumarase, with mitochondrial and cytosolic localizations, is a reliable marker for contamination of CLDs with mitochondrial components.

7.2.1.3 Solubilization of CLD-associated proteins for immunoblotting

In order to solubilize CLD-associated proteins, fresh CLD fractions should be mixed with 10% SDS (1:1, v/v) and incubated for 1–2 h at 37 °C in a sonicating water bath with constant agitation. Then, samples should be centrifuged in a microcentrifuge for 10 min at maximum speed at room temperature and the infranatants containing the solubilized proteins should be collected from beneath the floating lipid layer. For this purpose, it is very helpful to use a 200 µl tip inserted very carefully through the floating fat cake all the way down to the bottom of the tube. It is important not to disrupt the lipid cake; should this happen, recentrifugation should be performed. Transfer the infranatant to a 1.5 ml tube and add equivalent volumes of 2 × SDS electrophoresis sample buffer. Then, boil samples for 10 min and finally load them onto a discontinuous SDS–PAGE gel.

7.2.2 Isolation of LLDs from mouse liver

7.2.2.1 Preparation of the mouse

To prepare mice for LLD isolation, we fast mice overnight (12–16 h) before experiments because under fasting conditions, large amounts of LDs are accumulated in the liver. Instead of simply removing food from the mice, fasting should be done by transferring mice into a clean cage with free access to water. Usually up to four livers are used for an experiment. If the study requires comparing different feeding conditions, then much more material is required. The following method is performed using fasted mice.

7.2.2.2 Isolation of the liver and tissue homogenization

Anesthetize the mice to a state suitable for surgery by inhaling of metophane or isoflurane. Vital signs should be monitored during this procedure. Exsanguinate the mice via cardiac puncture and carefully remove the liver, which is immediately transferred to ice-cold TBS (Table 7.2). After rinsing with TBS, livers are weighed and homogenization buffer is added to make a final 20% (w/v) homogenate. For homogenization, we use a motor-driven Potter–Elvehjem homogenizer (Wheaton Science Products, Millville, NJ) at medium speed (levels 3–4) for 10 strokes in homogenization buffer. Proper homogenization is one of the most important steps for the preparation in order to efficiently break cells while maintaining the integrity of subcellular content. Alternative devices such as Polytron or loose-fitting Dounce

Table 7.2 Isolation of Lumenal Lipid Droplets from Mouse Liver

1. Harvest livers quickly and carefully
2. Clean livers from any other tissues
3. Rinse livers thoroughly in ice-cold TBS
4. Gently homogenize livers with Potter homogenizer in homogenization buffer on ice to make 20% homogenate
5. Transfer homogenates to 15 ml tubes
6. Centrifuge at $500 \times g$ for 10 min at 4 °C
7. Spin the supernatant at $15{,}000 \times g$ for 10 min at 4 °C
8. Spin the supernatant at $106{,}000 \times g$ for 1 h at 4 °C
9. Resuspend the pellet in ice-cold washing buffer
10. Spin at $106{,}000 \times g$ for 1 h at 4 °C and collect pellet (microsomes)
11. Resuspend microsomes in 1 mM Tris–HCl (pH 8.8)
12. Incubate on ice for 30 min
13. Gently homogenize to disrupt microsomes and release lumenal content
14. Spin at $106{,}000 \times g$ for 1 h at 4 °C and collect supernatant
15. Adjust supernatant to 50 mM Tris–HCl, 150 mM NaCl (pH 7.4)
16. Add anti-apoB polyclonal antibodies
17. Rotate end-over-end overnight at 4 °C
18. Add protein A Sepharose beads
19. Rotate end-over-end for at least 2 h at 4 °C
20. Pellet beads by centrifugation for 30 s at $6000 \times g$ and collect supernatant (post-IP)
21. Combine 2 ml of post-IP with an equal volume of glycerol
22. Transfer mixture to a Beckman 13.2 ml Ultra-ClearTM tube
23. Sequentially overlay with 4 ml of homogenization buffer and 4 ml of TBS
24. Centrifuge in Beckman SW40 rotor at 35,000 rpm ($160{,}000 \times g$) for 2 h at 8 °C
25. Carefully puncture the bottom of the tube to collect fractions
26. Collect 2 ml per fraction for a total of six fractions for analysis
27. Analyses

homogenizer can also be used but conditions should be optimized carefully. In order to monitor the proper separation, recovery, and integrity of subcellular fractions, an aliquot should be saved for each of the following purification steps. These aliquots will include: cytosol, microsomes, microsomal membrane, immunoprecipitate (IP), post-IP supernatant, microsomal lumen, and fractions #1–6.

7.2.2.3 Isolation of microsomes

Microsomes from C57BL/6J mouse liver homogenates are prepared by sequential centrifugation essentially as previously described (Lehner & Kuksis, 1993). Remove cellular debris by centrifugation at $500 \times g$ for 10 min in a table-top centrifuge and the resulting supernatant is centrifuged subsequently at $15{,}000 \times g$ for 10 min to pellet crude mitochondria. The $15{,}000 \times g$ supernatant contains microsomes and cytosol (plus CLDs), which can be separated by ultracentrifugation at $106{,}000 \times g$ for 1 h. The supernatant (cytosol plus CLDs) from this step is transferred into a new tube for further analysis and pellet (microsomes) is resuspended in washing buffer. To resuspend the pellet, we use the Potter homogenizer to gently disrupt the pellet before pipetting up and down since the pellet tends to stick to pipette tips. Washing is an

important step to remove proteins and CLDs peripherally associated with the microsomal membrane. Microsomal membranes are pelleted by ultracentrifugation at $106{,}000 \times g$ for 1 h, while peripheral proteins (cytosolic side) remain in the supernatant.

7.2.2.4 Release of microsomal lumenal content

To release microsomal lumenal contents, pelleted microsomes are resuspended in 1 mM Tris–HCl (pH 8.8) in a volume equivalent to 1/3 of the original homogenization buffer and incubate on ice for 30 min. This step swells microsomes by osmotic pressure and preserves the integrity of LLDs and the associated proteins. At the end of the incubation, the swelled microsomes are disrupted by homogenization with Potter by three strokes at low setting to release lumenal contents. The mixture is then centrifuged at $106{,}000 \times g$ for 1 h to obtain lumenal contents (supernatant) and microsomal membranes (pellet).

7.2.2.5 Separation of LLDs from VLDL precursors

At least two types of LDs are present in the microsomal lumen: the apoB-containing particles (including mature VLDL and VLDL precursors) and the apoB-free LLDs. To remove apoB-containing particles from LLDs, the microsomal lumenal contents obtained from the previous step are adjusted to 50 mM Tris–HCl, 150 mM NaCl (pH 7.4), and apoB-containing particles are immunoprecipitated using anti-apoB polyclonal antibodies (goat anti-apoB from Chemicon). It is important that the immunoprecipitation is performed in the absence of detergents in order to preserve the integrity of LLDs. Two forms of apoB are present in the rodent liver: apoB48 ($\sim$250 kDa) and apoB100 ($\sim$500 kDa). The antibodies we use recognize both forms of apoB. For each milliliter of lumenal content, usually 5 µl of antibodies are added and the mixture is incubated while rotating the tubes end-over-end at 4 °C overnight. Then 20 µl protein A Sepharose beads prewashed with PBS are added and incubated with the mixture end-over-end at 4 °C for 2 h to form protein–bead complex, which is subsequently pelleted by centrifugation for 30 s at $6000 \times g$. The IP is then washed $3\times$ with PBS and prepared for SDS–PAGE and immunoblotting. The supernatant (post-IP) is collected for further purification, which is described below.

7.2.2.6 Isolation of microsomal LLDs by density gradient ultracentrifugation

Two milliliters of post-IP supernatant are combined with an equal volume of glycerol and transferred to a transparent ultracentrifuge tube suitable for SW40 swing bucket rotor. We prefer the Beckman Ultra-Clear™ centrifuge tube, since it allows for good inspection of the gradient set up. The glycerol-adjusted samples are then overlayed with 4 ml of homogenization buffer and an additional layer of 4 ml of TBS. The gradient should be set with great care. We find using a long needle of 18G or narrower is helpful to gently deliver overlay solutions without disturbing the lower layer. Once the gradient is set, the samples are centrifuged in a Beckman SW40 rotor at 35,000 rpm ($160{,}000 \times g$) for 2 h at 8 °C. Be sure to use a swinging bucket and avoid fixed angle

rotors for this step since LLDs tend to stick to the wall of the centrifuge tube and thus cause cross-contamination in the various gradient fractions. Immediately after centrifugation, the bottom of the tube is carefully punctured with a fine needle and fractions are collected into collection tubes. Alternatively, a tube slicer can be used to collect fractions and minimize carry over from other fractions. Two milliliters per fraction are collected for a total of six fractions, labeled fractions #1–6, with #6 being the top (most buoyant) fraction. The fractions, together with aliquots from the various steps of preparation, are then used for analysis for their biochemical compositions and physical prosperities. The following are examples of analyses we performed.

7.2.2.7 Sample analysis

7.2.2.7.1 Analysis of protein composition by SDS–PAGE and immunoblotting

After purification, samples should first be analyzed for recovery and contamination by immunoblotting. We use ER-resident proteins such as PDI as markers for recovery of ER lumenal content, and immunoblot for apoB to monitor the degree of contamination of LLDs with apoB-containing VLDL precursors. SDS–PAGE and immunoblotting procedures are similar to standard procedure used for most proteins; however, it is important that 2–5% SDS (final concentration) should be included to dissolve lipids. The sample buffer we use for immunoblotting of LLD-associated proteins is listed in Section 1. Due to the low abundance of LLDs and large volume collected from the density gradient, fractions #4–6 are concentrated 15-fold using centrifugation-based protein concentrators (Pierce) with molecular weight cutoff of 10 kDa. By comparing proteins enriched in each fraction obtained from the gradient with aliquots saved during the preparation, fractions #4–6 are LLD fractions with distinct associated proteins. An ER lumenal lipase, Ces3/TGH, can be used as a marker for fraction #4 (corresponding to smaller LLDs), and apoE for fraction #6 (corresponding to larger LLDs).

Fractions can be analyzed for protein and lipid compositions using established methodologies described elsewhere.

7.2.2.7.2 Analysis of LD particle size by FPLC and native PAGE

These analyses are performed in combination with protein and lipid composition analysis. Information obtained from these analyses is useful not only to understand the physical property of LLDs, but more importantly, as a read out for protein and lipid compositions associated with the various subsets of LLDs. This information is especially beneficial when comparing between different experimental conditions, such as genetic backgrounds, nutritional states, or drug treatments.

Gel filtration chromatography. Combined LLD fractions are concentrated and applied to a Superose 6 size exclusion FPLC column (Pharmacia, Uppsala, Sweden). Elution of various TG-rich LLD subfractions is determined by on-line detections of TG content. Specifically, eluted fractions are mixed in-line with the Infinity™ Triglyceride Reagent (Thermo Fisher Scientific, Inc., Waltham, MA) using a post-column T-connector/Solvent Delivery Module (model 110B, Beckman Coulter, Mississauga,

Ontario, Canada) and passed through a CH-30 Column Heater (Eppendorf, Mississauga, Ontario, Canada) set at 37 °C. Reaction products are monitored at 500 nm in real time using a Programmable Detector Module (model 166, Beckman Coulter). Under this setup, fractions that elute from the 22nd to 25th minute contain VLDL-sized particles and fractions that elute from the 38th to 53th minute contain HDL-sized particles. To analyze protein contained in different fractions, we collect one fraction every 4 min (2 ml) from the 22nd to 58th minute, precipitate proteins with 2 volumes of ice-cold acetone for 30 min at −20 °C, resuspend the protein pellet in 50 μl of SDS–PAGE sample buffer, and analyze the protein content by SDS–PAGE and immunoblotting. Several reference proteins, including Ces3/TGH, MTP, and apoE, can be used to evaluate successful separation of LLDs of varies size. We found that Ces3/TGH is present in eluent from 26 to 58 min and peaks around 42 to 58 min, apoE is eluted at around 26–38 min, while MTP elutes later than apoE, at around 42–58 min.

Gradient native PAGE. Twenty five microliter aliquots of isolated LLDs are mixed with an equal volume of 2 × native PAGE loading buffer and applied to a 2–10% gradient nondenaturing polyacrylamide gel, casted with a gradient maker (Hoefer, Inc.). Proteins are resolved in native PAGE running buffer without SDS at 80–120 V using a BioRad Mini-PROTEAN system. Particle size is determined by comparison with the migration of purified lipoprotein standards listed in the table below:

Size Category	Molecular Mass (kDa)	Diameter (nm)
HDL1	440–669	12.2–17.0
HDL2	232–440	10.5–12.2
HDL3/preβ1	66–232	7.1–10.5

7.2.3 Use of BODIPY fatty acids for visualization of CLD dynamics (Table 7.3)

7.2.3.1 Preparation of mouse hepatocytes grown on the cover slip or glass bottom dish

This protocol uses primary mouse hepatocytes isolated by collagenase perfusion of the mouse liver. Details of the preparation can be found in previous publications (Yao & Vance, 1988). We use fasted mice. The isolation should be performed immediately before each experiment since cultured primary hepatocytes gradually lose their metabolic properties, such as expression of some key enzymes involved in lipid metabolism. Hepatocytes should not be older than 72 h and preferably used within 48 h after seeding (Table 7.3). We seed 0.2×10^6 cells for each well in a six-well plate. For proper imaging of cells by confocal microscopy, cells should be plated on glass cover slips with thickness compatible with the microscope objectives to be used. If a microscope has a culture chamber or adaptor for live-cell imaging, cells can be seeded onto a regular round cover slip placed at the bottom of a six-well plate;

Table 7.3 Visualization of Lipid Droplet Dynamics using BODIPY Fatty Acids

1. Coat cover slips with collagen solution and place in a six-well plate
2. Seed 0.2×10^6 freshly prepared hepatocytes per well
3. Incubate cells in serum-free DMEM overnight
4. Incubate cells with labeling medium A containing 558/568 C_{12} for at least 4 h
5. Setup microscope for imaging with 491 and 543 nm laser lines
6. Transfer cover slips onto a culture chamber placed on the microscope in an environment chamber thermostated at 37 °C and supplied with 5% CO_2
7. Find fields of interest and record stage location
8. Carefully rinse once with PBS without disturbing stage locations
9. Carefully add labeling media B (time 0)
10. Immediately start image acquisition every 1 min over a period of 30 min with Z-slices of 0.5 μm steps

the diameter of the cover slip should be suitable for the adaptor. Otherwise, a glass bottom culture dish (e.g., MatTek dishes) can be used instead. To coat cover slips or glass bottom dishes with collagen, pipette collagen solution (Sigma) into wells containing the cover slip, let the solution sit briefly, then remove the collagen solution, and rinse wells twice with PBS. Collagen solutions can be reused if kept sterile. Cells are then maintained in hepatocyte culture media at 37 °C in humidified air containing 5% CO_2 for at least 4 h to allow attachment of the cells to the collagen matrices.

7.2.3.2 Oleic acid and BODIPY fatty acid incubations of hepatocytes

It is recommended that endogenously preformed CLDs should be reduced as much as possible by incubating cells grown on collagen-coated cover slips in serum-free DMEM overnight. Even though preformed CLDs always exist in hepatocytes isolated from wild-type mice, this step helps reduce the stored TG pool and reduce the background.

To visualize preformed LDs using exogenous fatty acid source, the intracellular TG storage is first augmented by incubations with OA for at least 4 h. OA is complexed to BSA to avoid lipotoxicity induced by free fatty acids. To make a 20 × OA/BSA complex, dissolve 5 g of FA-free BSA in 50 ml DMEM and warm up the mixture to 56 °C in a water bath. Weigh out 106 mg OA in a glass beaker (100 ml) using analytical balance and warm up at 56 °C water bath for 2 min. This step should be done immediately before adding BSA in order to minimize oxidation. Add BSA solution to the warmed up OA and stir vigorously using a stirring bar for 2 min. The solution should clear, an indication that OA has been complexed with BSA. Filter-sterilize the solution while it is still hand-warm and store at 4 °C.

Labeling of preformed LDs is performed by mixing the red fluorescent fatty acid analogue, BODIPY 558/568 C_{12}, during OA loading (labeling medium A). After 4 h incubation, cells are washed with PBS and incubated with labeling media containing the fluorescent green fatty acid analogue, BODIPY FL C_{12}. For imaging and quantification of nascent LD formation under real-time conditions, we use labeling media B for this step since a quencher is included in the labeling medium B to absorb

background fluorescent signals in the media so that only signals from the fluorophores incorporated into LDs are collected. For this purposes, labeling medium B should be added into the culture chamber on the microscope, immediately before image capture (see Section 2.3.3).

In principle, the choice of fatty acid analogues can be reversed, that is, BODIPY FL C_{12} for preformed and BODIPY 558/568 C_{12} for nascent CLDs. We have demonstrated by both microscopy and TLC that these analogues are similar in their capabilities to incorporate into TG/LDs. However, we prefer using BODIPY FL C_{12} for nascent LD formation in live-cell imaging for the following reasons: (1) compared to BODIPY 558/568 C_{12}, BODIPY FL C_{12} gives brighter signal and sharper image; (2) as a consequence, lower laser power can be used for live-cell imaging, which is crucial to keep cells healthy during imaging and for avoiding artifacts in quantification due to bleaching of the fluorophore; (3) chemical quencher for BODIPY FL C_{12} is readily available in the commercial kit mentioned above.

7.2.3.3 Microscope setup and imaging

The spinning-disk confocal microscopes suitable for this experiment should be equipped with green and red laser lines for excitation of the fatty acid analogues. It is essential to have a temperature-controlled environment chamber. It is preferable to have CO_2 supply; however, cells can be maintained in medium containing 50 mM HEPES for at least 30 min without CO_2. Samples should be secured to the stage with an adaptor that completely restricts movement of the samples when reagents are added during imaging. For this reason, a culture chamber is preferable to a glass bottom dish, as the latter usually does not fit tightly to the stage adaptor. It is also extremely helpful to have multistage control and autofocusing to image multiple cells during the same imaging session. Taking Z stacks of cells is helpful to bypass focus fluctuations during long-term imaging.

For our setup, cells grown on cover slips are mounted onto a culture chamber (Chamlide, Seoul, Korea) and placed in an environment chamber thermostated at 37 °C and supplied with 5% CO_2. Labeling media B is carefully added to cells in the culture chamber immediately before image capture (time 0). Confocal microscopy is performed on a spinning-disk microscope (WaveFx from Quorum Technologies, Guelph, Canada) setup on an Olympus IX-81 inverted stand (Olympus, Markham, Canada). Images are acquired through a 60× objective (N.A. 1.42) with an EMCCD camera (Hamamatsu, Japan). Fluorescent fatty acid analogues BODIPY FL C_{12} and BODIPY 558/568 C_{12} are successively excited by a 491 nm (GFP channel) and a 543 nm (Cy3 channel) laser line (Spectral Applied Research, Richmond Hill, Canada), respectively. Z-slices of 0.5 μm steps are acquired using Volocity software (Improvision) through the cells using a piezo z-stage (Applied Scientific Instrumentation, Eugene, USA) with image capture every 1 min over a period of 30 min. Quantification of fluorescent intensity is done using Volocity software (Ver. 5.0.0) (see below).

7.2.3.4 Image processing and analysis

Image analysis can be performed by a variety of software, including open architecture software, such as Image J and CellProfiler, commercial imaging software, such as MetaMorph and Volocity, or a high-level language for data analysis and visualization, such as MATLAB. Each of the abovementioned software has its own strength and weakness, but in all cases, object segregation is the most challenging task in order to identify objects as well as possible. This is especially valid when CLDs are vastly different in their sizes, because it might be difficult, if possible at all, to precisely identify all objects. Parameters should be optimized so that when different samples are compared, it is reasonable to assume the same error for the object of interest in all samples. Therefore, relatively large data sets should be obtained for quantification.

For our processing and analysis, images captured with time-lapse microscopy are processed with Volocity software (PerkinElmer). Brightness, contrast, density, and blackness are adjusted to obtain sharp images, and photobleaching is corrected for quantification. For our data set, one can calculate the transfer of newly synthesized lipids (BODIPY FL C_{12}) to preformed LDs (BODIPY 558/568 C_{12}). Performed CLDs are defined as areas containing red fluorescent signal, selected by choosing objects within a defined density threshold in the Cy3 channel. A single cell is defined as the region of interest (ROI), and objects within the ROI are refined by setting object size and shape references. Touching objects are separated and objects selected under these criteria are defined as the preformed CLD area. Data from all time points are corrected for photobleaching. Fluorescence intensity within preformed CLD area at each point is quantified for both GFP and Cy3 channels and presented as the percentage of initial fluorescent intensity (at time "0"). The results are exported to Microsoft Excel for plotting as line graphs, in which the rate of incorporation can be reflected clearly: red signals in the preformed LD are relatively stable, suggesting of low turnover, while signals from the green channel increase gradually with time, suggesting continuous incorporation of the newly synthesized TG, either by lipolysis and reesterification of new LDs, or by fusion of newly synthesized and preformed LDs. When hepatocytes from different genetic background were compared, differences in fatty acid transfer rate from nascent to preformed CLDs can be clearly observed (Wang et al., 2010).

7.3 DISCUSSION

The importance of CLD metabolism in tissues other than adipose resulted in the need of methodologies that would lead to the recovery of highly purified CLD preparations. Due to the high proportion of neutral lipids and the relative low concentration of proteins, CLDs would appear to be easy to isolate compared with other organelles. However, a care has to be undertaken in homogenization and centrifugation protocols that allow preparations of highly purified CLD fractions.

Technically, the study of CLD metabolism in the liver should not represent a problem, provided mild homogenization protocols are followed that preserve the integrity of intracellular organelles and avoid contamination of CLDs with lumenal TG-rich lipoproteins and LLDs. Therefore, the study of hepatic CLDs components has added complexity compared to CLDs from other cells and tissues. A few studies have been performed to describe the proteome of hepatic CLDs (Fujimoto et al., 2004; Turro et al., 2006). An early method for isolating hepatic CLDs involved discontinuous density gradient centrifugation and yielded six discrete bands of lipid particles, rich in TG and cholesterol (Ontko, Perrin, & Horne, 1986). Unfortunately, due to the lack of knowledge of CLD-associated proteins (PAT family of proteins was discovered nearly a decade later), the purity and protein composition of the various fractions method has not been adequately validated.

Here, we describe a method for isolating CLDs from the liver, based on a method by Brasaemle and Wolins (2006), and a method to isolate LLDs. The use of a soft tissue homogenization is crucial to preservation of CLD integrity of LDs. Simple two-step low-speed centrifugation and a single ultracentrifugation step using a discontinuous density gradient yield highly purified CLD preparations. We have used this method to analyze a CLD proteome in fasted and re-fed conditions. The CLD proteome changes dramatically depending on the feeding state of the mouse and therefore it is highly advisable that CLDs are prepared from animals in a controlled metabolic state.

It is important to note that this method of CLD isolation can be applied to evaluate a wide range of metabolic processes such as lipid metabolism in different feeding states, biochemical determinations such as enzyme activities, particle size, etc. The analysis of the dynamic nature of these organelles not only provides the tools for the understanding of molecular mechanisms involved in CLD formation and mobilization, but also paves the road to development of novel therapies for treatment of pathological conditions.

The research on LLDs has been challenging for multiple reasons: (1) it is difficult to resolve LLDs from apoB-containing VLDL and its precursors; (2) it is difficult to compromise the integrity of the microsomal membranes without affecting the integrity of LLDs; (3) LLDs are present in low abundance; (4) contamination from ER-resident proteins needs to be avoided; (5) LLDs are too small to be visualized by light microscopy, and there had been only limited success with electron microscopy in studying LLDs. The protocol we developed overcomes most of these difficulties and represents a practical and effective way to purify LLDs.

To separate LLDs from apoB-containing VLDL and its precursors, we used immunoprecipitation to remove apoB-containing particles, which was proved successful since apoB was absent from the post-IP fraction, while the LLD-associated carboxylesterase 3/TGH was recovered in this fraction (Wang et al., 2007). However, care should be taken to maintain the integrity of particles during immunoprecipitation procedure; immunoprecipitation should be performed in the absence of detergents, as they would destroy lipid particles, and/or change protein and lipid composition of isolated LLDs. Similarly, the method using high pH carbonate

(0.2 M Na_2CO_3, pH 11) for extraction used for studying topologies of polytopic membrane proteins is unsuitable as this method might denature proteins and/or strip proteins from the LLD surface. We used hypotonic solution to swell and compromise microsomal membrane integrity to release LLDs. Because LDs do not contain aqueous phase within the core, they are not susceptible to hypotonic osmotic pressure. One of the challenges we have encountered in the preparation of LLDs is their low abundance. Our studies have suggested that less than 3% of the intrahepatic TG is contained in LLDs. This problem is compounded by the relatively low recovery. For obtaining sufficient amount of LLDs for subsequent studies, usually two to four mouse livers are necessary. However, it is challenging to adapt this method to cultured hepatocytes unless one uses radioactive tracers to monitor LLD recovery. As mentioned above, an alternative approach to release microsomal lumenal contents is through the use of high pH Na_2CO_3 (Sundaram et al., 2010). This method is more efficient at breaking microsomes than the hypotonic method we used, and therefore results in a higher recovery of lumenal contents. However, because Na_2CO_3 will also strip proteins and CLDs peripherally associated with membranes in addition to LLDs, this might result in contamination of LLDs with CLDs that remained associated with microsomes. Readers should choose carefully which procedure is preferable depending on the experimental needs. The following publications maybe referred to for comparison (Wang et al., 2007; Yao, Zhou, Figeys, Wang, & Sundaram, 2013).

To assess the purity of the isolated LLDs and to estimate the degree of contamination, protein composition needs to be determined. The LLD fractions should be free of apoB, transmembrane proteins, and CLD markers. However, many ER lumenal proteins are found in LLDs. This may be due to the ER–LD connection, as by high-confidence LD proteomics studies, many proteins were found to have dual localization in the ER and on LDs (Krahmer et al., 2013). Using mass spectrometry, we also found some cytosolic and mitochondria associated proteins in LLDs fraction. These are inevitable contaminations that exist in essentially all subcellular purifications. Thus, further combining LLD purification with a high-confidence proteomics approach such as SILAC would be beneficial to identify bona fide LLD proteins. This approach would further assist in identifying specific protein markers for LLDs. So far, proteins confirmed to be present on mouse liver LLDs are carboxylesterases 1 and 3, apoE, MTP (Wang et al., 2007), and apoCIII (Sundaram et al., 2010). However, these proteins are not exclusively associated with LLDs but are also present in their "lipid-free" form in the ER lumen.

The isolation and analysis of LLDs provided direct biochemical evidence that LLDs are true entities that possess different biochemical properties from that of CLDs and VLDL. This protocol can be adapted to a wide variety of applications to study the mechanism of LLD formation, the role of LLD-associated proteins in VLDL secretion, as well as genetic, nutritional, and pharmaceutical influence on these processes.

This protocol also provides an example of using BODIPY fatty acid analogues to study dynamics of LD formation and metabolism. BODIPY fatty acids are available

in different chain length, and both BODIPY-C_{16} and BODIPY-C_{12} are reported to incorporate well into cells (Thumser & Storch, 2007). We have only used BODIPY-C_{12} since with the inclusion of the BODIPY head group the analogue mimics the natural chain length of OA. BODIPY-C_{12} is esterified into both phospholipids and neutral lipids and labels the same LDs as those stained with conventional LD dyes (BODIPY 493/503 and Nile Red), thus showing properties similar to natural long-chain fatty acids (Wang et al., 2010).

We used primary hepatocytes in this protocol due to our specific need to compare cells of different genetic backgrounds; however, this method can be easily adapted to other cell types. When a new cell type is used, several control experiments should be performed to ensure that the analogues are metabolized as would be expected from native fatty acids. Controls should include: (1) confirmation of esterification into phospholipids and neutral lipids by TLC; (2) monitoring dynamics of analogue uptake and incorporation, preferably compare these with radiolabeled OA; (3) colocalization with conventional neutral lipid dyes.

This method can also be used to study the localization of nascent CLD formation, interactions of proteins, and nascent LDs immediately after fatty acid loading of cells. However, these applications require either transient expression of fluorescently tagged proteins, or immunostaining of a protein of interest. Immunofluorescence staining of LD-associated proteins is challenging since lipids cannot be fixed by general fixatives. Some recently published protocols addressed this problem (DiDonato & Brasaemle, 2003; Ohsaki, Maeda, & Fujimoto, 2005). Primary hepatocytes are known for being difficult to transfect. Liposome-based transfection reagents, such as Lipofectamine 2000, are able to deliver plasmid DNAs into hepatocytes when the ratio of transfection reagent DNA is optimized; however, the transfection efficiency is generally very low, less than 15%. We found that a cell type-specific reagent, Targefect-Hepatocytes, together with its enhancer Virofect (Targeting Systems, CA), provides a much higher transfection efficiency (Wang et al., 2010).

CONCLUSION

We have provided detailed protocols and expected outcomes for the purification and analysis of hepatic LDs, including CLDs and LLDs. We have also presented a cell biological method for monitoring LD dynamics in hepatocytes. These protocols can be extended to study liver LD metabolism under different metabolic states.

Acknowledgments

Supported by grants from the Canadian Institutes of Health Research and Natural Sciences and Engineering Research Council of Canada. Huajin Wang is supported postdoctoral fellowship from the Canadian Institutes of Health Research. Richard Lehner is an Alberta Innovates-Health Solutions Scientist.

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