Isolating lipid droplets from multiple species

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The lipid droplet (LD) is a cell organelle that has been linked to human metabolic syndromes and that can be exploited for the development of biofuels. The isolation of LDs is crucial for carrying out morphological and biochemical studies of this organelle. In the past two decades, LDs have been isolated from several organisms and investigated by microscopy, proteomics and lipidomics. However, these studies need to be extended to more model organisms, as well as to more animal tissues. Thus, a standard method that can be easily applied to these new samples with the need for minimal optimization is essential. Here we provide an LD isolation protocol that is relatively simple and suitable for a wide range of tissues and organisms. On the basis of previous studies, this 7-h protocol can yield 15–100 μg of protein-equivalent high-quality LDs that satisfy the requirements for current LD research in most organisms.

INTRODUCTION

LDs exist in almost all organisms from bacteria through to humans, and they have been referred to as oil bodies, lipid bodies, adiposomes, oil droplets and fat bodies[1–3]. Imbalances of lipid metabolism can lead to many metabolic disorders in humans, such as obesity, fatty liver, type 2 diabetes and cardiovascular diseases[4,5]. Several model systems[1,6], including bacteria, yeast, green algae, *Caenorhabditis elegans*, *Drosophila*, plants and several types of mammalian cells and tissues, have been used to study LD biogenesis and functions. In addition, bacterial and green algal LDs have received considerable attention, as they hold potential for use in the development of biofuels[7,8]. In the past decade, our laboratory and other groups have purified LDs from various systems and studied their proteomes, showing that they are more complex organelles than originally anticipated, and that they may be involved in lipid metabolism, membrane biosynthesis, membrane trafficking and signal transduction[9–14].

Development of LD isolation methods

Techniques for isolating LDs can be traced back to the 1970s or even earlier. Additional methods for isolating LDs for proteomic and/or functional studies from various species have been established in the past two decades (**Table 1**). The development of these methods has been driven by the need for increased purity and biological activity of the isolated LDs. As almost all cells from current model systems can form LDs, a standard method is needed that is simple, suitable for different organisms and capable of yielding a reproducible amount of high-quality LDs. Our group has contributed to research on LDs by establishing a simple and stable method that has successfully been used to isolate LDs from mammals[15–18], *C. elegans*[19], yeast[20] and bacteria[21]. Source-specific adaptations are required for tissue homogenization and cell disruption, which are described below (see also **Table 2**).

Here we provide a detailed protocol that is based on our own previously published methods[15,18,19,21]. We believe that this protocol will facilitate the isolation of high-quality LDs from a variety of systems.

Applications and advantages of the protocol

To our knowledge, this is the first detailed protocol for isolating LDs that has been shown to be effective on a range of source materials. It is relatively simple to use, and microgram quantities of proteins from high-quality (pure and biologically active) LDs can be obtained. The yield and quality of LDs using this protocol have good reproducibility.

Limitations of the protocol

As this protocol contains several washing steps for LDs after ultracentrifugation, in some cases very small LDs may be lost during both the ultracentrifugation and washing steps. In addition, supersized LDs may be disrupted during homogenization and ultracentrifugation. In eukaryotes, small amounts of membrane structures that are in physical contact with LDs may be co-isolated with LDs, which is undesirable if the aim is to focus on LD-associated proteins.

Overview of the protocol

LDs have low density because of their neutral lipid core. The rationale behind this protocol is based on the fact that LDs float on top of all aqueous gradients after centrifugation. The processes and buffers used for different organisms are basically the same. Briefly, samples containing LDs are first washed and homogenized in a buffer containing 250 mM sucrose to protect intracellular organelles. The homogenate is then ultracentrifuged to separate the LDs from other cellular fractions. The LD fraction is collected and washed several times to remove any contamination until the purity of the isolated LDs is satisfactory for quality analysis by biochemical and morphological studies. Note that some parts of the protocol will probably need to be adapted and optimized, as described in Experimental design below.

Experimental design

Sample homogenization. A cell suspension is first prepared from tissue samples and multicellular organisms, such as *C. elegans*, *Drosophila* and plants. The suspension can be prepared

TABLE 1 | Species from which LDs have been successfully isolated.

Species			References
Bacteria		*Rhodococcus opacus*	24
		R. ruber	24
		Mycobacterium bovis	25
		R. jostii	21
Green algae		*Chlamydomonas reinhardtii*	26
			27
Yeast		*Saccharomyces cerevisiae*	20,28,29
Nematode		*Caenorhabditis elegans*	19
Fruit fly		*Drosophila melanogaster*	30,31
Plants		*Arachis hypogea* L.	32
		Allium cepa L.	33
		Brassica capitata L.	33
		Gossypium hirsutum L.	33
		Arabidopsis thaliana	34
		Sesamum indicum L.	35
		Brassica napus	36–38
Mammals	Mammalian cells	Fibroblasts	15,17
		Adipocytes	22
		Hepatocytes	39,40
		Macrophage	41
		Epithelia	16,17,42
		Skeletal muscle cells	18
		Beta cells	43
	Mammalian tissues	Rat renal papillae	44
		Human aorta	45
		Rat liver	46
		Bovine mammary tissue	47
		Rabbit renal medulla	48
		Rat adrenal	49
		Rat hepatic stellate cells	50
		Mouse mammary gland	51
		Mouse liver	18,51
		Mouse skeletal muscle	18

by homogenization in buffer A (see Reagent Setup) containing appropriate protease inhibitors and inhibitors of kinases, phosphatases, lipases or other enzymes depending on experimental requirements. Usually, we recommend using a Dounce homogenizer to disrupt mammalian tissues such as skeletal muscle and heart tissues, and a Polytron to break tough samples such as *C. elegans*, which have a thick cuticle. However, supersized LDs, such as LDs from adipose tissue, may be damaged using either method because of mechanical shearing. All samples should be fresh. Frozen samples should not be used, because LDs fracture under freezing conditions.

Cell disruption. The purpose of this step is to disrupt the cells under high pressure to release their contents. Cell suspensions prepared by homogenization, and samples of unicellular organisms such as bacteria, yeast, green algae, and cultured cell lines, are disrupted in this step using a French press cell or a nitrogen bomb (also in buffer A with inhibitors). The type of homogenizer, the pressure applied and the treatment time are the crucial factors for obtaining intact LDs and other cellular organelles, and they may differ from sample to sample. For organisms with a cell wall, a French press cell is the better choice as it can provide higher pressure, whereas a Nitrogen bomb is more suitable for mammalian cells. Our empirical applied pressure and treatment times are summarized in **Table 2**. The use of inhibitors to protect LDs from damage and modification is also essential during this step, and the inhibitor(s) used is dependent on the proteins (or other molecules) of interest.

Ultracentrifugation. The most important physical property of LDs is their low density; they float on top of all aqueous gradients after centrifugation. The speed of ultracentrifugation mainly depends on the size of the LDs; larger LDs will float at lower speeds. A speed of 10,000*g* for 1 h is generally required for LDs to float well. Although very small LDs may not be able to move to the top of the gradient under these conditions, increasing the speed and duration of centrifugation not only damages large LDs but may also strip proteins from LDs. Examples of effective ultracentrifugation conditions that result in less damage and loss of LDs are given in the PROCEDURE and **Table 2**. However, one should keep in mind that the size of the LDs is the key factor to consider when deciding on centrifugation conditions. One may need to optimize the centrifugation conditions on the basis of the size of the LDs in the starting material.

Washing. The collected LD fraction may contain some contaminating proteins from two sources: the cytosol and the broken membranes that nonspecifically adhere to the LDs during homogenization. Washing is needed to reduce the contamination. The number of washing steps to use can be determined by whether there is any membrane pellet left in the bottom of the tube. However, trace amounts of these proteins will remain in the LD fraction even after washing many times. One can then adjust the pH of the washing buffer to 11 to disrupt any nonspecific binding of these proteins to LDs[22]. LDs, especially small ones, may be lost at each washing step. If small LDs are being studied, increasing the centrifugation speed to 100,000*g* for washing may be necessary.

TABLE 2 | Starting materials and sample treatment conditions used for isolating LDs from various organisms.

Sample	Starting amounts	Sample collection (Step 1)	Homogenization (Steps 3 and 4)	Ultracentrifugation (Step 8)	Typical yield of LD protein (μg)	Typical size range of LDs (μm)
Caenorhabditis elegans	4×10^5 worms	Wash the worms three times with 50 ml of PBS plus 0.001% (vol/vol) Triton X-100 and then once with 50 ml of buffer A. Resuspend the worms in 10 ml of buffer A with 0.2 mM PMSF	Homogenize the worms four times on ice with a Polytron at 14,000 r.p.m., 1 min per time with 30-s intervals. Centrifuge at 1,000*g* for 30 s, keep the supernatant and proceed to Step 5	10,200–10,600*g* for 1 h at 4 °C	~30	0.5–2
Mouse skeletal muscle	Muscle dissected from hind legs of 20 C57BL/6 mice	Remove the fascia and connective tissues, then cut into small pieces and resuspend in 12 ml of buffer A with 0.2 mM PMSF	Homogenize on ice 20 times with a tight-fitting Dounce. Centrifuge at 100*g* for 10 min at 4 °C, keep the supernatant and proceed to Step 5	182,000*g* for 1 h at 4 °C	~15	0.5–1
Mouse liver	Livers of two C57BL/6 mice		Homogenize on ice ten times with a loose-fitting Dounce. Centrifuge at 100*g* for 10 min at 4 °C, keep the supernatant and proceed to Step 5	2,000*g* for 30 min at 4 °C	~100	1–5
CHO-K2 cells	Cells from ten 150-mm plates (100% confluent)	Wash the dishes with 10 ml of ice-cold PBS. Scrape the cells and resuspend in 100 ml of PBS, and centrifuge at 1,000*g* for 10 min at 4 °C to collect cells	None required. Proceed directly to Step 5	182,000*g* for 1 h at 4 °C	~20	0.1–1
Bacteria/yeast	400 ml of liquid culture in stationary phase	Collect the cells by centrifuging the culture at 4,000*g* for 10 min. Wash the cells twice with 30 ml of PBS and collect cells	None required. Proceed directly to Step 5	10,200–10,600*g* for 1 h at 4 °C for yeast and 182,000*g* for 1 h at 4 °C	~25	0.1–1

Assessment of LD quality by western blotting and data analysis. An important way to check the purity of LDs obtained is to perform western blotting to determine the distribution of marker proteins between the LD fraction and other cellular fractions. With regard to the normalization of data in western blotting, we think that it is better to present the data with respect to the amount of protein used rather than as a percentage of the amount of starting material used, as it is unclear how many LDs are lost during the purification process. Therefore, we recommend using equal protein loading to determine the enrichment of marker proteins to judge the purity of LDs[15,18,19,21]. However, as a parallel analysis, it is useful to sample and compare fractions after collection of LDs and before the washes by loading an equal percentage of the total fractions.

MATERIALS

REAGENTS

- Suitable source material (e.g., tissues or organs from C57B/L6 mice; see also **Table 2** for a list of starting materials for which this protocol has been successfully used) **! CAUTION** All experiments with animals must be carried out according to all relevant governmental and institutional regulatory guidelines.
- Inhibitors (user specified; see Experimental design)
- NaCl (Sigma-Aldrich, cat. no. S7653)
- KCl (Sigma-Aldrich, cat. no. P9333)
- $MgCl_2$ (Sigma-Aldrich, cat. no. 208337)
- KH_2PO_4 (Sigma-Aldrich, cat. no. P5655)
- Na_2HPO_4 (Sigma-Aldrich, cat. no. S5136)
- Sucrose (Sigma-Aldrich, cat. no. S9378)
- Tricine (Sigma-Aldrich, cat. no. T0377)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- DMSO (Sigma-Aldrich, cat. no. D2650)
- PMSF (Sigma-Aldrich, cat. no. P7626) **! CAUTION** PMSF is acutely toxic. Handle it with protective gloves.
- Chloroform (Sigma-Aldrich, cat. no. C2432) **! CAUTION** Chloroform is acutely toxic. Handle it in a fume hood. Dispose of chloroform in accordance with local regulations.
- Acetone (Sigma-Aldrich, cat. no. 650501) **! CAUTION** Acetone is acutely toxic and is an unstable explosive. Avoid flames and handle it in a fume hood. Dispose of acetone in accordance with local regulations.
- Hexane (Sigma-Aldrich, cat. no. 296090) **! CAUTION** Hexane is acutely toxic and is an unstable explosive. Avoid flames and handle it in a fume hood. Dispose of hexane in accordance with local regulations.
- Diethyl ether (Sigma-Aldrich, cat. no. 296082) **! CAUTION** Diethyl ether is acutely toxic and is an unstable explosive. Avoid flames and handle it in a fume hood. Dispose of diethyl ether in accordance with local regulations.
- Acetic acid (Sigma-Aldrich, cat. no. 320099)
- Methanol (Sigma-Aldrich, cat. no. 322415) **! CAUTION** Methanol is acutely toxic and is an unstable explosive. Avoid flames and handle it in a fume hood. Dispose of methanol in accordance with local regulations.
- Deionized water
- Ice
- Tris base (Sigma-Aldrich, cat. no. T1503)
- Glycerol (Sigma-Aldrich, cat. no. G5516)
- SDS (Sigma-Aldrich, cat. no. L4390)
- 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M6250)
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126)
- Iodine (Sigma-Aldrich, cat. no. 207772)
- LipidTox Red (Life Technologies, cat. no. H34476)
- Nile Red (Life Technologies, cat. no. N1142)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)

EQUIPMENT

- Nitrogen bomb (Parr Instrument, cat. no. 4639; cell disruption bomb)
- Polytron homogenizer (Cole-Parmer, LabGEN 700 homogenizer)
- Dounce homogenizer (Wheaton, cat. no. 357542)
- French press cell (JNBIO, cat. no. JN-3000, high-pressure homogenizer)
- Ultracentrifuge (Beckman Coulter, Optima L-100 XP ultracentrifuge)
- Ultracentrifuge (Beckman Coulter, Optima MAX ultracentrifuge)
- TLA100.3 rotor (Beckman Coulter, cat. no. 349490)
- TLA100.3 1.5-ml tube (Beckman Coulter, cat. no. 362333)
- SW40 Ti rotor (Beckman Coulter, cat. no. 331302)
- SW40 14-ml tube (Beckman Coulter, cat. no. 331374)
- Particle analyzer (Beckman Coulter, Delsa Nano C particle analyzer)
- Temperature-controlled benchtop centrifuge (Eppendorf, cat. no. 5417R)
- Centrifuge tubes (1.5 ml, Axygen, cat. no. NCT-150)
- Cell scrapers (Sarstedt, cat. no. 83.1830)
- Confocal microscope (Olympus, cat. no. FV1000)
- Transmission electron microscope (FEI, Tecnai 20)
- Pipette and pipette tips
- ImageJ software (http://rsb.info.nih.gov/ij/)

REAGENT SETUP

PBS Dissolve 8 g of NaCl, 0.2 g of KCl, 3.63 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml of deionized water. Adjust the pH to 7.4 with HCl. Bring the volume to 1 liter; sterilize the solution and store it at 4 °C in preparation for Step 1 of the PROCEDURE. This solution can be stored indefinitely at 4 °C.

Sucrose (2.5 M) Add 800 g of sucrose to 400 ml of deionized water and stir until most of the sucrose is dissolved. Bring the volume to 1 liter and stir until all of the sucrose is dissolved. This solution can be stored at room temperature indefinitely. **▲ CRITICAL** It will take a long time to dissolve this quantity of sucrose. This solution should be prepared in advance and can be stored for 3 months at 4 °C.

Buffer A Buffer A is 20 mM tricine and 250 mM sucrose (pH 7.8). Dissolve 1.79 g of tricine in 400 ml of deionized water. Adjust the pH to 7.8 with KOH. Add 50 ml of 2.5 M sucrose. Bring the volume to 500 ml and sterilize the buffer. Keep buffer A at 4 °C in preparation for Step 1 of the PROCEDURE. This solution can be stored for 3 months at 4 °C.

Buffer B Buffer B contains 20 mM HEPES, 100 mM KCl and 2 mM $MgCl_2$ (pH 7.4). Dissolve 0.95 g of HEPES, 1.49 g of KCl and 0.038 g of $MgCl_2$ in 180 ml of deionized water. Adjust the pH to 7.4 with KOH. Bring the volume to 200 ml and sterilize the buffer. Keep buffer B at 4 °C in preparation for Step 7 of the PROCEDURE. This solution can be stored for 3 months at 4 °C.

PMSF (1,000×, 0.2 M) Dissolve 0.35 g of PMSF in 10 ml of DMSO. Divide the solution into 1-ml aliquots. The aliquots should be protected from light and stored at −20 °C. PMSF can be stored for 1 year. **! CAUTION** PMSF is acutely toxic. Handle it with protective gloves.

SDS sample buffer (2×) SDS sample buffer is 125 mM Tris, 20% (vol/vol) 4% (wt/vol) SDS, glycerol, 4% (vol/vol) 2-mercaptoethanol and 0.04% (wt/vol) bromophenol blue (pH 6.8). Dissolve 1.51 g of Tris in 50 ml of deionized water. Adjust the pH to 6.8 with HCl. Add 4 g of SDS, 20 ml of glycerol, 10 ml of 2-mercaptoethanol and 0.8 g of bromophenol blue. Stir until all dissolve and then bring the volume to 100 ml. Store for 1 month at room temperature. **! CAUTION** 2-Mercaptoethanol is highly toxic; handle it with protective gloves.

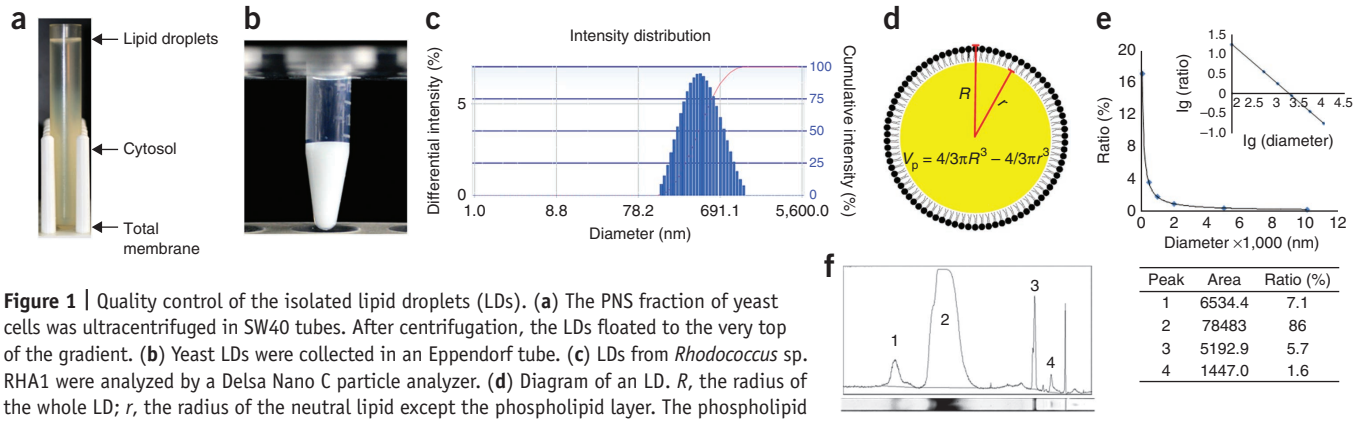
PROCEDURE

Sample collection and/or homogenization ● TIMING 1–2 h

1| Collect and wash the samples. The amount of starting material will depend on how many LDs are needed and on the gradient capacity; typical starting amounts and conditions required for the listed yield of LDs are shown in **Table 2** for a range of starting materials.

2| Suspend the sample in buffer A plus 0.2 mM PMSF and keep it on ice for 20 min.

3| Homogenize the samples from multicellular organisms and tissues into small pieces using a Polytron or into a cell suspension with a Dounce homogenizer; typical homogenization conditions for a range of materials are given in **Table 2**. The entire process should be performed on ice with ice-cold buffer A containing 0.2 mM PMSF. For cultured cells or single-cell organisms (e.g., yeast, bacteria), no homogenization is necessary; omit this step and proceed straight to cell disruption (Step 5).
▲ CRITICAL STEP To protect against protein degradation, it is crucial to keep the sample cold and to add protease inhibitors. Add PMSF to the buffer just before use, as PMSF is unstable in the aqueous phase.
▲ CRITICAL STEP Be aware that very large LDs can be easily broken by mechanical force when homogenizing with either a Polytron or a Dounce.



Peak	Area	Ratio (%)
1	6534.4	7.1
2	78483	86
3	5192.9	5.7
4	1447.0	1.6

Figure 1 | Quality control of the isolated lipid droplets (LDs). (**a**) The PNS fraction of yeast cells was ultracentrifuged in SW40 tubes. After centrifugation, the LDs floated to the very top of the gradient. (**b**) Yeast LDs were collected in an Eppendorf tube. (**c**) LDs from *Rhodococcus* sp. RHA1 were analyzed by a Delsa Nano C particle analyzer. (**d**) Diagram of an LD. *R*, the radius of the whole LD; *r*, the radius of the neutral lipid except the phospholipid layer. The phospholipid ratio is calculated as V_p / $4/3\pi R^3 \times 100\%$. (**e**) The relationship between LD diameter and phospholipid ratio. The log-log plot of these two parameters is shown in the inset chart. (**f**) Lipid analysis of LDs from *Rhodococcus* sp. RHA1 using TLC and ImageJ. 1, unknown neutral lipid; 2, triacylglycerol; 3, diacylglycerol; 4, phospholipid. Panels **c** and **f** are reproduced from Ding *et al.*[21].

4| Centrifuge the sample after homogenization to collect the sample; typical centrifugation conditions for a range of materials are given in **Table 2**.

Cell disruption ● TIMING 1–2 h

5| Disrupt cell suspensions under high pressure. For bacteria and yeast samples (from Step 2), disrupt the cells three times with a French press cell at 1,000 bar or 1,500 bar, respectively, at 4 °C. For samples from multicellular organisms and tissues (from Step 4), including cultured mammalian cells (from Step 2), use a nitrogen bomb at a pressure of 35 bar for 15 min on ice to disrupt cells.

6| Centrifuge the homogenate at 3,000*g* for 10 min at 4 °C to remove nuclei, cell debris and unbroken cells. The supernatant is the postnuclear supernatant (PNS) fraction. A 500-µl aliquot of PNS should be retained on ice for use as a control for determining purification efficiency at Step 17D.

Floating the LDs by ultracentrifugation ● TIMING ~1 h

7| Transfer 10 ml of the PNS fraction from Step 6 into a SW40 tube and then load 2 ml of buffer B on top of the PNS.
▲ CRITICAL STEP The volume of buffer B on top of the PNS can be increased. The larger the volume of buffer B, the purer the LDs will be. Others have also loaded a sucrose gradient on top of the sample before loading buffer B in order to force LDs to move through the gradient, thus enhancing the washing effect[23]. It may also be effective to use a higher pH, such as pH 11, to disrupt interactions between LDs and loosely attached proteins and membrane fragments[22].

8| Centrifuge the sample using an ultracentrifuge (**Fig. 1a**). The speed and time may vary according to the size of the LDs from different organisms. Typical conditions used in our lab are given in **Table 2** for a range of starting materials.
▲ CRITICAL STEP If the speed is too high, large LDs will be disrupted and stick to other cell components causing membrane contamination. If the speed is too low, small LDs will be lost.
? TROUBLESHOOTING

LD collection ● TIMING 1–2 h

9| Carefully collect LDs from the top band of the gradient formed in Step 8 and transfer them into an Eppendorf tube using a 200-µl pipette. Aim to collect as many LDs as possible in the lowest possible volume of buffer (**Fig. 1b**). This is the LD fraction. Retain the fraction on ice before and during the following washing steps. An aliquot can be saved at this point as crude LD sample if you are using equal percentage of the total fractions to normalize data. In addition, aliquots can be taken for LD quality analysis in Step 17. The LDs can be retained on ice for up to 10 h.
▲ CRITICAL STEP To avoid losing LDs on the surface of the pipette tip, soak the tip in buffer B first and collect LDs at a volume of less than 10 µl at a time. The buffer underneath the LDs may contain cytosol proteins and membranes. Therefore, the more buffer collected, the greater the contamination.
? TROUBLESHOOTING

10| Take 1 ml of solution from the middle of the gradient formed in Step 8 and centrifuge at 270,000*g* for 1 h at 4 °C in a microcentrifuge tube using a TLA100.3 rotor. Collect and retain a 500-µl aliquot of supernatant as the cytosol fraction for determining purification efficiency at Step 17D.

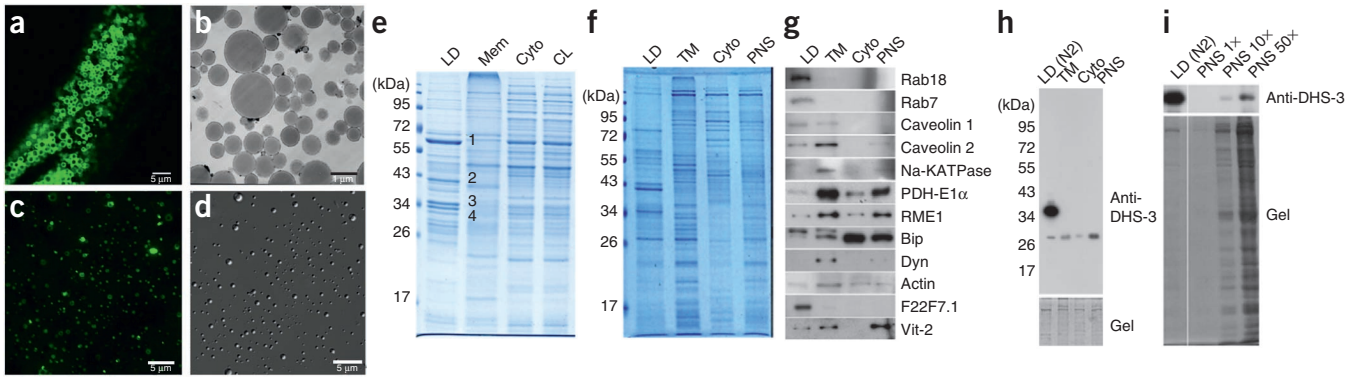


Figure 2 | Verification of isolated lipid droplets (LDs). (**a**) *C. elegans* overexpressing short-chain dehydrogenase (DHS-3), viewed by confocal microscopy. (**b**) Purified LDs from CHO K2 cells were fixed, dehydrated, embedded, sectioned, stained and examined by electron microscopy. (**c**,**d**) Purified LDs from *C. elegans* overexpressing DHS-3 viewed by fluorescence microscopy. (**e**) Proteins from *Rhodococcus* sp. RHA1 LDs, membrane (Mem), cytosol (Cyto) and whole-cell lysates (CL) were separated by SDS-PAGE followed by Colloidal Blue staining. Reproduced from Ding *et al.*[21]. (**f**) Proteins from *C. elegans* LDs (LD), total membrane (TM), cytosol and the PNS were separated by SDS-PAGE followed by colloidal blue staining. (**g**,**h**) Equal amounts of proteins from the four fractions of *C. elegans* were blotted with the indicated antibodies. PDH-E1α, pyruvate dehydrogenase complex subunit E1α; RME1, receptor-mediated endocytosis 1; Dyn, dynamin. (**i**) More PNS was used to determine the enrichment of the marker protein. Panels **f**–**i** are reproduced from Zhang *et al.*[19].

11| Wash the pellet in the SW40 tube from Step 8 three times with 1 ml of buffer B and then resuspend the pellet in 1 ml of buffer B. Retain a 50-μl aliquot as the total membrane fraction for determining purification efficiency at Step 17D.

Washing the LD fraction ● TIMING ~1 h

12| Centrifuge the Eppendorf tube from Step 9 at 20,000*g* for 5 min at 4 °C to separate the LDs from the buffer.
▲ **CRITICAL STEP** The sample should be kept on ice or at 4 °C throughout Steps 12–16.

13| Remove and discard the underlying solution and pellet using a gel-loading tip. If the volume of LDs is greater than 10 μl, divide the LDs into two tubes to obtain better washing and lipid-protein separation in subsequent steps.
▲ **CRITICAL STEP** The pellet at the bottom of the tube contains some membranes. It must be removed completely.

14| Resuspend LDs in 200 μl of buffer B.

15| Repeat Steps 12–14 two more times.

16| If you are preparing LD samples for proteomic identification used in Step 17D, resuspend the LDs in 1 ml of buffer B and centrifuge at 270,000*g* for 10 min at 4 °C using a TLA100.3 rotor. Carefully remove the visible pellet (if present) at the bottom of the tube using a gel-loading tip. Otherwise, proceed directly to Step 17.
▲ **CRITICAL STEP** Step 16 is only used for preparing samples for proteomic identification, as the extra ultracentrifugation can damage the LDs.

Quality control ● TIMING variable

17| Monitor the quality of isolated LDs by biochemical and morphological methods. Option A describes measuring the size of LDs using a particle analyzer (**Fig. 1c**); option B describes analyzing the phospholipid ratio of LDs by thin-layer chromatography (TLC; **Fig. 1d**–**f**); option C describes analyzing LDs by light microscopy (**Fig. 2a**,**c**,**d**); option D describes comparing the protein profiles of LDs, total membranes, cytosol and the PNS (**Fig. 2e**,**f**); and option E describes analyzing LDs by western blotting (**Fig. 2g**–**i**). Quality control of purified LDs can also be carried out by electron microscopy (**Fig. 2b**); negative staining, positive staining and ultrathin sectioning of LDs can all be used. Detailed methods are given in our previous reports[15,19]. Of these, ultrathin sectioning is the most accurate but the most time-consuming approach.

(A) Measurement of LD size using a particle analyzer ● TIMING ~1 h

(i) Dilute 5 μl of purified LDs in 3 ml of buffer B in a quartz cuvette.
(ii) Invert the cuvette several times to obtain a homogenous sample.
(iii) Measure the size of the LDs using a Delsa Nano C particle analyzer (or equivalent) according to the manufacturer's instructions.

(B) Analysis of the phospholipid ratio by TLC ● **TIMING ~1 h**

(i) Separate neutral lipids in hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol) and phospholipids in chloroform/methanol/acetic acid/H_2O (75:13:9:3, vol/vol/vol/vol).

(ii) Visualize the plate using iodine vapor.

(iii) Quantify the lipids by grayscale scanning (NIH ImageJ software) according to the software manual.

? TROUBLESHOOTING

(C) Analysis of LDs by light microscopy ● **TIMING ~2 h**

(i) Stain LDs with LipidTOX Red or Nile red, if necessary, following the manufacturer's instructions.

(ii) Place the LDs on a glass slide and view the sample with an Olympus FV1000 confocal microscope or equivalent equipment.

▲ **CRITICAL STEP** It is better to view the sample after waiting for 15 min, as the LDs will be more closely attached to the coverslip.

(D) Comparison of protein profiles among LDs, total membranes, cytosol and PNS ● **TIMING 1–2 d**

(i) Separate the proteins and lipids of LD samples from Step 15 or 16 with 1 ml of chloroform/acetone (1:1, vol/vol), collect the lipids in the organic phase, air-dry the pellet and dissolve the pellet in 50–100 µl of 2× SDS sample buffer.

▲ **CRITICAL STEP** If the pellet is somewhat yellow, add 1 ml of chloroform/acetone (1:1, vol/vol) to the pellet again, and vortex the mixture to dissolve the lipids. A volume of 1 ml of chloroform/acetone is suitable for extracting lipids and precipitating proteins from 50 µg of protein-equivalent LDs.

? TROUBLESHOOTING

(ii) Add 500 µl of 2× SDS sample buffer to the 500-µl aliquot of the PNS fraction (retained at Step 6).

(iii) Mix the 500-µl aliquot of the cytosol fraction (retained at Step 10) with 500 µl of 2× SDS sample buffer.

(iv) Pellet the 50-µl aliquot of total membrane (retained at Step 11) and separate the lipids and proteins with 1 ml of chloroform/acetone (1:1, vol/vol), and dissolve the protein pellet in 100 µl of 2× SDS sample buffer.

(v) Separate each of the samples obtained in Steps 17D(i–iv) by 10% (wt/vol) SDS-PAGE followed by colloidal blue or silver staining using standard methods for comparing the protein profile. Alternatively, the samples can be used to perform proteomic analysis or western blotting (see Step 17E).

? TROUBLESHOOTING

■ **PAUSE POINT** The samples can be stored at −20 °C for proteomic analysis or western blotting (Step 17E).

(E) Analysis of LDs by western blotting ● **TIMING 1–2 d**

(i) Separate the LD proteins using 10% (wt/vol) SDS-PAGE according to standard methods.

(ii) Transfer the proteins to a PVDF membrane by following standard procedures.

(iii) Blot the target proteins using appropriate antibodies according to standard methods.

(iv) Detect the signals using an enhanced chemiluminescence (ECL) system according to the manufacturer's instructions.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
8, 9	LD aggregation	LDs may be disrupted	Lower the centrifugation speed
9	The quantity of LDs is much lower than expected	The quantity of LDs in the samples is too low	Prepare the sample well to increase the quantity of LDs
		The sample is not homogenized well enough	Repeat the homogenization step two or three times more
17B	The ratio of phospholipids is too high	Membrane contamination	Repeat Step 16
17D(i)	LD proteins cannot be pelleted	The ratio of chloroform is too high	Add more acetone
	There is still some yellow material in the pellet during isolation of LD proteins	Lipids have not been extracted completely	Re-extract the sample with acetone one more time

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
17D(v)	Protein bands are smeared	Proteins may be degraded	Add PMSF at each step and keep the sample on ice all the time
		Proteins may not be dissolved well, as they are very hydrophobic	Increase the SDS concentration in the sample buffer
		Proteins may not be reduced and denatured well	Increase the concentrations of the reducing agent and the SDS in the sample buffer

● TIMING

Steps 1–4, sample collection and/or homogenization of the sample into cell suspension: 1–2 h
Steps 5 and 6, cell disruption under high pressure: 1–2 h
Steps 7 and 8, separation of LDs: ~1 h
Steps 9–11, collection of LDs: 1–2 h
Steps 12–16, removal of contamination from LDs: ~1 h
Step 17A, measurement of LD size: ~1 h
Step 17B, analysis of lipid ration: ~1 h
Step 17C, analysis of LDs by light microscopy: ~2 h
Step 17D, comparison of protein profiles: ~1–2 d
Step 17E, western blotting analysis: ~1–2 d

ANTICIPATED RESULTS

Because of their low densities, LDs should float on top of the gradient in the ultracentrifuge tube after centrifugation during Step 8 (**Fig. 1a**). The collected LDs should be well suspended and will appear like a milky solution in Step 9 (**Fig. 1b**). If this is not the case, it is likely that most of the LDs are broken, in which case sample homogenization and/or disruption conditions for releasing LDs should be adjusted to use a lower force. Lower centrifugation conditions (lower force and/or shorter time) may be needed. In addition, more attention should be paid to careful collection and washing of LDs.

The sizes of LDs obtained using this protocol will vary. For example, LD size, if analyzed with a Delsa Nano C particle analyzer, should have a bell-shaped distribution of a certain range (depending on LD source; **Fig. 1c**).

The purity of the LDs should be checked during and after isolation. There should be no visible pellet at the bottom of the tube when washing the LDs during Step 12. The presence of a pellet indicates that there may be some membrane contamination. One or two more washes may be needed at this step. Theoretically speaking, the volume of the whole LD (V_w) is equal to $4/3\pi R^3$, the volume of the neutral lipid (V_n) is equal to $4/3\pi r^3$ (R, the radius of the whole LD; r, the radius of the neutral lipid except the phospholipid layer), the volume of the phospholipid (V_p) is equal to $V_w - V_n$, and the phospholipid ratio is equal to $V_p/V_w \times 100\%$ (**Fig. 1d**). **Figure 1e** describes the relationship between LD size and the phospholipid ratio. The phospholipid ratio in the whole LD should range from 17% (if the diameter is 100 nm) to 0.18% (if the diameter is 10 μm). If the phospholipid ratio is much higher, as measured by TLC, there may be some membrane contamination. **Figure 1f** shows that the phospholipid ratio in isolated LDs of *Rhodococcus* sp. RHA1 is nearly 1.6%, indicating that these LDs are pure. In addition, there should be almost no membrane contamination in preparations of pure LDs when examined by confocal microscopy (**Fig. 2a**,**c**,**d**) or electron microscopy (**Fig. 2b**). When the purity is checked by polyacrylamide gel electrophoresis, proteins in the LD fraction, as visualized by colloidal blue staining (**Fig. 2e**,**f**), should be unique and should not be visible in the membrane, cytosol or PNS fractions. If western blotting is used to test purity, LD marker proteins should be highly enriched in the LD fraction compared with the membrane, cytosol and PNS fractions, and marker proteins for other fractions should be detected mainly in those fractions and not in isolated LDs when equal quantities of proteins are loaded (**Fig. 2g**,**h**). When the LD fraction is of high purity, more protein of other cellular fractions needs to be loaded to detect LD marker protein in those fractions (**Fig. 2i**).

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