A single centrifugation method for isolating fat droplets from cells and tissues

Lydia-Ann L. S. Harris, Trevor M. Shew, James R. Skinner, and Nathan E. Wolins

Center for Human Nutrition, Washington University School of Medicine, St. Louis, MO

Abstract  Fat droplets (FDs) have important roles in cellular energy regulation. Isolating FDs from either cells or tissue continues to be important for studying these organelles. Here, we describe a procedure wherein whole homogenates of cultured cells or tissue are fractionated with a single centrifugation step in a standard microcentrifuge. This procedure reproducibly yields three fractions highly enriched in either FDs, soluble cellular components, or sedimentable organelles/membranes.—Harris, L-A. L. S., T. M. Shew, J. R. Skinner, and N. E. Wolins. A single centrifugation method for isolating fat droplets from cells and tissues. J. Lipid Res. 2012. 53: 1021–1025.

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The incidence of obesity has increased and consequently so has obesity-associated metabolic disease. Obesity is characterized by excessive fat stores. Fat is stored in fat droplets (FDs). These organelles are composed of a core of triglyceride and other neutral lipid surrounded by a unique protein coat. Evidence is amassing that these organelles are involved in nutrient flux through cells and animals and that the FD coat proteins are regulators of nutrient flux. Fractionating FDs away from cytosol and sedimentable organelles has been a useful tool in studying FDs (1–6). However, these methods were laborious because the large format diluted the samples and generated many fractions, making analysis more cumbersome. To improve the ease of FD analysis, we have developed a simple and efficient procedure to generate fractions enriched in either FDs, cytosol, or sedimentable organelles, starting from cell culture or whole tissue.

We utilized two properties conferred by the fat core of FDs to fractionate them away from the cytosol and sedimentable organelles. First, because FDs lack an aqueous core, they are unaffected by osmotic changes. Thus, we utilized osmotic shock followed by mechanical shear to disrupt structural elements linking organelles. Second, the fat in FDs causes them to float in an aqueous solution. Thus, we used centrifugation to float FDs away from the cytosol and sedimentable organelles. We developed a procedure using these strategies and demonstrate that this procedure reproducibly generates FD (floating), cytosol (soluble), and sedimentable organelle (pellet) enriched fractions from cells or tissue.

MATERIALS AND METHODS

Solutions, supplies, and equipment required

Lysis buffer (10 mM HEPES and 1 mM EDTA, titrated to pH 7.4 with NaOH), 60% (wt/wt) sucrose dissolved in lysis buffer, phosphate buffered saline (PBS), 2 ml centrifuge tubes (Fisher Scientific, Pittsburgh, PA catalog number 05-408-138), food coloring dye Brilliant Blue FCF, a refrigerated centrifuge that can generate 20,000 g and the large (6 1/4 inch) Master Grooming Tools Ergonomic Professional Nail Clippers (pet nail clippers to cut the 2 ml tubes). For cultured cells: 27 gauge 1 inch or longer needles on a 1 ml syringe. For tissues: dissecting tools, panty hose/gauze and a Potter-Elvehjem tissue homogenizer with a Teflon pestle or other means of homogenization.

Propagation of cells

The rat hepatoma McArdle RH-7777 cell line was grown in Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Mouse tissue isolation

Livers were isolated from a fed female and an 18 h fasted male adult C57BL6 mouse. Brown adipose tissue (BAT) was isolated from a fed adult male C57BL6 mouse. All experimental protocols were reviewed and approved by the Washington University Animal Studies Committee.

Fractionation of cultured cells

The process was as follows: cells were washed with 1× PBS. In a small volume of PBS, the cells were scraped from the plate and transferred into a 2 ml centrifuge tube (all subsequent manipulations

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occur in this tube). The cells were pelleted by centrifuging at 1,000 g for 1 min and the supernatant was discarded. The cell pellet was resuspended in 200 µl of 60% sucrose dissolved in lysis buffer. After thorough mixing by vortexing, the sample was incubated on ice for 10 min. Next, 800 µl ice-cold lysis buffer was added, mixed well, and incubated on ice for another 10 min. The cells were disrupted by five passages through a 27 gauge needle (1 inch or longer). The tip of the needle must be blunted to avoid damaging the microcentrifuge tube. Samples were pulse-centrifuged to 100 g to ensure that nothing remained on the upper sides of the tube. A mix was prepared consisting of 2 µl of food dye per ml of lysis buffer. Six hundred microliters of this mix was carefully layered on top of the cell homogenate (Fig. 1A) and centrifuged for 2 h at 20,000 g at 4°C. If the homogenate is fractionated, the centrifuged tube will have a pellet and floating material as seen in Fig. 1B. The tube was frozen thoroughly. A −80°C freezer works well because colder tubes cut more cleanly.

To isolate the individual fractions, the frozen tube was placed in a tube rack. While firmly grasping the top of the tube, the pet nail clipper was used to cut the frozen tube just below the dye layer. This is usually ~600 µl from the bottom (Fig. 1C). The bottom piece of the centrifuge tube contains the soluble and pellet fractions. These uncovered fractions are susceptible to spillage and contamination. The ice cylinder was forced out the top of the tube by holding the top and pushing from the cut end with a cylindrical rod (Fig. 1D). To collect the floating fraction, we cut an ~4 to 6 mm piece from the top of the ice cylinder and transferred this piece of ice to a preweighed microfuge tube (Fig. 1E). To collect the soluble and pellet fractions, we allowed the contents of the bottom piece of the tube to thaw completely. Then the soluble fraction was transferred to a fresh tube. The pellet was resuspended in lysis buffer to a total volume of 500 µl. One way to accomplish this is to first resuspend the pellet in 300 µl of lysis buffer, transfer to a new tube, weigh and then bring the volume to 500 µl (Assume lysis buffer is 1 mg/µl). Lysis buffer was added to bring the floating fraction to 500 µl, again assuming that lysis buffer is 1 mg/µl.

Fractionation of tissue

Tissues were placed in ice cold PBS immediately after harvesting, then blotted dry and weighed. Tissue was minced with a razor blade on a cold surface. We routinely use 50 to 100 mg of tissue. Minced tissue was transferred to a Potter-Elvehjem homogenizer, 200 µl of 60% sucrose was added to the tissue sample and incubated on ice for 10 min. We added 800 µl lysis buffer, mixed, and incubated on ice for another 10 min. We homogenized with five strokes of the Teflon pestle. The homogenate was carefully pipetted through gauze or panty hose and into a 2 ml centrifuge tube. Do not add more than 1 ml to the tube. A mix was prepared consisting of 2 µl of food dye per ml of lysis buffer. Six hundred microliters of this mix was carefully layered on top of the cell homogenate (Fig. 1A) and centrifuged for 2 h at 20,000 g at 4°C. If the homogenate is fractionated, the centrifuged tube will have a pellet and floating material as seen in Fig. 1B. The tube was frozen thoroughly. A −80°C freezer works well because colder tubes cut more cleanly. From here on, the procedure is the same as described in the previous section (Fractionation of cultured cells).

Immunohistochemistry and colorimetric assays

Antibodies. CGI-58 rabbit antiserum was a gift from Dawn Brasaemle and was described previously (7). Antibodies against glycogen synthase and GAPDH were purchased from Proteintech Group, Inc. (Chicago, IL, catalog numbers 10566-1-AP and 10494-1-AP, respectively). The antibody against lactate dehydrogenase was purchased from Abcam Inc. (Cambridge, MA, catalog number ab52488). Anti-Caveolin 1 was purchased from BD Transduction LaboratoriesTM (San Jose, CA, catalog number 610060). Calnexin antibodies purified from rabbit antiserum were purchased from Enzo Life Sciences (Farmingdale, NY, catalog number SPA-860). Guinea pig antiserum against perilipin 2 was purchased from Fitzgerald Industries (Concord, MA, catalog numbers RDI00010) and probed with unlabeled primary antibodies and infrared fluorescing secondary antibodies. The infrared fluorescing antibodies bound to membranes were imaged with the LI-COR Odyssey system (LI-COR Biotechnology, Lincoln, NE).

Protein and triglyceride quantification. Total protein and triglyceride content of fractions were measured as described previously (8).

General considerations

The goal of fractionation is usually to understand cellular processes and thus it is necessary to free cellular structures of interest while preserving these structures. Further, it is an absolute requirement that freed structures retain properties that allow their separation. This makes the disruption of cells a critical step. We use osmotic shock followed by mechanical shear to accomplish this. Needle shearing works well for cultured cells and piston homogenizers work well for brown fat (BAT) and liver. The gauge of the needle can be varied to suit cells that are tough, small, or have unusually large FDs. Also, tubes can be damaged by sharp needles resulting in tube failure and lost samples. Thus, we recommend...
Inhibitor Cocktail Tablets (catalog number 1183617001) in the lysis buffer were compatible with this procedure. Finally, the total volume of the homogenate is 1 ml and therefore the resulting volume of soluble fraction is also 1 ml. The pellet and floating fractions can be brought to any desired volume. However, comparing equal percents of fractions is informative to assess the distribution of organelles across the fractions. To accomplish this with the procedure as described (floating and pellet fraction being 0.5 ml and soluble fraction being 1 ml) requires that twice as much of the soluble fraction be analyzed as the floating and pellet fractions.

RESULTS

To assess the effectiveness of this method, BAT, liver, and cultured liver cells were fractionated. To determine the extent of organelle enrichment, fractions were immunoblotted with antibodies against the FD marker CGI-58, the soluble marker glycogen synthase, the membrane markers caveolin 1 and calnexin, and perilipins 3 and 5. Protein was assayed in these fractions. Less than 4% of protein is in the floating fractions, 32% in soluble fraction, and 64% in the pellet. A triglyceride (TAG) assay reveals that over 80% is in the FD fractions.
cytosolic proteins lactate dehydrogenase and GAPDH, and the ER transmembrane protein calnexin. These markers show that this method produces fractions highly enriched in either FDs, cytosol, or sedimentable organelles from these tissues or cultured cells (Figs. 2–4).

Previous methods of fat droplet isolation typically required large amounts of starting material and generated many fractions. This made analysis of multiple samples cumbersome and in most cases results from a single preparation under a given condition were reported (4–6). The small scale and generation of only three fractions described here make replicates feasible. This allows assessment of the reproducibility of this procedure. The perilipin 2 content in the FD fraction massively increases with fatty acid treatment; however, no change is seen in the signals for calnexin and GAPDH (Fig. 4B). FD fractions from separate fractionations have similar signal levels, showing that the extent of the increase can be accurately and reproducibly assessed (Fig. 4A). These data show reproducibility of both partitioning of organellar marker proteins between fractions and the amount of organellar proteins recovered in these enriched fractions.

Data from these three simple experiments reproduce several previous observations. In BAT, the majority of cellular TAG floats, whereas only about 4% of cellular protein floats, showing FDs are TAG rich and protein poor (Fig. 2B). In the BAT fractions, calnexin is detectable in the floating fraction whereas the non-ER membrane protein caveolin 1 is restricted to the pellet. This result contributes to the amassing evidence for an ER-FD linkage. In addition, perilipin 2 increases with liver fat; perilipins 3-5 are largely cytosolic; and perilipin 3 moves to FDs when intracellular fat is expanding (Figs. 2, 3). This demonstrates that this method is at least as informative as previously described more cumbersome fractionation protocols.

DISCUSSION

Here we demonstrate a simple, reproducible, fast, and versatile fractionation procedure that separates FDs, cytosol, and sedimentable organelles in both cultured cells and whole tissue. Fractionation is done in a single tube retaining all of the cellular contents in these three fractions.

This method is advantageous over previous fractionation procedures (2, 5, 9). Previous methods generated a low speed pellet that is generally discarded without characterization. Thus, a fraction of the cells is lost to analysis. Further, this manipulation likely increases variability. This single centrifugation procedure is reproducible (Fig. 4) and minimizes work and sample loss. The microcentrifuge format minimized dilution of cellular material, making it favorable for the fractionation of small samples. Also in this small format, particles pellet after migrating a few millimeters rather than a few centimeters. This allows lower g-forces to separate buoyant FDs from sedimentable organelles. Furthermore, generating only three fractions simplifies analysis. Finally, our procedure does not require an ultracentrifuge and tube slicer.

The fact that cells are fractionated into only floating, soluble, and membrane fractions makes data analysis conceptually obvious. The least skewed assessment is to compare equal percentages of each fraction (see General considerations section). FDs only have proteins on their cytosolic surface, making them protein poor. Thus, comparisons normalized to total protein, particularly in leaner cells or tissue, will result in hundreds-fold more of the floating fraction being compared with the membrane and soluble fractions based on percentage of starting material. Also, a tiny percentage of abundant proteins contaminating the protein poor FDs can comprise a considerable amount of the protein in the FD fraction.

This procedure has specific limitations. Freezing is required to collect FDs and thus it is not compatible with freeze-sensitive analyses. The pellet fraction contains all sedimentable material including cytoskeletal elements. Gradients where membrane bound organelles migrate up the gradient separate these organelles from such nonmem-

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**Fig. 3.** Comparison of the partitioning of proteins in the liver of a fed and fasted mouse. Fed and fasted mouse livers were fractionated and fractions analyzed as described in Fig. 2. The lipid droplet marker used was perilipin 2, the cytosolic marker was lactate dehydrogenase, and the ER membrane marker was calnexin.

**Fig. 4.** Fractionation of cultured rat liver cells. Cells cultured in lean media were compared with cell cultures treated with 0.8 mM oleate for 60 min, 300 min, or overnight. A: Only the floating fractions are shown here and two replicates were done for each time point. There is an increase in the perilipin 2 content of the floating fractions with increased length of oleate treatment. The results for each replicate are very similar, indicating the reproducibility of this isolation method. B: This method works well for cultured cells. Calnexin is seen primarily in the membrane fraction, GAPDH in the soluble fraction, and perilipin 2 is mostly in the FD fraction. It is apparent that overnight treatment with oleate results in small amounts of perilipin 2 localizing in the membrane (pellet) and soluble fractions.
brane bound sedimentable structures such as cytoskeletal elements. Also, this method does not separate different FD pools. For example, it is not possible to discern protein differences between nascent droplets and the constitutive mature pool. Finally, fractionations are not pure and the degree of enrichment varies for each experiment. For each preparation, organelar marker assessment will likely be necessary to interpret results of the experiment.

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