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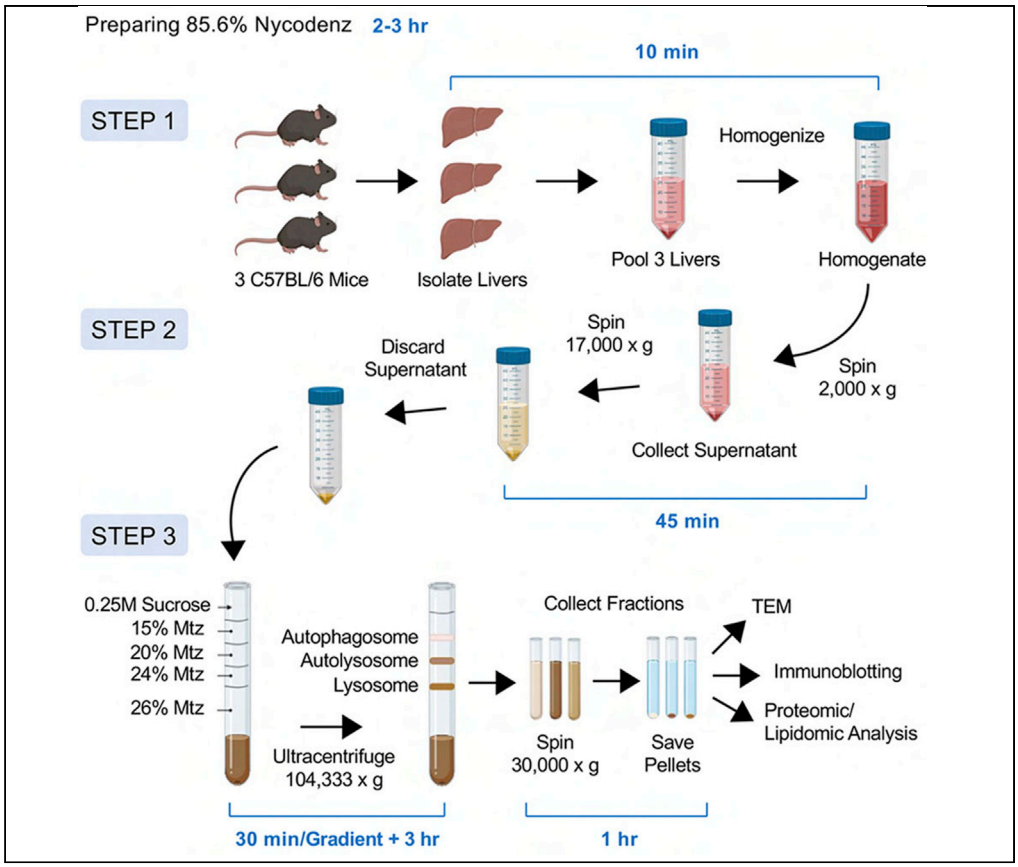
Isolation of autophagic fractions from mouse liver for biochemical analyses



Isolation of autophagosomes, autolysosomes, and lysosomes allows mechanistic studies into the pathophysiology of autophagy — a lysosomal quality control pathway. Here, we outline a Nycodenz density gradient ultracentrifugation approach for high-yield isolation of autophagic fractions from mouse liver. These fractions can be used for immunoblotting, transmission electron microscopy, and proteomic and lipidomic analyses.

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Highlights

Protocol to isolate distinct autophagic fractions from mouse liver

This protocol utilizes Nycodenz density gradient ultracentrifugation

This approach yields autophagosomes, autolysosomes, and lysosomes

These fractions can be used for biochemical and proteo-lipidomic analyses

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Isolation of autophagic fractions from mouse liver for biochemical analyses

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SUMMARY

Isolation of autophagosomes, autolysosomes, and lysosomes allows mechanistic studies into the pathophysiology of autophagy—a lysosomal quality control pathway. Here, we outline a Nycodenz density gradient ultracentrifugation approach for high-yield isolation of autophagic fractions from mouse liver. These fractions can be used for immunoblotting, transmission electron microscopy, and proteomic and lipidomic analyses.

For complete details on the use and execution of this protocol, please refer to Toledo et al. (2018).

BEFORE YOU BEGIN

Our protocol is an adaptation to generate autophagic vesicles from mouse liver using an approach developed in rat liver (Marzella et al., 1982). Prior to using this protocol, 85.6% Nycodenz needs to be prepared. The prepared Nycodenz solution can be aliquoted and stored for long-term at −20°C protected from light in aluminum foil. At least 20 min prior to preparing the Nycodenz gradient, an aliquot must be thawed in a 37°C water bath in an aluminum foil to protect from light.

In our experience (Toledo et al., 2018, Martinez-Lopez et al., 2013), the preparation time for a solution of 85.6% Nycodenz depends on the volume that one plans to prepare, with smaller volume preparations typically requiring less amount of time. We advise that you prepare Nycodenz in large batches to avoid batch variability. Given the time intensive nature of this isolation process, we also advice that Nycodenz be prepared in advance of the date of experiment. Based on our initial standardizations with Beckman Coulter 13.2 mL ultracentrifuge tubes suitable for the SW 41 Ti Swinging-Bucket Rotor, 2.55 mL of 85.6% Nycodenz is required per sample. Also, in our experience, the freezing and thawing of Nycodenz does not appear to compromise the integrity of the gradients. The following protocol provides instructions for the preparation of 200 mL of 85.6% Nycodenz. Depending on the amount required, adjustments can be made for preparation of larger or smaller amounts.

Preparation for 200 mL of 85.6% Nycodenz

Timing: 2–3 h

Because the Nycodenz solution is light sensitive, the beakers used to measure the amount and subsequently dissolve the Nycodenz should be appropriately covered in aluminum foil around the sides and the top.

1. Thoroughly rinse two autoclaved 400 mL beakers, a stir bar, and a spatula with double distilled water to remove any remaining detergent residue.

   Note: Detergents can dissolve and disrupt the autophagic membranes.

2. To prepare 200 mL of Nycodenz gradient, weigh 171.2 g of Nycodenz into one of the rinsed beakers. Fill the other foil coated beaker with a stirring bar and 100 mL of sterile water (Sigma or any other tissue culture grade water).
3. Place the beaker on a magnetic stirrer and begin stirring with water alone.
4. In very small amounts, use the spatula to add the Nycodenz slowly to the solution.

   Note: When added in large amounts to solution, Nycodenz forms clumps at the bottom of the beaker that are difficult to dissolve. To prevent this, with the spatula, add very small amounts of the Nycodenz to the water. If clumps form, continue stirring until they dissolve. Do not add Nycodenz until clumps are dissolved. This dissolution step may take 1.5–2 hr.

5. Once all the Nycodenz has been dissolved, use a couple of milliliters of sterile tissue culture grade water to rinse the sides of the beaker.
6. Adjust the pH to 7.3 using 0.01 N NaOH.

   Note: Because the Nycodenz is dissolved in water and is not a buffered solution, small amounts of the base will lead to large changes in the pH. Adjusting the pH to 7.3 may take time.

7. Measure the volume of the prepared solution in a graduated cylinder that has been thoroughly rinsed with sterile tissue culture grade water and adjust Nycodenz solution to final volume with sterile tissue culture grade water.
8. Aliquot 30 mL of 85.6% Nycodenz into 50 mL Falcon™ tubes covered with aluminum foil.
9. Store the prepared 85.6% Nycodenz at −20°C.

KEY RESOURCES TABLE

REAGENT or RESOURCES	SOURCE	IDENTIFIER
Antibodies		
ATG5-ATG12 conjugate (used at 1:1000 dilution)	Novus Biologicals	NB110-53818
LC3B (used at 1:1000 dilution)	Cell Signaling Technology	2775S
LAMP1 (used at 1:3000 dilution)	DSHB	1D4B
Cathepsin D	Abcam	ab75852
Chemicals, peptides, and recombinant proteins		
Nycodenz	Accurate Chemical & Scientific Corporation	AN1002424
Sucrose	AmericanBio	AB01900-0100
Sigma Water	Sigma Aldrich	W4502
Phosphatase Inhibitor Cocktail 2	Sigma Aldrich	P5726
Phosphatase Inhibitor Cocktail 3	Sigma Aldrich	P0044
Protease Inhibitor	Roche	11873580001
Sodium Chloride	Teknova	S4445

(*Continued on next page*)

Continued

REAGENT or RESOURCES	SOURCE	IDENTIFIER
Potassium Chloride Dibasic	American Bioanalytics	7447-40-7
Potassium Phosphate, dibasic, anhydrous	American Bioanalytics	7558-79-4
Experimental models: organisms/strains		
C57/BL6 Mice	Jackson Laboratory	000664
Other		
Thermo Scientific Samco Fine Tip Transfer Pipettes	Fisher Scientific	13-711-25
Wheaton Potter-Elvehjem Tissue Grinder	DWK Life Sciences	358013
Ultra-Clear Centrifuge Tubes (13.2 mL)	Beckman Coulter	344059
Ultra-Clear Centrifuge Tubes (5 mL)	Beckman Coulter	C14279
Falcon™ 50 mL Conical Centrifuge Tubes	Thermo Fisher	14-432-22

Additional reagents including 500 mL of 0.25 M sucrose and 1 L of Phosphate-buffered saline (PBS) (each prepared in sterile tissue culture grade water) can be prepared and stored at 4°C one day prior to autophagic fraction isolation.

STEP-BY-STEP METHOD DETAILS

Extraction of liver tissue from mice

Timing: 10 min

This section details the isolation of liver from mice followed by gentle homogenization. Although we describe here the method to isolate autophagic fractions from murine liver, this method can be adapted for isolation of similar fractions from cultured cells.

Note: There are no pause points in this method.

1. Place 500 mL of 0.25 M sucrose and PBS on ice.
2. Begin by taking two 50 mL Falcon™ tubes and filling them with 50 mL of 0.25 M sucrose. To each Falcon™ tube, add 500 µL of each protease and phosphatase inhibitor (cocktails 2 and 3) referenced in the key resources table. Keep the two 50 mL tubes on ice.

   Note: The 100 ml of 0.25 M sucrose with protease and phosphatase inhibitors made in this step will be used in each step that requires 0.25 M sucrose with inhibitors. The remaining 400 ml of sucrose will be used in the wash and centrifugation steps that require sucrose but not inhibitors.

3. For each sample, we typically pool three livers of mice of any sex or age into one 50 mL Falcon™ tube containing 15 mL of 0.25 M sucrose. In the non-obesogenic state, three livers approximately amounts to 3–4 g.
4. Once isolated, livers are gently rinsed in a 50 mL beaker containing ice-cold PBS to remove excess blood.

   Note: In our experience, the PBS perfusion is not required. The removal of excess blood in steps 13 and 16 is sufficient.

5. Using a razor blade, we carefully remove the gallbladder to eliminate contamination of the sample with bile/biliary detergents, and we dice the liver into smaller pieces. The dicing of livers facilitates the homogenization process (Figures 1A and 1B).
6. Place the pooled liver pieces into the 50 mL Falcon™ tube containing 0.25 M sucrose and kept on ice (Figure 1C).

   ⚠ CRITICAL: For every proceeding step, the liver samples should be kept on ice.

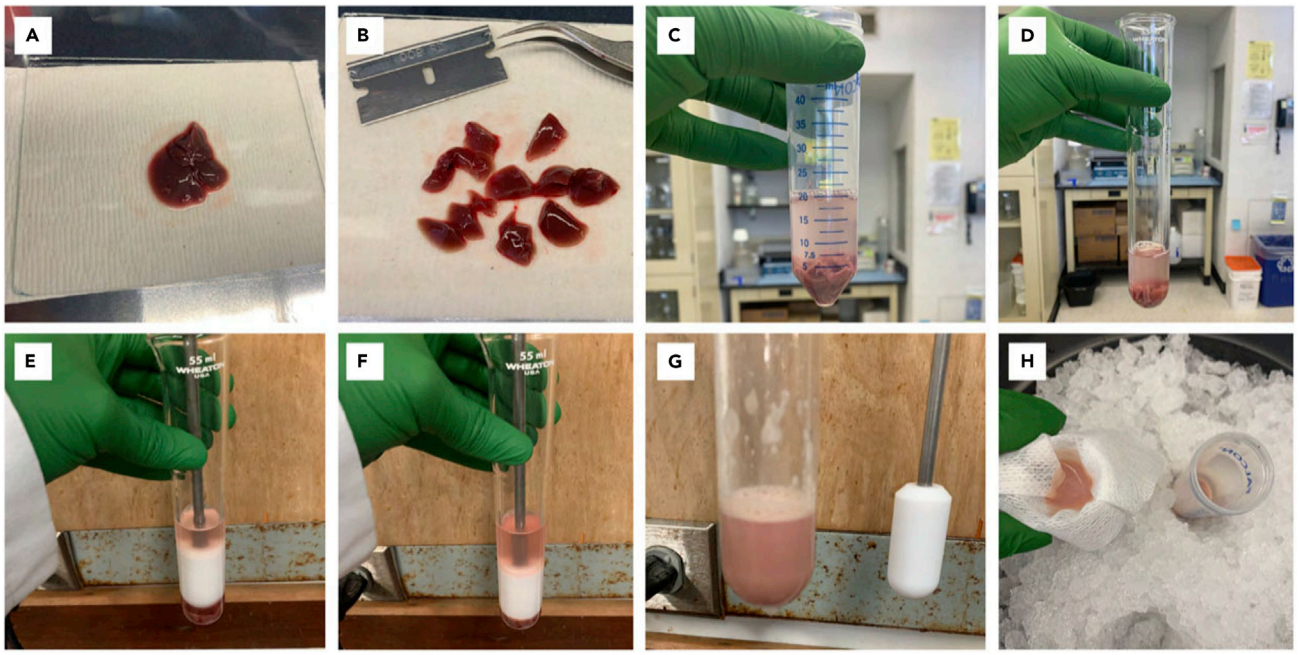

Figure 1. Steps depicting liver extraction and homogenization

(A and B) Photographs depicting the extraction and dicing of liver into small pieces to facilitate homogenization.

(C) Addition of fresh 0.25 M sucrose with protease and phosphatase inhibitors, cocktails 2 and 3, to the diced livers.

(D–G) (D) Transfer of the liver pieces in 0.25 M sucrose to a WHEATON® SAFE-GRIND® Potter-Elvehjem tissue grinder for homogenization, as depicted through (E–G).

(H) Filtering of the liver homogenate through a 4×4 2-ply gauze into a clean Falcon™ tube to eliminate as much as possible the lipids floating on top of the homogenate.

7. Gently pour out the 15 mL of 0.25 M sucrose from the pooled liver samples and replace with fresh 0.25 M sucrose containing protease and phosphatase inhibitors.

   ⚠ CRITICAL: Protease and phosphatase inhibitors (cocktails 2 and 3) are crucial for proceeding homogenization step. In our experience, the lack of inhibitors during this step vastly decreases the protein yield.

8. We homogenize the liver using a WHEATON® SAFE-GRIND® Potter-Elvehjem tissue grinder with a polytetrafluoroethylene (PTFE) pestle tip, which is ideal for soft tissues such as the liver. We homogenize the samples in 15 mL of 0.25 M sucrose using a wall-mounted electric motor rotating the PTFE pestle tip at maximum speed inside the cold room within a ~4°C–7°C temperature range (Figures 1D–1G). Ten strokes per sample is sufficient. We typically, ensure by eye that there are no clumps or residual pieces of liver. On the other hand, excessive homogenization may rupture the lysosomal fraction.

   Note: The goal is to evenly homogenize the livers take caring not to exceed more than 10 strokes to prevent rupturing the autophagosome and lysosome membranes. In the absence of a motorized setup, we advise an empirical approach to first determine the minimum number of strokes required to evenly homogenize livers, while keeping the tissue grinder completely submerged in ice. We visually determine there are no large pieces of liver. These steps are time sensitive hence it is imperative to complete the homogenization process in a timely manner.

9. Once the livers are homogenized, we filter the liver homogenate through a 4×4 2-ply gauze into a clean Falcon™ tube (Figure 1H). The steps described below aide in the removal of liver lipids that float to the top of the homogenate.

a. Place the gauze over the top of the 50 mL Falcon™ tube and use a clean spatula to create a small dip/cup shape into the gauze. This step will prevent the spillover of the homogenate when poured into the tube.
b. Pour the homogenate into gauze, which will then passively flow through into the Falcon™ tube. Lift the sides of the gauze and use a spatula to secure the sides of the gauze to facilitate filtration.
c. Repeat steps **a through c** until all the liver homogenate has been filtered.
d. After the entire homogenate flows through the cheese cloth into the Falcon™ tube, the cheese cloth is gently squeezed with forceps to eliminate any residual homogenate into the tube while the sequestered fat in the cheese cloth is discarded.

10. Note: Remove 100–150 μL of homogenate for western blotting. Proceed with the next step, using the remaining homogenate for the autophagic fractions

Sequential centrifugations

Timing: 45 min

This section outlines the sequential centrifugation steps designed to generate the different organelle fractions and discard nuclear and cellular debris. The 0.25 M sucrose and liver fractions should be kept on ice.

11. Once filtered, we centrifuge the liver homogenate at 2,000 × *g* for 5 min at 4°C on an Eppendorf 5810R centrifuge using the F34-6-38 rotor.
12. The supernatant contains endoplasmic reticulum (ER), cytosol, and all autophagic and lysosomal fractions.
13. The supernatant is then collected into a new 50 mL tube and kept on ice.

⚠ CRITICAL: It is difficult to remove all of the supernatant containing the autophagic fractions without disturbing the pellet. Steps 22–24 are included to improve the yield of the starting material by washing the pellet and retrieving as much of the autophagic fractions as possible.

14. Fill the 50 mL Falcon™ tube containing the pellet with enough fresh ice-cold 0.25 M sucrose to fill the tube to 35 mL.

⚠ CRITICAL: Add the sucrose gently to avoid disruption of the pellet.

15. Centrifuge the pellet containing fresh 0.25 M sucrose again at 2,000 × *g* for 5 min at 4°C.
16. Remove as much of the supernatant as possible and add to the original 50 mL tube containing the initial supernatant.
17. Discard the 50 mL tube containing the pellet.
18. Spin the 50 mL tube containing the supernatant at 12,000 rpm (17,000 g) for 12 min at 4°C on an Eppendorf 5810R centrifuge using the F34-6-38 rotor.

Note: The pellet obtained from this spin will contain the autophagic fractions.

19. Remove the supernatant without disturbing the pellet. Pellets should be kept on ice.
20. Add fresh 0.25 M sucrose to the 50 mL tube and resuspend the pellet.
21. Spin the 50 mL tube again at 12,000 rpm for 12 min at 4°C.
22. Eliminate as much of supernatant as possible via vacuum suction. This pellet will be used in subsequent steps (see below) to isolate each of the autophagolysosomal fractions.

Preparation of Nycodenz gradient

Timing: 30 min per gradient + 3 h centrifugation

In this step, we will prepare the differential density gradient. This gradient will allow us to isolate the autophagic fractions.

23. To prepare a Nycodenz gradient, label 4 of the 15 mL tubes, 26%, 24%, 20%, and 15%, respectively.
24. Calculate the total amount of Nycodenz needed per sample as per chart below.

	85.6% Nycodenz	Water, pH 7.0
26%	1.2 mL	2.8 mL
24%	0.55 mL	1.45 mL
20%	0.45 mL	1.55 mL
15%	0.35 mL	1.65 mL

Note: The values provided in the chart above are the gradient amounts for one sample. When preparing the gradient, make sure to prepare enough Nycodenz for the number of samples that are being prepared. For example, to prepare 2 samples, the total amount of 26% Nycodenz will be 8 ml. Therefore, one will need to prepare 8 ml by adding 2.4 ml (1.2×2) of 85.6% Nycodenz and 5.6 ml (2.8×2) of water.

Note: The 26% Nycodenz layer is the only layer where 4 ml is added for each sample gradient. The 24%, 20%, and 15% Nycodenz layers only require 2 ml (See Figure 2).

25. Resuspend pellet (obtained from step 30 in the previous section) in 950 µL of 0.25 M sucrose containing protease inhibitors.
26. Add 1.4 mL of 85.6% Nycodenz and mix well.
27. Pipet the samples into a 13.2 mL Beckman Coulter ultracentrifuge tube.

Note: Please add the sample to the bottom of the tube without touching the sides. Final density of the sample is 51% Nycodenz. The gradient added above the sample will progressively be of lower density.

28. Using a 1 mL extended fine tip transfer pipet, slowly add 26% Nycodenz. Four mL of 26% Nycodenz should be added to each gradient (Methods video S1)

⚠ CRITICAL: Key to preparing the gradient is ensuring that the layers below are not disrupted when the Nycodenz is being poured. This is ensured by slowly adding a new layer by apposing the tip of the transfer pipet to the side of the 13.2 ml Beckman Coulter tube and adding the new layer immediately above the existing layer (Methods video S1).

29. Slowly using a fine tip, pipet the 24% Nycodenz layer on the top of the 26% layer.
30. Repeat this step for the 20% and 15% Nycodenz layers, respectively, with the 15% layer at the top as indicated in Figure 1.
31. Once the Nycodenz layers are added, add a small amount of 0.25 M sucrose to the top.
32. Add the Beckman Coulter 13.2 mL tubes to the Ti41 Beckman buckets and balance the buckets using 0.25 M sucrose.
33. Load the buckets onto the SW41 Ti ultracentrifuge rotor and centrifuge the gradients for 3 h at 104,333 × *g* at 4°C. We use the Beckman Coulter Optima XPN 90 ultracentrifuge for this step.

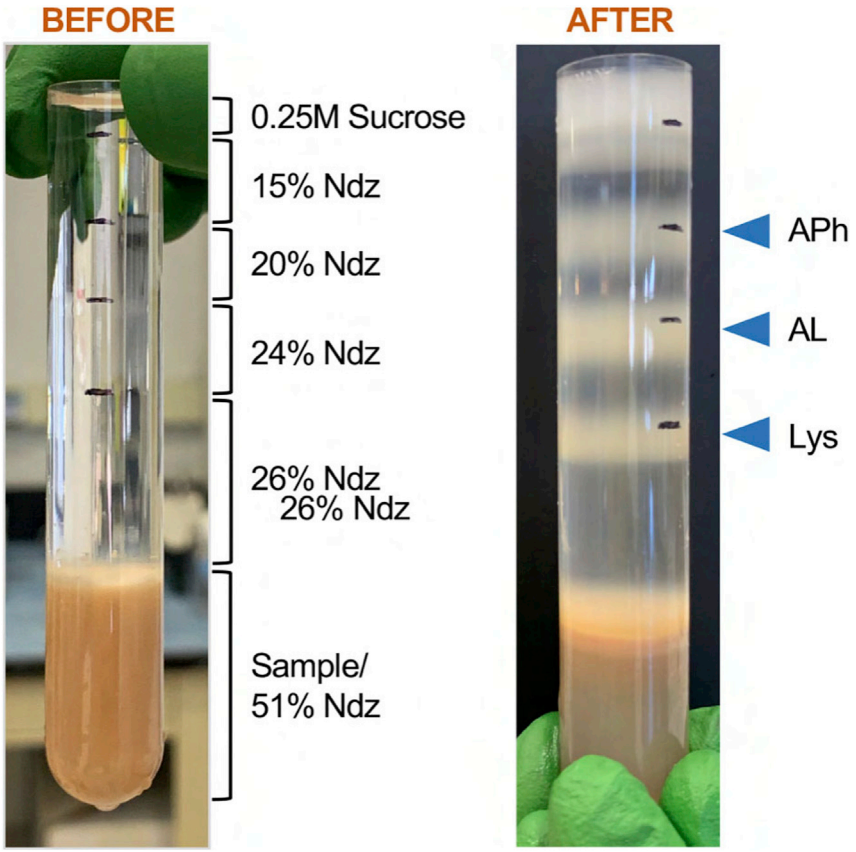

Figure 2. Nycodenz (Ndz) gradients before and after ultracentrifugation

Nycodenz (Ndz) gradients before (left panel) and after (right panel) ultracentrifugation at 24,700 rpm (104,333 g) for 3 h. Brackets in left panel indicate the sample with 51% Ndz (bottom layer), and different Ndz gradients (26%, 24%, 20%, and 15%), and 0.25 M sucrose (top layer). Arrows in right panel depict the bands representing autophagosomes (APh), autolysosomes (AL), and lysosomes (Lys). See also Methods video S1.

Isolation of autophagic fractions

Timing: 1 h

After the completion of the ultracentrifugation, remove the 13.2 mL tubes from the buckets and use 1 mL transfer pipets to collect the different autophagic fractions. See Figure 2, depicting the various bands representing autophagosomes, autolysosomes, and lysosomes after the ultracentrifugation step.

34. The first step entails use of gentle suction to remove the top layers until the autophagosome fraction is reached. A transfer pipette is used to slowly suction out the autophagosome fraction into 5 mL Ultra-Clear centrifuge tubes.
35. Repeat this step to individually collect the autolysosomal and lysosomal fractions in separate tubes
36. Once the samples are collected in the 5 mL Ultra-Clear centrifuge tubes, add ice-cold 0.25 M sucrose with inhibitors made in step 10 to the tubes. Fill the tubes until 0.5 cm from the top of the tubes.

⚠ CRITICAL: The autophagic fractions are suspended in viscous Nycodenz, which keeps the fractions suspended in solution and prevents them from pelleting. To ensure that the

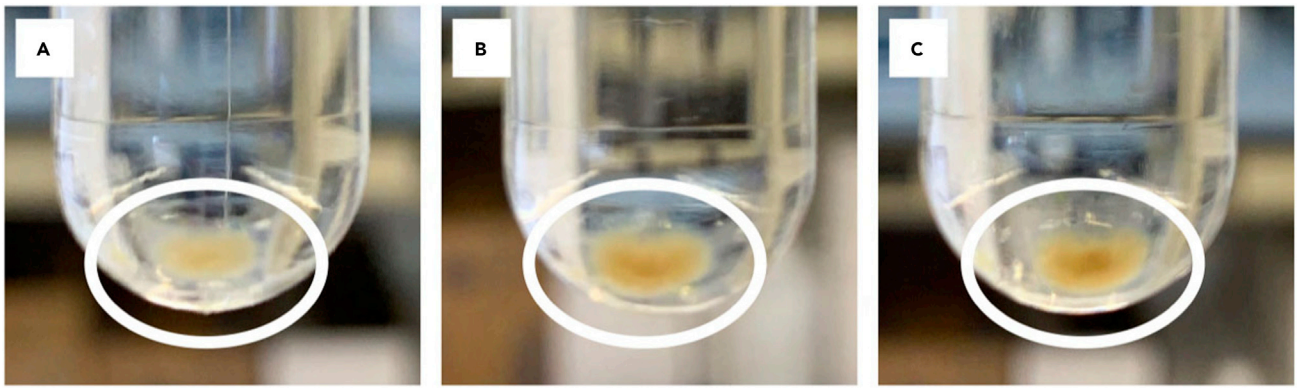

Figure 3. Appearance of isolated autophagic fractions collected after step 46

Photographs depicting the pellets of fractions of (A) autophagosomes (APh), (B) autolysosomes (AL), and (C) lysosomes (Lys) after the dilution of the Nycodenz with 0.25 M sucrose.

fractions can be pelleted, it is necessary to dilute the Nycodenz using 0.25 M sucrose. Hence step 45 remains critical for collection of each of the fractions.

37. The fractions in the 5 mL polypropylene tubes are spun down at 30,000 × *g* for 1 h at 4°C in a TLA-110 rotor on a bench top Beckman Optima TLX Ultracentrifuge.

Note: Pelleting the fractions at this speed renders the sample suitable for biochemical analysis such as immunoblotting, co-immunoprecipitation, and proteo-lipidomics but may not preserve the architecture of the fractions. To maintain the architecture of the autophagic fractions, it is advised to pellet the cells at 21,000 × *g* for 1 h. The appearance of the pellets is shown in Figure 3.

EXPECTED OUTCOMES

The protocol generates three fractions, i.e., autophagosomes (APh), autolysosomes (AL), and lysosomes (Lys). In our experience, livers from fasted mice yield approximately between 250–300 μg of APh, 400–500 μg of AL, and 500–700 μg of lysosomes. Since starvation stimulates autophagy, the amount of autophagosomes generated is substantially higher in 12 h fasted mice. In the fed state, the protocol may still yield approximately 100 μg of APh. Shown here in Figure 4 are representative blots for the various autophagic fractions generated via the method described above. Autophagosomes are double-membraned organelles, which sequester cargo but have not yet fused with acidic lysosomes. Hence at the biochemical level, autophagosomes are enriched in the membrane-bound lipidated form of MAP1LC3B, i.e., LC3B-II, and should contain very little, if at all, soluble cytoplasmic LC3B-I or markers for phagophores, e.g., ATG5-ATG12 conjugate (Figure 4). Because autophagosomes have not yet fused with LAMP1-positive acidic organelles, in principle, these fractions should have substantially lower amounts of LAMP1 or lysosomal proteases, i.e., Cathepsins, when compared to autolysosomes and lysosomes (Figure 4). By contrast, autolysosomes and lysosomes will contain higher levels of LAMP1 and Cathepsins. Since starvation drives autophagy, fractions from starved livers typically show higher levels of LAMP1, LC3B-II, and Cathepsins when compared to livers of fed mice (Figure 4). However, please note that assessment of autophagy activity can only be determined by assessments of autophagy flux in presence of lysosomal inhibitor injected several hours prior to collection of livers (Yamada and Singh, 2012). Hence, immunoblotting for LC3B, LAMP1 and various Cathepsins helps differentiate between each of the autophagic subfractions and validate each of these distinct fractions. These fractions can be used for biochemical, proteomic and lipidomic analysis (Rodriguez-Navarro et al., 2012). Autophagic fractions isolated from cultured cells using this protocol can be used for transmission electron microscopic analyses (Sahu et al., 2011) and for study of autophagosome-lysosome membrane fusion via an in vitro vesicular fusion assay (Koga et al., 2010).

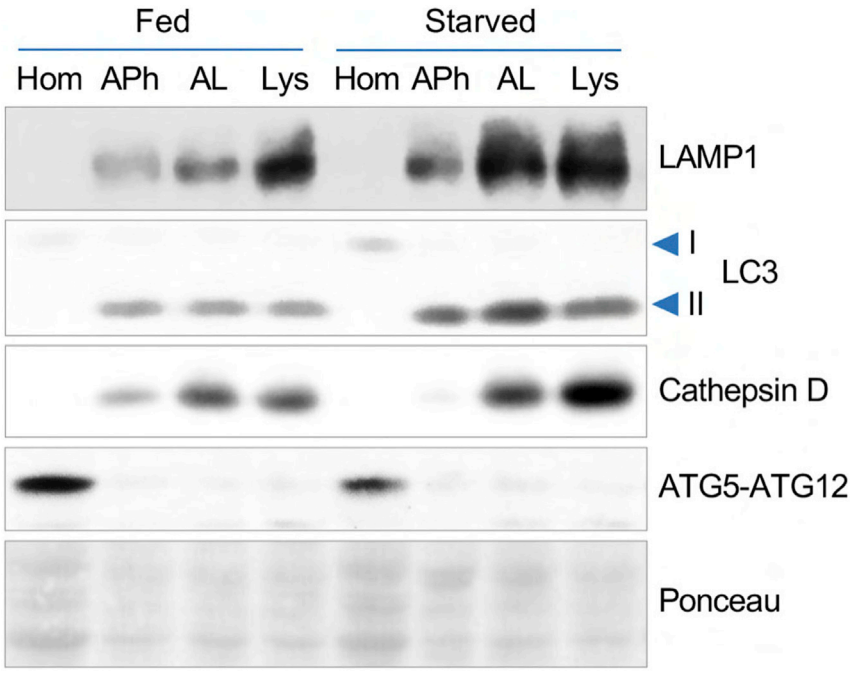

Figure 4. Validating autophagic fractions by immunoblotting

Immunoblots of indicated proteins from homogenates (Hom), autophagosomes (APh), autolysosomes (AL) and lysosomes (Lys) from livers of Fed and Starved mice. Cytosolic LC3B-I and membrane bound LC3B-II are indicated by arrows. Ponceau is the loading control.

LIMITATIONS

A limitation to this protocol is the number of mice needed to obtain one sample. We typically use three livers to generate one autophagic fraction. Hence, for the purpose of reproducibility and statistical analyses, a large number of mice are needed to generate sufficient number of replicates. Offsetting this limitation, it is possible to isolate these fractions from cells in culture; however, as one might expect, several plates of cells are typically pooled together to generate a single sample (Bejarano et al., 2014). A second minor limitation is that Nycodenz is expensive, and its preparation takes time. In addition, the overall isolation process is time intensive. In fact, the quality and integrity of the fractions depend on the skill and speed of the individual. Preparation of one gradient alone may take up to 30 min. Hence, adequate training and practice is needed in establishing the approach in a laboratory naïve to these methods. Finally, due to the possibility of increased protein degradation by the lysosomal acid-sensitive enzymes during the homogenization process, care should be taken to carry out the isolation process at 4°C in presence of protease inhibitors in the 0.25 M sucrose solution.

TROUBLESHOOTING

Problem 1

Issues with 85.6% Nycodenz Preparation (before you begin; step 4)

Nycodenz will form clumps when added in large amounts to water. These clumps are difficult to bring to solution are best avoided.

Potential solution

To prevent clumping, we advise that Nycodenz be added in very small amounts to water, taking care that through continuous stirring Nycodenz is solubilized before new Nycodenz powder is added. In

case clumps do form, then the stirring should be continued until they dissolve prior to adding any more of the Nycodenz powder.

Problem 2

Issues with Preparation of Gradients (steps 23–31)

The most time intensive and sensitive part of the protocol is preparing the Nycodenz gradients. It is critical to be able to clearly visualize and differentiate between the different gradient layers prior to the 3 h centrifugation (Figure 2). The inability to see clear delineated gradient layers may indicate that the components of the gradient were incorrectly prepared. One possible cause is failure to prepare the Nycodenz solution correctly.

Potential solution

A simple solution is to take great care in preparing the 85.6% Nycodenz solution. In fact, we suggest that 85.6% Nycodenz solution is prepared in advance of the isolation process. This problem can be resolved by careful gradient preparation and layering of gradients in the centrifuge tube as depicted in Methods video S1.

Problem 3

Issues with Pelleting the Autophagic Fractions (step 36)

One may encounter difficulties in pelleting the isolated autophagic fractions, as alluded to earlier. Because Nycodenz is a viscous solution, we have found that diluting the Nycodenz solution with 0.25 M sucrose is imperative to efficiently pellet the APh, AL and Lys fractions. If one forgets to dilute the Nycodenz, then instead of firm pellets, the fractions will remain in suspension.

Potential solution

When adding 0.25 M sucrose to the collected autophagic fraction, one must ensure that the sucrose mixes with the samples. Because Nycodenz has greater viscosity than sucrose, the sucrose may simply settle on top of Nycodenz. To ensure proper pelleting of autophagic fractions, one must ensure that the 0.25 M sucrose mixes with Nycodenz to decrease its viscosity.

Problem 4

Low Yield of Fractions (step-by-step method details; step 3)

Decreased protein yield in autophagic fractions is a common problem. Although, we offset this problem by incorporating multiple livers in each sample, additional measures as indicated below are helpful.

Potential solution

It is crucial to use 0.25 M sucrose with protease and phosphatase inhibitors at each step indicated below. We have found that use of inhibitors at certain steps are critical for high protein yield. The use of inhibitors is required during liver extraction. As livers are being extracted, it is important that the isolated livers are placed in a 50 mL Falcon™ tube with 0.25 M sucrose with inhibitors on ice. Although one should try to not leave the livers sitting for too long, the isolation and homogenization of all three livers may take time. The inhibitors ensure no protein degradation occurs during this time. The use of inhibitors is essential for the homogenization step. In our experience, this is another step where the use of inhibitors vastly increases protein yield of the fractions. Even though we recommend this step is done either in a cold room within a ~4°C–7°C temperature range or on ice, the samples may warm-up. To ensure no protein degradation occurs, inhibitors should be used. The resuspension of the pellet containing the autophagic fractions should also be done in presence of inhibitors. These fractions undergo a 3 h centrifugation, and it is possible that protein degradation will occur in this step.

Problem 5

Overfilling the 5 mL Ultra-Clear Tubes (step 36)

A step where sample may be lost is during the last pelleting step in the 5 mL tubes. The 5 mL Ultra-Clear tubes have an open-top and when filled to the brim, spill into the centrifuge, resulting in significant protein loss.

Potential solution

We recommend filling the tubes up until 0.5 cm from the top of the tubes. This is enough volume to prevent the tubes from collapsing during the centrifugation from too little volume and does not result in sample loss during the spin.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rajat Singh (rajat.singh@einsteinmed.org).

Materials availability

This Study did not generate new unique reagents.

Data and code availability

The published article includes all data sets generated or analyzed during this study. For complete details on the use and execution of this protocol, please refer to Toledo et al., 2018.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100730.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, writing – original draft, writing – review & editing, funding acquisition, and resources, H.B. and R.S.; investigation, H.B; supervision and validation, R.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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