Isolating Lysosomes from Rat Liver

Paul R. Pryor

Centre for Immunology and Infection, Hull York Medical School and Department of Biology, University of York, York YO10 5DD, United Kingdom

This protocol describes the generation of a fraction enriched in lysosomes from rat liver. The lysosomes are rapidly isolated using density-gradient centrifugation with gradient media that retain the osmolality of the lysosomes such that they are functional and can be used in in vitro assays.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution’s Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

β-hexosaminidase substrate solution <R>
Ficoll (20%) <R>
Na₂CO₃ (1 M) <R>
Nycodenz (20%) <R>
Nycodenz (45%) <R>
Rat (male Wistar; 200–250-g) <R>
STM fractionation buffer (prechilled to 4°C) <R>
Triton X-100

Equipment

Beaker for collecting and cutting liver tissue
Centrifuges, rotors, and tubes
- Benchtop ultracentrifuge (e.g., Beckman Optima series) with rotor (e.g., Beckman TLA-110) and tubes
- Floor-standing centrifuge (e.g., Beckman Avanti J-26XP) with JA20 rotor or equivalent and tubes
- Floor-standing ultracentrifuge (e.g., Beckman Optima-XE series) with rotor (vertical; e.g., Beckman VTi50) and sealable tubes (39-mL) (e.g., Beckman 342414)
Centrifuge tube heat sealer
Dounce homogenizer

1Correspondence: paul.pryor@york.ac.uk

© 2016 Cold Spring Harbor Laboratory Press
Cite this protocol as Cold Spring Harb Protoc; doi:10.1101/pdb.prot084814
Drill (2500 rpm)
Fluorimeter
Fraction collector
Homogenizer (55 mL; Potter–Elvehjem with serrated Teflon pestle)
LP3 tubes
Peristaltic pump
Measuring cylinder (plastic; see Step 9.ii)
Scale for weighing liver tissue
Scalpel
Scissors for dissection
Serrated forceps
Syringe (50-mL) attached to a 14-gauge needle
Syringe needle (long, wide-bore; stainless steel 315) (Sigma-Aldrich Z261386)
Vortex mixer

METHOD

Preparation of the Homogenate

1. Quickly kill a male Wistar rat by cervical translocation.
2. With a strong pair of dissecting scissors, open the chest cavity and nick the hepatic portal vein.
3. Use a 50-mL syringe attached to a 14-gauge needle to inject 50 mL of ice-cold STM buffer into the rat via the left ventricle of the heart.
   *This injection perfuses the liver with STM. The liver should turn from a dark red to a light pink color.*
   *The muscles of the rat will rapidly contract when perfused with cold buffer. Therefore, grip the needle firmly with a pair of serrated forceps to prevent the needle from dislodging during injection.*
4. Quickly dissect the liver from the carcass, removing as much connective tissue as possible.
5. Place the liver into a beaker or into ice-cold STM buffer if transportation is required.
   *Perform all subsequent steps at 4°C.*
6. Weigh the liver.
   *A typical liver from a 250-g rat weighs ~14 g.*
7. Place the beaker on ice and finely mince the liver with a pair of scissors. Transfer the minced liver into fresh prechilled STM buffer; use 3 mL of STM per gram of minced liver.
8. Pour the liver–STM mixture into a 55-mL Potter–Elvehjem vessel, and keep it on ice.
9. Homogenize the liver as follows. Carry out this step in a cold room if possible.
   i. Attach the serrated Teflon pestle to a household drill mounted onto a laboratory bench.
      *The drill should be mounted so that the pestle is vertical.*
   ii. Place the vessel containing the liver–STM mixture into a wide plastic measuring cylinder that is packed with ice water.
      *The measuring cylinder is normally shortened, allowing enough of the vessel to protrude from the top of the cylinder so that the vessel can be gripped to stop its rotation during homogenization.*
   iii. Using a drill speed of 2400 rpm, push the vessel upward once and allow the pestle to reach the bottom of the vessel.
   iv. Homogenize the liver with three additional strokes of the pestle (down and up three times).
10. Transfer the homogenate into centrifuge tubes and place them in a JA20 rotor (or equivalent). Centrifuge the tubes at 960g (average) for 10 min at 4°C.
11. Carefully decant the homogenate (postmitochondrial supernatant), keeping the loose pellet toward the top of the tube. Discard the pellets. 

Proceed immediately to Step 12.

Density-Gradient Centrifugation

Depending upon the size of the liver, two to four gradients may be required. Each gradient can accommodate 14 mL of homogenate.

12. Prepare the Nycodenz gradients in 39-mL sealable ultracentrifuge tubes:

i. Add 21 mL of 20% Nycodenz to each tube.

ii. Use a long wide-bore needle and syringe to underlay the 20% Nycodenz layer with 4 mL of 45% Nycodenz.

iii. Use a peristaltic pump to deliver 14 mL of the homogenate onto the top of each gradient. A syringe may be used instead of the pump, if used carefully.

iv. Seal the tubes with an appropriate heat sealer.

13. Centrifuge the gradients in a vertical rotor at 206,360 \( g \) (average) for 60 min, adjusting the acceleration and de-acceleration to low settings to ensure gentle acceleration and breaking for 3 min at the beginning and end of the centrifugation cycle.

After centrifugation, membranous material should be visible on the 45% Nycodenz/20% Nycodenz interface.

14. Cut open the tops of the tubes with a sharp scalpel.

15. Pump the material from the bottom of each gradient through a long wide-bore needle that extends to the bottom of the tube. Using a peristaltic pump and a fraction collector, pump at a rate of 1 mL/min and collect 1-mL fractions.

The lysosomes are generally found in fractions 4–8. Contaminating membranes will be present; however, these fractions will be devoid of endosomes. If you wish to determine the precise location of the lysosomes, carry out a lysosomal enzyme assay as described in Step 16. Otherwise proceed to Step 17.

16. Assay each fraction for \( \beta \)-hexosaminidase activity as follows.

i. Set up duplicate LP3 tubes for each fraction to be assayed. Each fraction will be assayed for enzyme activity in the presence and in the absence of Triton X-100.

ii. Add 5–25 \( \mu L \) of the fraction to each of the duplicate tubes.

iii. Add 100 \( \mu L \) of \( \beta \)-hexosaminidase substrate solution containing 0.1% Triton X-100 to one tube, and add 100 \( \mu L \) of \( \beta \)-hexosaminidase substrate without 0.1% Triton X-100 to the other tube.

iv. Precisely 3 min later, add 1 mL of 1 M \( \text{Na}_2\text{CO}_3 \) to each tube.

v. Vortex the tubes briefly and measure the fluorescence using a fluorimeter (360 nm excitation, 445 nm emission).

If the lysosomal membranes are intact, very little enzymatic activity should be detected in the assays performed without Triton X-100. In the presence of Triton X-100, the membranes become permeable to the substrate, and enzyme activity will be observed. This comparison allows an estimation of how latent (intact) the lysosomes are, and therefore if they can be used in functional experiments.

17. Pool fractions 4–8 (or those fractions determined to have lysosomes in Step 16). Dilute the pooled fractions to 18 mL with STM.

18. Centrifuge the samples in a benchtop ultracentrifuge at 38,203 \( g \) (average) for 15 min. Discard the supernatants.

The pellets are enriched in lysosomes and endolysosomes and may be used in functional assays (Pryor et al. 2000, 2004). Proceed to Step 19 to isolate or remove the endolysosomes.
Isolation of Endolysosomes

19. Resuspend the pellets from Step 18 in STM using a Dounce homogenizer. Use a volume of 24 mL of STM for the lysosomes derived from each rat liver used as starting material.

20. Prepare Ficoll/Nycodenz gradients in 39-mL sealable ultracentrifuge tubes.
   i. To each tube add 14.5 mL of 20% Ficoll.
   ii. Use a long wide-bore needle and syringe to underlay the 20% Ficoll layer with 14.5 mL of 20% Nycodenz.
   iii. Use the long wide-bore needle and syringe to underlay the 20% Nycodenz layer with 4 mL of 45% Nycodenz.
   iv. Deliver 6 mL of lysosomes onto the top of each gradient.
   v. Seal the tubes with an appropriate heat sealer.


22. Cut open the tubes and collect fractions as in Steps 14 and 15.
   *The lysosomes will be found at the bottom of the gradient and the endolysosomes at the 20% Ficoll/20% Nycodenz interface.*

23. Pool the fractions containing the lysosomes and separately pool the fractions containing the endolysosomes. Dilute each pool threefold with STM.

24. Collect the organelles from each pool by centrifugation as in Step 18.
   *Storage is not recommended.*

DISCUSSION

This protocol relies on the property that lysosomes are relatively dense compared with other organelles and therefore can be isolated from the bottom of the Nycodenz step gradient. The use of a vertical rotor in an ultracentrifuge allows isopycnic centrifugation to be achieved in 1 h, which is useful if you are trying to reduce degradation of any lysosome luminal contents.

In practice this protocol can be adapted for tissue culture cells, subject to optimizing the homogenization step. In this study, we find that the lysosomes from NRK and HeLa cells will migrate to the bottom of the 20% Nycodenz gradient. These preparations tend to be free of endosomes, but not free of the endoplasmic reticulum. A simpler approach for isolating lysosomes from tissue culture cells using magnetic separation is given in Protocol: Purification of Lysosomes Using Supraparamagnetic Iron Oxide Nanoparticles (SPIONs) (Rofe and Pryor 2014).

RECIPES

**β-Hexosaminidase Substrate Solution**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (for 100 mL)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>100 mM (pH 5)</td>
<td></td>
</tr>
<tr>
<td>Na₃-citrate</td>
<td>1.91 g</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>0.72 g</td>
<td></td>
</tr>
<tr>
<td>Substrate (4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside; Sigma-Aldrich M2133)</td>
<td>19 mg</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.6 g</td>
<td>0.27 M</td>
</tr>
</tbody>
</table>

Aliquot and store at −20°C.
Isolating Lysosomes from Liver

**Ficoll (20%)**
Ficoll (25%)
STE sucrose buffer
Dilute 25% Ficoll to 20% with STE.

**Ficoll (25%)**
Dissolve Ficoll 400-DL (lyophilized and dialyzed, e.g., Sigma-Aldrich F9378) to a concentration of 25% in double distilled water. Store at −20°C.

**Nycodenz (20%)**
Nycodenz (45%)
STE sucrose buffer
Dilute 45% Nycodenz to 20% with STE.

**Nycodenz (45%)**
45% (w/v) Nycodenz
10 mM TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid) (pH 7.4)
1 mM EDTA (pH 7.4)

**STE Sucrose Buffer**
250 mM sucrose
10 mM TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid) (pH 7.4)
1 mM EDTA (pH 7.4)

**STM Fractionation Buffer**
250 mM sucrose
1 mM MgCl₂
10 mM TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid) (pH 7.4)

Store the buffer at −20°C, and keep on ice until use.

**ACKNOWLEDGMENTS**

Dr. Barbara Mullock developed this protocol in Prof. Paul J Luzio’s laboratory, University of Cambridge. The author would like to thank them for passing on their knowledge and experience in lysosome biology.

**REFERENCES**


Cite this protocol as Cold Spring Harb Protoc; doi:10.1101/pdb.prot084814
Isolating Lysosomes from Rat Liver

Paul R. Pryor

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot084814

Email Alerting Service
Receive free email alerts when new articles cite this article - click here.

Subject Categories
Browse articles on similar topics from Cold Spring Harbor Protocols.
- Cell Biology, general (1254 articles)
- Preparation of Cellular and Subcellular Extracts (92 articles)
- Subcellular Fractionation (71 articles)

To subscribe to Cold Spring Harbor Protocols go to: http://cshprotocols.cshlp.org/subscriptions