

# Chapter 2

## Isolation of Peroxisomes from Mouse Brain Using a Continuous Nycodenz Gradient: A Comparison to the Isolation of Liver and Kidney Peroxisomes

Miriam J. Schönenberger and Werner J. Kovacs

### Abstract

In the central nervous system (CNS) peroxisomes are present in all cell types, namely neurons, oligodendrocytes, astrocytes, microglia, and endothelial cells. Brain peroxisomes are smaller in size compared to peroxisomes from other tissues and are therefore referred to as microperoxisomes. We have established a purification procedure to isolate highly purified peroxisomes from the central nervous system that are well separated from the endoplasmic reticulum and mitochondria and are free of myelin contamination. The major difficulty in purification of brain peroxisomes compared to peroxisomes from liver or kidney is the presence of large amounts of myelin in the CNS, which results in contamination of the subcellular fractions. Hence, the crucial step of the isolation procedure is the elimination of myelin by the use of a sucrose gradient, since without the elimination of myelin no significant enrichment of purified peroxisomes can be achieved. Another difficulty is that in brain tissue the abundance of peroxisomes decreases significantly during postnatal development. We provide a detailed protocol for the isolation of peroxisomes from mouse central nervous system as well as a protocol for the isolation of peroxisomes from the liver and kidney using a continuous Nycodenz gradient.

**Key words** Peroxisomes, Microperoxisomes, Brain, Liver, Kidney, Central nervous system, Myelin, Fractionation, Nycodenz gradient

---

### 1 Introduction

Peroxisomes are ubiquitous and highly dynamic organelles whose number, size, and function are dependent on cell type and metabolic needs. They play essential roles in reactive oxygen species and lipid metabolism. The importance of peroxisomal metabolism for mammalian physiology is illustrated by peroxisome biogenesis disorders (i.e., Zellweger spectrum diseases) in which functional peroxisomes are absent or disorders caused by single peroxisomal enzyme and membrane transporter deficiencies [1]. These disorders result in severe neurological dysfunction

associated with abnormal CNS neuronal migration, abnormal white matter (demyelination, dysmyelination, hypomyelination), abnormal Purkinje cell dendritic arborization, loss of axonal integrity, neuroinflammation, and other neurodegenerative processes [2]. Mouse models of Zellweger syndrome have been useful in addressing the cause of Zellweger syndrome neuropathology. In particular, studies on mice with either ubiquitous or brain-specific deletion of *Pex2*, *Pex5*, and *Pex13* have demonstrated a range of neuropathological changes similar to those of Zellweger syndrome patients.

In the brain, peroxisomes have been detected in all neural cell types, namely in neurons, oligodendrocytes, and astrocytes and in microglia and endothelial cells [2]. Brain peroxisomes are smaller in size (0.1–0.2  $\mu\text{m}$  diameter) compared to liver peroxisomes (0.3–0.9  $\mu\text{m}$  diameter) and are therefore referred to as microperoxisomes. Several studies showed that peroxisome abundance in the CNS changes during development (reviewed in [2]). During rat brain development, peroxisomal activity remains constant in the cerebral cortex (a typical gray matter region), whereas microperoxisomes are especially abundant in myelin-forming oligodendrocytes prior to the appearance of myelin sheaths and for several days thereafter (during postnatal days 17–31) [3]. Subsequently, there is a decrease in the overall frequency of microperoxisomes and in the rate of synthesis of lipid precursors [4]. In the mouse CNS, myelin formation is most pronounced during the first 3 weeks after birth. Cholesterol, the main lipid component of myelin, is rapidly synthesized during this period. We found that activities for catalase, a marker enzyme for peroxisomes, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), a myelin/oligodendrocyte marker enzyme, and the cholesterol biosynthetic enzymes isopentenyl diphosphate:dimethylallyl diphosphate isomerase and 3-hydroxy-3-methylglutaryl coenzyme A reductase showed a similar postnatal development with high activities in brain stem, cerebellum, and spinal cord accompanying the phase of myelination [5]. A systematic comparison by immunohistochemistry, western blot analysis, and catalase activity measurements found the maximum level two days after birth, and subsequently the abundance of peroxisomes decreased significantly [6]. In addition, the study of Ahlemeyer et al. [6] is indicative of a selective alteration of the enzyme/protein composition of brain peroxisomes during postnatal development. In summary, these data suggest that the high peroxisomal activity in the first weeks of postnatal life might relate to lipid synthesis accompanying rapid myelin formation and to the elaboration of plasma membranes for growing neurons in early postnatal brain.

As with most subcellular organelles, methods for the isolation of peroxisomes have come largely from work with rat and mouse liver. The major difficulty in purification of brain peroxisomes

compared to peroxisomes from liver or kidney is the presence of large amounts of myelin in the CNS, which results in contamination of the subcellular fractions. The crucial step of the isolation procedure is the elimination of myelin by the use of a sucrose gradient, since without the elimination of myelin no significant enrichment of purified peroxisomes can be achieved [5].

---

## 2 Materials

### 2.1 General Materials

1. Nycodenz (Cat. No. 1002424) was obtained from Axis-Shield (Oslo, Norway).
2. Motor-driven Potter-Elvehjem tissue grinder with loose-fitting Teflon pestle (clearance 0.045–0.065 mm).
3. OptiSeal polyallomer centrifuge tubes (e.g., Beckman; Cat. No. 362183; 25 × 86 mm or 1 × 3.5 in.; 36.2 mL capacity).

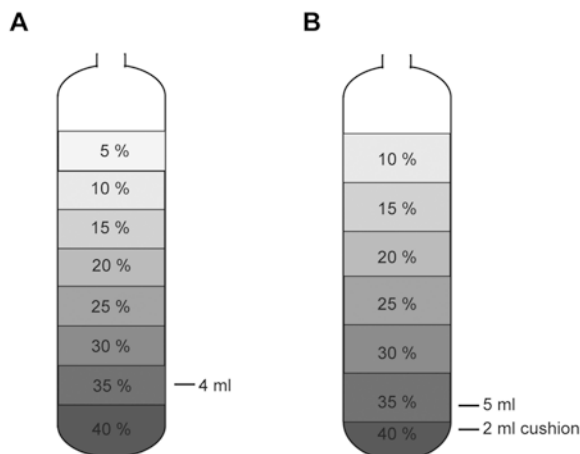
To avoid damage of peroxisomes, clean all glassware, centrifuge tubes, and equipment without detergents, and prepare buffers in detergent-free glassware.

### 2.2 Solutions

1. Homogenization buffer (HB): 5 mM MOPS (pH 7.4), 250 mM sucrose, 1 mM EDTA, 0.1% (v/v) ethanol. Adjust pH to 7.4 with NaOH. Store at 4 °C. Add protease inhibitors prior to usage. We used the cOmplete EDTA-free protease inhibitor cocktail from Roche Diagnostics.
2. TVBE buffer: 1 mM NaHCO<sub>3</sub>, 1 mM EDTA, 0.1% (v/v) ethanol, 0.01% (v/v) Triton X-100. Adjust the pH of the cold buffer (4 °C) to 7.6 with HCl. Store at 4 °C.

### 2.3 Preparation of Nycodenz Gradient

1. Gradient buffer: Homogenization buffer without protease inhibitors.
2. Prepare 25 mL of a 50% Nycodenz solution in HB to prepare one Nycodenz gradient. Dissolve 12.5 g of Nycodenz in 15 mL of HB by shaking at 37 °C. Once it is dissolved, adjust the volume to 25 mL.
3. Nycodenz gradient for the isolation of brain peroxisomes: prepare 4 mL each of 5, 10, 15, 20, 25, 30, 35, and 40% Nycodenz solutions. Nycodenz gradient for the isolation of liver and kidney peroxisomes: prepare 5 mL each of 10, 15, 20, 25, 30, 35% Nycodenz solutions and 2 mL of a 40% Nycodenz solution for the cushion. Pour the gradient as a step gradient one day in advance of the isolation in an OptiSeal polyallomer centrifuge tube and allow linearizing overnight at 4 °C (Fig. 1). Pour the gradient slowly with a long glass Pasteur pipette and avoid air bubbles.



**Fig. 1** Scheme of the preparation of Nycodenz gradients for the isolation of peroxisomes from the brain (a) and liver/kidney (b). Pour the gradient as a step gradient one day in advance of the organelle isolation in an OptiSeal polyallomer centrifuge tube and allow linearizing overnight at 4 °C

## 2.4 Enzyme Assays for Purity Validation

### 2.4.1 Catalase Assay

1. Catalase substrate: 10 mL 0.2 M imidazole buffer (pH 7.0), 90 mL H<sub>2</sub>O, 100 mg bovine serum albumin (Fraction V, fatty acid-free), 35  $\mu$ L 30% H<sub>2</sub>O<sub>2</sub>. Adjust the pH of the cold buffer (4 °C) to 7.0 and store at 4 °C.
2. TiOSO<sub>4</sub> solution: 9.1 mL TiOSO<sub>4</sub> solution (Sigma Cat. No. 89532; 27–31% H<sub>2</sub>SO<sub>4</sub> basis, ~5% Ti basis), 3.6 mL H<sub>2</sub>SO<sub>4</sub> (95–97%), 87.3 mL H<sub>2</sub>O.

### 2.4.2 Esterase Assay

1. Reaction buffer: 20 mM potassium phosphate buffer (pH 7.4), 0.1% Triton X-100, 0.01 mM EDTA.
2. Substrate solution: Dissolve 32.6 mg o-nitrophenyl acetate in 1 mL methanol (prepare the substrate solution fresh; keep on ice).

### 2.4.3 Glutamate Dehydrogenase Assay

1. Reaction buffer: 50 mM potassium phosphate buffer (pH 7.4), 50 mM NH<sub>4</sub>Cl, 0.1% Triton X-100, 0.5 mM ADP (dissolved in potassium phosphate buffer (pH 7.0)), 2  $\mu$ g/mL rotenone (200  $\mu$ g/mL in ethanol), 0.2 mM NADH (prepare a fresh 5 mM stock solution).
2. Substrate solution: 0.5 M  $\alpha$ -ketoglutarate in potassium phosphate buffer (pH 7.0).

### 2.4.4 Phosphoglucose Isomerase

1. Reaction buffer: Prepare fresh by mixing the following stock solutions (volumes are given for one reaction): 1.954 mL 0.1 M Tris buffer (pH 8.0), 34  $\mu$ L 0.1 M fructose-6-phosphate (prepared in Tris buffer), 10  $\mu$ L 0.1 M NADP, 2  $\mu$ L glucose-6-phosphate dehydrogenase (1 unit/2  $\mu$ L in Tris buffer).

2.4.5 2',3'-Cyclic  
Nucleotide  
3'-Phosphodiesterase  
(CNPase)

1. 0.05 M Tris-HCl (pH 7.5).
2. 1% (v/v) Triton X-100.
3. Reaction buffer: 0.2 M imidazole (pH 6.2).
4. Substrate solution: 30 mM adenosine 2',3'-cyclic monophosphate (2',3'-cAMP) in 0.2 M imidazole buffer (pH 6.2). Store frozen in small aliquots.
5. Stop solution: glacial acetic acid.
6. 15 mM adenosine 2'-monophosphate (2'-AMP).
7. 15 mM adenosine 3'-monophosphate (3'-AMP).
8. Thin-layer chromatography (TLC) standard: 50  $\mu$ L 0.2 M imidazole buffer (pH 6.2), 25  $\mu$ L 30 mM 2',3'-cAMP, 50  $\mu$ L 15 mM 2'-AMP, 50  $\mu$ L 15 mM 3'-AMP, 25  $\mu$ L H<sub>2</sub>O.
9. Cellulose MN 300 plates (Macherey-Nagel, Düren, Germany) for thin-layer chromatography.

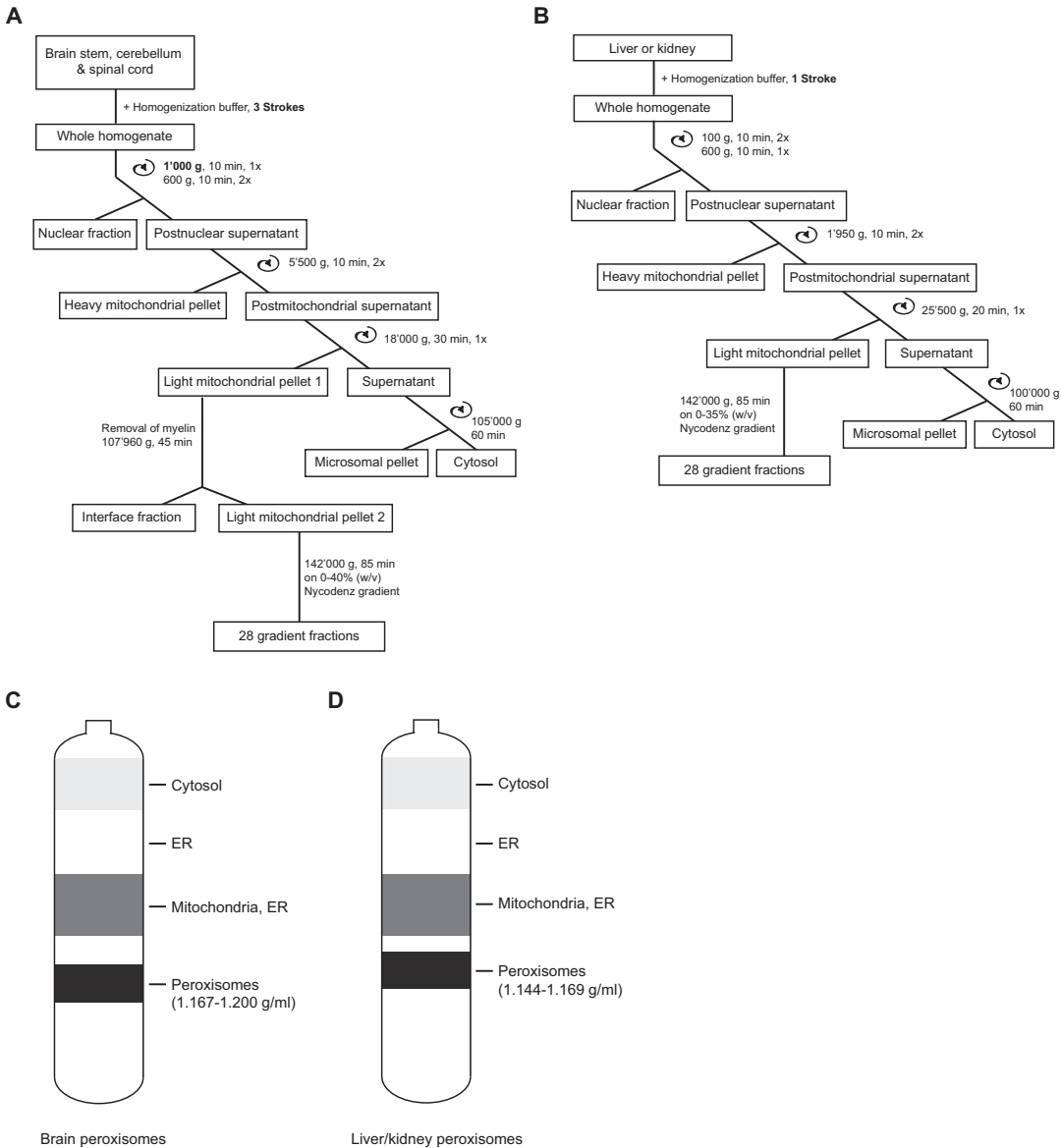
### 3 Methods

The protocol for the isolation of CNS peroxisomes described here was established using CNS tissue from 15-day-old mice [5], a time point of active myelination and when peroxisomes are still abundant in the brain. We used CNS regions that are rich in myelin and therefore lipid metabolism plays an important role. Note that the amount of myelin in the brain of adult mice is much higher, whereas peroxisome abundance is lower. Hence, elimination of myelin will be crucial to isolate highly purified peroxisomes from adult brain.

#### 3.1 Isolation of Peroxisomes from the Central Nervous System Using a Continuous Nycodenz Gradient

All steps should be performed at a temperature of 4 °C unless otherwise specified. The fractionation scheme is illustrated in Fig. 2a. Best results were obtained using ~2 g brain tissue (*see Note 1*).

1. For the isolation of peroxisomes, euthanize 15-day-old mouse pups using CO<sub>2</sub> and remove immediately brain stem, cerebellum, and spinal cord. After determining its weight, mince the tissue into pieces of approximately 2.5 mm<sup>3</sup> and suspend in ice-cold homogenization buffer (HB) at a ratio of 3 mL/g tissue in the precooled glass vessel of the Potter-Elvehjem homogenizer.
2. Homogenize the tissue slowly with three up and down strokes using a motor-driven Potter-Elvehjem tissue grinder and a loose-fitting Teflon pestle rotating at 1000 rpm.
3. Centrifuge the homogenate at 1000  $\times g$  for 10 min at 4 °C to sediment nuclei and cellular debris.
4. Store the supernatant on ice and resuspend the pellet in HB. Rehomogenize the pellet by three strokes of 1000 rpm and centrifuge at 600  $\times g$  for 10 min.



**Fig. 2** (a) Fractionation scheme for the isolation of peroxisomes from brain. Myelin-free light mitochondrial fractions prepared by differential centrifugation are further purified by equilibrium density centrifugation on a linear Nycodenz gradient, and fractions are collected [1 (*top*)-25 (*bottom*)]. (b) Fractionation scheme for the isolation of peroxisomes from liver and kidneys. Light mitochondrial fractions prepared by differential centrifugation are further purified by equilibrium density centrifugation on a linear Nycodenz gradient, and fractions are collected [1 (*top*)-28 (*bottom*)]. (c) Schematic drawing of the distribution of organelles on the Nycodenz gradient for the isolation of brain peroxisomes after centrifugation. (d) Schematic drawing of the distribution of organelles on the Nycodenz gradient for the isolation of liver and kidney peroxisomes after centrifugation. Note that the ER distributes also over the gradient fractions containing mitochondria

5. Combine the supernatant with the first one and resuspend the pellet in HB. Rehomogenize the pellet by three strokes of 1000 rpm and centrifuge at  $600 \times g$  for 10 min.

6. Combine the supernatants (postnuclear supernatant; PNS) and dilute it to 10% (w/v) with HB. Store a small sample of the PNS at  $-80^{\circ}\text{C}$  for protein and enzyme determinations. Discard the final pellet that consists mainly of nuclei, large myelin fragments, and tissue debris.
7. Centrifuge the PNS at  $5500 \times g$  for 10 min at  $4^{\circ}\text{C}$  to obtain a heavy mitochondrial pellet (M) and a postmitochondrial supernatant (PMS). Resuspend the M pellet manually with a glass rod in 1 mL/g HB and spin again at  $5500 \times g$  for 10 min. The combined supernatants represent the PMS. Resuspend the M pellet manually in 1 mL/g HB.
8. Centrifuge the PMS at  $18,000 \times g$  for 30 min at  $4^{\circ}\text{C}$  to obtain a light mitochondrial pellet (L1). Remove the supernatant carefully and centrifuge at  $105,000 \times g$  for 1 h at  $4^{\circ}\text{C}$  to obtain a microsomal pellet (P) and a final supernatant (contains cytosolic proteins). Resuspend the microsomal pellet in 1 mL/g HB.
9. Resuspend the pellet L1 in 0.85 M sucrose in 5 mM Mops, pH 7.4, 1 mM EDTA, and 0.1% (v/v) ethanol. Resuspend the pellet L1 carefully with a glass rod, add the buffer dropwise until a homogenous suspension is gained, and then adjust the volume to 19 mL and transfer to an open-top polypropylene or Ultra-Clear centrifuge tube (tubes for SW27 rotor or similar). Overlay with an equal volume of 0.25 M sucrose in 5 mM Mops, pH 7.4, 1 mM EDTA, and 0.1% (v/v) ethanol and centrifuge at  $107,960 \times g$  for 45 min at  $4^{\circ}\text{C}$  (Beckman SW27 rotor or similar) to remove any remaining myelin. The majority of myelin will be at the interface (interface fraction) (*see Note 2*).
10. Remove the upper layer (0.25 M sucrose) and myelin at the interface. Resuspend the pellet and combine it with the lower layer (0.85 M sucrose) and dilute the suspension with HB to a final concentration of 0.25 M sucrose. Centrifuge at  $18,000 \times g$  for 30 min at  $4^{\circ}\text{C}$  to obtain the myelin-free light mitochondrial pellet (L2).
11. Resuspend the pellet L2 carefully in HB with a glass rod, add the buffer dropwise until a homogenous suspension is gained (*see Note 6*).
12. Layer the resuspended L2 dropwise with a plastic Pasteur pipette on the top of a 0–40% (w/v) continuous linear Nycodenz gradient. Seal the tube with a cap and make sure to avoid air bubbles. Centrifuge at  $142,000 \times g$  for 85 min at  $8^{\circ}\text{C}$  in a Beckman VTI50 or a Sorvall TV-850 vertical rotor with slow acceleration/deceleration. Remove the cap from the centrifuge tube and collect fractions (1.25 mL) in an Eppendorf tube from the bottom of the tube with a two-way needle. The peroxisomes band close to the bottom of the gradient (*see Note 7*).

### **3.2 Isolation of Liver and Kidney Peroxisomes Using a Continuous Nycodenz Gradient**

The method described here was adapted from [7, 8] using livers and kidneys from 4-week-old and adult mice [9]. All steps should be performed at a temperature of 4 °C unless otherwise specified. The fractionation scheme is illustrated in Fig. 2b (*see Note 1*).

1. Starve mice overnight before the isolation experiment (*see Note 3*).
2. For the isolation of liver peroxisomes, the mouse is anesthetized using Ketamin/Rompun (*see Note 3*). Wait until the mouse is completely anesthetized and shows no signs of response. Open the abdominal cavity and perfuse the liver with ice-cold PBS or 0.8% saline solution through the portal vein using a pump system generating a constant buffer speed of 13 mL/min. Perfusion is carried out until blood is drained away completely. For the isolation of kidney peroxisomes euthanize mice with CO<sub>2</sub> and dissect both kidneys directly without perfusion of the organ.
3. After determining its weight, mince the tissue on an ice-cold metal block with razorblades into pieces of approximately 2.5 mm<sup>3</sup> and suspend in ice-cold homogenization buffer (HB) at a ratio of 3 mL/g tissue directly in the precooled glass vessel of the Potter-Elvehjem homogenizer (*see Note 4*).
4. Homogenize the tissue very slowly with one up and down stroke (each 1 min) using a motor-driven Potter-Elvehjem tissue grinder and a loose-fitting Teflon pestle rotating at 1000 rpm (*see Note 5*).
5. Centrifuge the homogenate at 100 × *g* for 10 min at 4 °C to sediment nuclei and cellular debris.
6. Store the supernatant (Postnuclear supernatant 1, PNS1) on ice and resuspend the pellet in 2 g/mL HB. Rehomogenize the pellet by one stroke of 1000 rpm and centrifuge again at 100 × *g* for 10 min at 4 °C. Combine the supernatant with PNS1 and discard the pellet.
7. Centrifuge the PNS1 once at 600 × *g* for 10 min at 4 °C. Resuspend the nuclear pellet in 1 mL HB (P1). Combine the PNS1 and dilute it to 10% (w/v) (postnuclear supernatant 2; PNS2). Store a small aliquot of the postnuclear supernatant (PNS2).
8. Centrifuge the PNS2 at 1950 × *g* for 10 min. Store the supernatant (postmitochondrial supernatant, PMS) on ice. Resuspend the pellet in 1 mL HB and centrifuge again at 1950 × *g* for 10 min. Combine both supernatants (PMS) and save a small aliquot. Resuspend the second pellet, which is the heavy mitochondrial fraction (M), manually in 1 mL HB.



9. Centrifuge the PMS at  $25,500 \times g$  for 20 min at 4 °C to obtain a light mitochondrial pellet (LM). Remove the supernatant carefully and resuspend the pellet carefully with a glass rod, add the buffer dropwise until a homogenous suspension is gained. Start resuspension of the pellet with 1 mL HB and only once a homogenous suspension is gained add 4 mL of HB (*see Note 6*).
10. Centrifuge the supernatant from the  $25,500 \times g$  spin at  $100,000 \times g$  for 1 h at 4 °C to obtain a microsomal pellet (P) and a final supernatant (contains cytosolic proteins). Resuspend the microsomal pellet in 1 mL HB and store at -80 °C.
11. Add the LM dropwise with a plastic Pasteur pipette on the top of a 0–35% (w/v) continuous linear Nycodenz gradient. Seal the tube with a cap and make sure to avoid air bubbles. Centrifuge at  $142,000 \times g$  for 85 min at 8 °C in a Beckman VTI50 or a Sorvall TV-850 vertical rotor with slow acceleration/deceleration. Remove the cap from the centrifugation tube and collect fractions (1.25 mL) in an Eppendorf tube from the bottom of the tube with a two-way needle at RT (*see Note 7*).

### 3.3 Assays for Marker Enzymes of Organelles

#### 3.3.1 Catalase Assay for Peroxisomes

Catalase activity is assayed based on the procedure described [10–12]. The titanium oxysulfate method is the method of choice for peroxisomes and provides very reliable data. The catalase assay is usually carried out at 0 °C. However, since catalase activities in the subcellular fractions and gradient fractions from brain tissues are much lower compared to liver and kidney, the assay is carried out at 37 °C. Dilute samples with TVBE buffer. Keep the substrate solution at 0 °C.

1. Place 10  $\mu$ L of 2% Triton X-100 in glass test tubes (disposable soda-lime culture tubes;  $75 \times 10 \times 0.6$ ) in an ice bath.
2. Add 10  $\mu$ L of appropriately diluted samples (*see Note 8*). Also set up a reagent control using TVBE and Triton X-100. Perform assays in duplicate.
3. Mix well and incubate for at least 1 min.
4. Add 1 mL of substrate solution (substrate should be chilled to 0 °C), adding the substrate solution to successive tubes at timed 10 s intervals. Incubate each sample for exactly the same time at 0 °C.
5. Stop the reaction by adding 1 mL of  $\text{TiOSO}_4$  solution, adding the solution to successive tubes at timed 10 s intervals.
6. Vortex immediately and transfer the glass tubes to room temperature.
7. Wait for at least 10 min for full color development.
8. Blank the spectrophotometer with  $\text{H}_2\text{O}$  and measure the absorbance of the samples and the reagent control at 410 nm

(*see* **Note 8**). The absorbance of the reagent control should be around 1–1.3.

9. Calculate the activity according to the following formula:

$$\text{Enzyme activity (BU/mL)} = (1 + x \text{ mL Sample} + x \text{ mL Triton X-100})/50 \times 1/(\text{incubation time in min}) \times 1/(\text{Sample volume in mL}) \times \log (\text{Reagent control OD}/\text{Sample OD}) \times \text{Dilution factor}.$$

### 3.3.2 Esterase Assay for Endoplasmic Reticulum

1. Esterase is measured according to [13]. Mix 930  $\mu\text{L}$  reaction buffer with 50  $\mu\text{L}$  of appropriately diluted sample. Use TVBE buffer for the blank. Start the reaction by adding 20  $\mu\text{L}$  substrate solution. The assay is performed at 25  $^{\circ}\text{C}$ .
2. Measure the absorption at 420 nm directly after starting the reaction for at least 2 min.
3. Calculate the activity according to the following formula:

$$\text{Enzyme activity (U/mL)} = \Delta A (\text{Sample} - \text{Blank})/\text{min} \times 1/3.06 \times \text{Reaction volume (mL)}/\text{Sample volume (mL)} \times \text{Dilution factor}.$$

### 3.3.3 Glutamate Dehydrogenase for Mitochondria

The activity of glutamate dehydrogenase is measured according to [14].

1. Bring the reaction buffer to room temperature (25  $^{\circ}\text{C}$ ) and carry out all operations at this temperature.
2. In a cuvette, add 50  $\mu\text{L}$  of sample to 940  $\mu\text{L}$  of reaction buffer and mix well.
3. Blank the spectrophotometer against air. Record the absorbance at 340 nm until the endogenous rate disappears, and then add 10  $\mu\text{L}$  substrate solution.
4. Mix well and continue to record the absorbance at 340 nm until a linear increase in value can be measured over a period of 1–2 min.
5. Calculate the activity according to the following formula:

$$\text{Enzyme activity (U/mL)} = \text{Rate} \times \text{Reaction volume (mL)}/\text{Sample volume (mL)} \times 1/6.22 \times \text{Dilution factor}.$$

### 3.3.4 Phosphoglucose Isomerase for Cytosol

Phosphoglucose isomerase is used as a marker for the cytosolic fraction and measured according to [15].

1. Bring the reaction buffer to room temperature (25  $^{\circ}\text{C}$ ) and carry out all operations at this temperature. Use 0.1 M Tris buffer (pH 8.0) to dilute samples.
2. In a cuvette, add 50  $\mu\text{L}$  of sample to 2 mL of reaction buffer and mix well (*see* **Note 9**).

3. Blank the spectrophotometer against air. Record the absorbance at 340 nm until a linear increase in value can be measured over a period of 1–2 min.
4. Calculate the activity according to the following formula:

$$\text{Enzyme activity (U/mL)} = \frac{\text{Rate} \times \text{Reaction volume (mL)}}{\text{Sample volume (mL)} \times 1/6.22 \times \text{Dilution factor}}$$

3.3.5 2',3'-Cyclic  
Nucleotide  
3'-Phosphodiesterase  
(CNPase) Assay for Myelin

The enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (EC 3.1.4.37, CNPase), a marker enzyme for myelin, is assayed according to [16]. Early work on CNPase demonstrated that pretreatment of samples using detergents resulted in an increase in the measured specific activity of the enzyme and allowed more reproducible results to be obtained.

1. Activation of samples (subcellular fractions and gradient fractions): Add 100  $\mu\text{L}$  sample ( $\sim 0.5 \text{ mg/mL}$  protein = 50  $\mu\text{g}$  protein total) to 50  $\mu\text{L}$  0.05 M Tris-HCl (pH 7.5) and 100  $\mu\text{L}$  1% Triton X-100. Incubate for 10 min at 0–4  $^{\circ}\text{C}$ . Dilute with water so that the enzyme activity can be determined using 100  $\mu\text{L}$  portions without further dilution.
2. Mix 50  $\mu\text{L}$  0.2 M imidazole buffer (pH 6.2) with 100  $\mu\text{L}$  of appropriately diluted sample (1–100  $\mu\text{g}$  protein; routinely used 1–25  $\mu\text{g}$  protein). Start the reaction by adding 50  $\mu\text{L}$  of 30 mM 2',3'-cAMP (1.5  $\mu\text{mole}$  substrate). Incubate for 5–30 min at 30  $^{\circ}\text{C}$  (*see* **Note 10**).
3. Stop the reaction by the addition of 20  $\mu\text{L}$  glacial acetic acid.
4. Centrifuge the reaction mixture at  $\sim 16,000 \times g$  for 10 min.
5. Spot 10  $\mu\text{L}$  of the reaction mixture on a MN 300 cellulose TLC plate and develop for about 4 h in 80:18:2 (v/v/v) saturated  $(\text{NH}_4)_2\text{SO}_4$ :0.5 M sodium acetate:isopropanol. Run 10  $\mu\text{L}$  of a mixture of 2'-AMP, 3'-AMP, and 2',3'-cAMP as standard.
6. Dry the TLC plate and visualize the spots under UV light, and circle the 2'-AMP, 3'-AMP, and 2',3'-cAMP spots with a pencil. Scrape the circled areas and transfer into small test tubes.
7. Dissolve the scraped spots in 1–2 mL of 10 mM HCl by vortex mixing for 5 s, centrifuge to pellet the cellulose, and measure the absorption of the supernatant fractions at 260 nm. The absorbance values are corrected for the blank cellulose spot absorbance.
8. The specific activity is defined as  $\mu\text{moles}$  of 2'-AMP ( $\text{Product}_{260}$ ) formed from 2',3'-cAMP ( $\text{Substrate}_{260}$ ) per minute per mg protein. Calculate the activity according to the following formula:

$$\text{U/mg} = \frac{\text{Product}_{260}}{(\text{Product}_{260} + \text{Substrate}_{260}) \times 1.5 \mu\text{mole}} \times 1/t [\text{min}] \times 1/\text{protein} [\text{mg}].$$

### 3.4 Immunoblotting for Purity Validation

To verify the relative purity of the organelles, subject equal volumes of the gradient fractions to SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes. To characterize the subcellular fractions, subject equal amounts of protein to SDS-polyacrylamide gel electrophoresis. After blocking for 1 h in a Tris-buffered saline containing 0.05% Tween 20 and 1% bovine serum albumin, probe membranes with indicated antibodies listed in Table 1 overnight at 4 °C. Incubate the membranes with secondary antibodies conjugated to horseradish peroxidase and visualize using enhanced chemiluminescence.

## 4 Notes

1. Save and store a small sample (e.g., 250 µL) of supernatants and resuspended pellets at –80 °C for protein and organelle marker enzyme determinations. Determine the volume of each supernatant and resuspended pellet to be able to calculate total enzyme content.

**Table 1**

**Organelle marker antibodies for western blot analysis of subcellular and Nycodenz gradient fractions**

Organelle	Protein	Species	Dilution	Antibody source (Product number, company)
Peroxisomes	Pex14p	Rabbit	1:1000	10594-1-AP, Proteintech
Peroxisomes	Catalase	Rabbit	1:8000	219010, Calbiochem
Peroxisomes	PMP70	Sheep	1:1000	Gift from S. Gould
Peroxisomes	Pex3p	Rabbit	1:1000	10946-1-AP, Proteintech
Peroxisomes/Cytosol	Pex5p	Rabbit	1:1000	BD 6115941
Peroxisomes	Pex16p	Rabbit	1:1000	14816-1-AP, Proteintech
Mitochondria	Vdac	Rabbit	1:5000	AB10527, Millipore
Mitochondria	Tom20	Rabbit	1:1000	sc11415, Santa Cruz
Mitochondria	Trap1	Mouse	1:2000	sc-135944, Santa Cruz
Endoplasmic reticulum	Grp78	Goat	1:500	sc-1051, Santa Cruz
Endoplasmic reticulum	Grp94	Rat	1:200	RT-102-P1, Neomarkers
Myelin	CNPase	Mouse	0.5 µg/mL	MAB326R, Chemicon
Myelin	MBP	Mouse	1:1000	808401, BioLegend
Myelin	PLP	Mouse	1:750	MCA839G, Serotec
Cytosol	Hsp90	Mouse	1:1000	ADI-SPA-830-D, Enzo Life Sciences

2. The open-top centrifuge tubes should be filled as full as possible.
3. Make sure to obtain permission for animal use and experiments (e.g., Institutional Animal Care and Use Committee, Government Commission of Animal Care).
4. For an optimal separation of peroxisomes from mitochondria and ER do not use more than 1 g and not less than 600 mg tissue.
5. Try to avoid air bubbles.
6. This is a crucial step, since peroxisomes are fragile and if the pellet is resuspended too harshly the peroxisomes will break up.
7. Measure catalase activity in the collected gradient fractions the same day before storing them at  $-80^{\circ}\text{C}$ .
8. Dilute the samples in TVBE buffer to a concentration, which avoids a complete discoloring of the reaction solution and to obtain absorbance values between 0.350 and 0.850. The sample volume can be increased if necessary, but always use the same volume of sample and 2% Triton X-100.
9. The assay can also be performed with 25  $\mu\text{L}$  sample and 1 mL reaction buffer.
10. We recommend performing the assay in duplicate with either two different protein concentrations or two different incubation times.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## References

1. Raymond GV, Watkins P, Steinberg S, Powers J (2009) Peroxisomal disorders. In: Lajtha A, Tettamanti G, Goracci G (eds) Handbook of neurochemistry and molecular neurobiology: neural lipids. Springer Science+Business Media, Berlin, Germany, pp 631–670
2. Berger J, Dorninger F, Forss-Petter S, Kunze M (2015) Peroxisomes in brain development and function. *Biochim Biophys Acta* 1863:934–955
3. Adamo AM, Aloise PA, Pasquini JM (1986) A possible relationship between concentration of microperoxisomes and myelination. *Int J Dev Neurosci* 4:513–517
4. Arnold G, Holtzman E (1978) Microperoxisomes in the central nervous system of the postnatal rat. *Brain Res* 155:1–17
5. Kovacs WJ, Faust PL, Keller GA, Krisans SK (2001) Purification of brain peroxisomes and localization of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Eur J Biochem* 268:4850–4859
6. Ahlemeyer B, Neubert I, Kovacs WJ, Baumgart-Vogt E (2007) Differential expression of peroxisomal matrix and membrane proteins during postnatal development of mouse brain. *J Comp Neurol* 505:1–17
7. Völkl A, Fahimi HD (1985) Isolation and characterization of peroxisomes from the liver of normal untreated rats. *Eur J Biochem* 149:257–265
8. Biardi L, Sreedhar A, Zokaei A, Vartak NB, Bozeat RL, Shackelford JE, Keller GA, Krisans SK (1994) Mevalonate kinase is predominantly localized in peroxisomes and is defective in patients with peroxisome deficiency disorders. *J Biol Chem* 269:1197–1205
9. Walter KM, Schönenberger MJ, Trötz Müller M, Horn M, Elsässer H-P, Moser AB, Lucas MS, Schwarz T, Gerber PA, Faust PL, Moch H, Köfeler HC, Krek W, Kovacs WJ (2014) Hif-2 $\alpha$  promotes degradation of mammalian peroxisomes by selective autophagy. *Cell Metab* 20:882–897

10. Chantrenne H (1955) Effects of a catalase inhibitor on the induced formation of catalase in yeast. *Biochim Biophys Acta* 16:410–417
11. Baudhuin P, Beaufay H, Rahman-Li Y, Sellinger OZ, Wattiaux R, Jacques P, De Duve C (1964) Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, d-amino acid oxidase and catalase in rat-liver tissue. *Biochem J* 92:179–184
12. Patel CC, Mohan MS (1960) Nature of the colour-forming species in peroxy titanium sulphate. *Nature* 186:803–804
13. Beaufay H, Amar-Costesec A, Feytmans E, Thinès-Sempoux D, Wibo M, Robbi M, Berthet J (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver: I. Biochemical methods. *J Cell Biol* 61:188–200
14. Schmidt E (1974) Methods of enzymatic analysis. In: Bergmeyer HV(ed), 2nd English edn, vol 2. Verlag-Chemie, Weinheim, Germany, pp 650–656
15. Noltmann EA (1966) Phosphoglucose isomerase: I. Rabbit muscle (crystalline). *Methods Enzymol* 9:557–565
16. Sprinkle TJ, McMorris FA, Yoshino J, DeVries GH (1985) Differential expression of 2':3'-cyclic nucleotide 3'-phosphodiesterase in cultured central, peripheral, and extraneural cells. *Neurochem Res* 10:919–931

Peroxisomes

Methods and Protocols

Schrader, M. (Ed.)

2017, XVII, 347 p. 63 illus., 36 illus. in color., Hardcover

ISBN: 978-1-4939-6935-7

A product of Humana Press