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Golgi Isolation

Danming Tang, **Yanzhuang Wang**[1]

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109

Abstract

The Golgi apparatus is a membranous organelle that modifies and packages proteins and lipids into transport carriers and sends them to the proper locations in the cell. The study of Golgi structure and function can be facilitated by the isolation of this organelle from homogenates of tissues or cells. Liver cells have abundant Golgi membranes because they actively secrete proteins and lipids; therefore, liver tissue is often the preferred source. In this protocol, Golgi membranes are purified from rat liver homogenate by two sequential sucrose gradients. The relative yield of the prepared Golgi stacks is then assessed by measuring the increase in activity of a Golgi marker enzyme, β−1,4-galactosyltransferase, over that of the total liver homogenate. A typical preparation can yield Golgi membranes that are purified 80- to 100-fold over the homogenate, and the majority (60%–70%) retain their stacked nature.

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

Ethyl alcohol (190 proof, ACS/USP grade)

β−1,4-Galactosyltransferase (GalT) assay mixture <R>

Gradient buffers A–E for Golgi isolation <R>

Protein Assay Dye Reagent Concentrate (Bio-Rad 500–0006)

Phosphotunastic acid (PTA)/HCl (1% phosphotungstic acid/0.5 M HCl) <R>

Rats (Sprague–Dawley females with body weights of 150–200 g)

Scintillation fluid (Opti-Fluor; PerkinElmer 6013199)

[1]Correspondence: yzwang@umich.edu.

Sodium dodecyl sulfate (SDS) (5% w/v)

Tris (2 M)

Water from a Milli-Q filtration system (Millipore)

Equipment

AR200 Automatic Digital Refractometer (Reichert Technologies 13950000) Centrifuge (benchtop)

CO_2 for killing rats

Conical flask (250-mL)

Eppendorf tubes (screw-capped)

Falcon tubes (50-mL)

Heat block at 37°C

Liquid nitrogen

Pasteur pipette (plastic)

Scintillation counter

Scissors

Spectrophotometer

Stainless-steel laboratory test sieve (150-μm mesh; Endecotts 200SBW.150)

Stainless-steel receiver (Endecotts 200BRASREC)

SW-41 rotor

Ultracentrifuge (L8–70M or Optima LE-80K from Beckman Coulter, Inc.)

Ultraclear tubes for SW-41 rotor (size 14 mm × 89 mm; Beckman Coulter, Inc., cat. no. 344059)

METHOD

This method was modified from protocols described earlier by Hui et al. (1998) and Wang et al. (2006).

Preparation of Golgi

1. Fast six female Sprague–Dawley rats for 24 h, but provide water during this time period.

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2. Quickly kill the rats using CO_2, followed by cervical dislocation. Rapidly transfer the livers into a large volume of ice-cold buffer C (without protease inhibitors) to cool the liver and wash off the blood.

3. Weigh the livers.

   A maximum of 50 g of wet liver weight can be used for 12 gradients. Do not overload the gradients.

4. Transfer the liver into fresh ice-cold buffer C containing protease inhibitors (complete EDTA-free Protease Inhibitor Cocktail Tablets and pepstatin A). Mince the livers into small pieces (~4–5 mm in diameter) with a pair of scissors.

5. Using the base of a 250-mL conical flask, homogenize the tissue by gently pressing the minced liver through a 150-μm mesh stainless-steel sieve with a circular motion. If needed, add a small amount of buffer C (containing protease inhibitors) to the liver to make it easier to press the tissue through the mesh. Collect the homogenate in a 50-mL Falcon tube.

   To maintain the morphology of the Golgi stacks, take caution and be gentle when homogenizing the tissues. he final volume should be ~50 mL. Keep 200 μL of the homogenate on ice for the enzyme assay (to be used in Steps 14 and 16).

6. In each of 12 SW-41 Ultraclear tubes, prepare the following gradient.

   i. Add 6 mL of buffer D to the bottom of the tube.

   ii. Overlay buffer D with 4.5 mL of liver homogenate.

   iii. Overlay the homogenate with 1.8 mL of buffer B, and balance the tubes to within 0.01 g.

7. Centrifuge at 29,000 rpm (103,800*g*) in a SW-41 rotor for 60 min at 4°C.

8. Aspirate and discard the lipids and the cytosol at the top. Use a plastic Pasteur pipette to collect Golgi that accumulate at the 0.5 M sucrose (homogenate/buffer C)/0.86 M sucrose (buffer D) interface.

   The Golgi membranes appear as a cloudy band (see Troubleshooting). In contrast, the lipids at the very top are colored white, and the cytosol is colored red from the hemoglobin. Avoid lipid contamination when taking the Golgi fractions, because the presence of lipids can disrupt the morphology of the Golgi stacks.

9. Pool the Golgi membranes into a 50-mL tube (the total volume should be ~15 mL and the refractive index should be ~1.3706 [0.77 M sucrose]). Keep 200 μL on ice for the enzyme assay (to be used in Steps 14 and 16). Adjust the Golgi sample to 0.25 M sucrose (refractive index 1.3456) using buffer A. Increase the volume to 48 mL with buffer B if necessary and confirm that the refractive index is still 1.3456.

   It is critical that the sucrose concentration is accurate.

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10. Prepare six additional gradients. For each gradient, overlay 1 mL of buffer E with 2 mL of buffer C and then 8 mL of the diluted Golgi from Step 9. Balance the tubes with buffer B.

11. Centrifuge at 8000 rpm (7900*g*) in a SW-41 rotor for 30 min at 4°C.

12. Aspirate and discard the top layer. Collect the membranes (thin cloudy band) at the 0.5 M sucrose (buffer C)/1.3 M sucrose (buffer E) interface. Gently mix the Golgi membranes (about 1.5 mL–2 mL) with about 1 volume of buffer A to adjust the sucrose concentration to 0.5 M (refractive index 1.3574).

    The final volume of the Golgi membranes is normally 3–4 mL, and the typical protein concentration (as measured in Step 16) is normally 1–2 mg/mL.

13. Aliquot and freeze samples in liquid nitrogen, then store at −80°C.

    Samples can be thawed and frozen twice without significant loss of enzymatic activity or loss of morphology. Purified rat liver Golgi can be further used in several cell-free assays, such as the vesicle transport assay and the Golgi disassembly and reassembly assay (see Discussion).

Determination of Yield

14. Prepare 1:20 dilutions of the homogenate (Step 5 above) and intermediate (Step 9 above) and Golgi (Step 12 above) fractions using water.

15. Add 80 μL of GalT assay mixture (in duplicate) to screw-capped tubes containing 20 μL of the diluted samples or water (blanks). Vortex and incubate at 37°C for 30 min.

16. While the GalT reactions are incubating, measure the protein concentration in each of the three samples using the Protein Assay Dye Reagent Concentrate from Bio-Rad, and then determine the total amount (mg) of protein that is present in each sample.

17. Stop the reactions by adding 1 mL of ice-cold PTA/HCl to each tube. Centrifuge at 13,000 rpm in a benchtop microcentrifuge for 10 sec.

18. Aspirate and discard the supernatants. Add 1 mL of PTA/HCl. Resuspend the pellets by vortexing and centrifuge as in Step 17.

19. Aspirate and discard the supernatants. Add 1 mL ice-cold 95% ethanol, and resuspend the pellets as in Step 18.

20. Centrifuge as in Step 17 and discard the supernatant. Resuspend the pellets in 50 μL of 2 M Tris followed by 200 μL of 5% SDS. Shake or vortex until dissolved.

21. Combine 10 μL of assay mixture (containing 2.5 nmol of UDP-galactose), 40 μL of water, and 200 μL of 5% SDS in a fresh tube.

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This standard will be used to determine the specific activity (SA) of the [^{3}H]UDP-galactose in the mixture (see Step 23).

22. Add 1 mL of scintillation fluid to each sample, and vortex. Use the tritium channel in a scintillation counter to measure the disintegrations per minute (dpm) of each sample and blank.

23. Calculate the SA of the [^{3}H]UDP-galactose in the mixture (in dpm/nmol) by subtracting the dpm of the blank from the dpm of the standard, and then dividing by 2.5 nmol (which is the amount of UDP-galactose in the standard; see Step 21).

24. For each sample, calculate the GalT activity concentration as follows:

$$\text{GalT activity concentration(nmol/h/mL)} = \frac{\text{average dpm} - \text{blank dpm}}{\text{SA}} \times \frac{1}{0.02\text{mL}} \times \frac{1}{0.5\text{h}} \times 20\text{(dilution factor)}\,.$$

25. For each sample, calculate the total GalT activity as follows:

Total GalT activity (nmol/h) = GalT activity concentration (nmol/h/mL) × volume (mL).

26. Determine the yield of Golgi membranes by dividing the total GalT activity in the Golgi fraction by the total GalT activity in the homogenate and multiplying the resulting value by 100%.

The average yield in 10 of our preparations was 13.7% ± 0.4% (mean ± S.E.M.). See Troubleshooting.

27. For each sample, calculate the SA of GalT (in nmol/h/mg) by dividing the value obtained in Step 25 by the value obtained in Step 16.

28. Determine the purification fold by calculating the factor by which the SA of GalT increases in the Golgi fraction over the homogenate.

The average purification fold in 10 of our preparations was 97.4 ± 4.2 (mean ± S.E.M.). See Troubleshooting.

TROUBLESHOOTING

Problem (Step 8): The purity of Golgi membranes is low.

Solution: Highly purified Golgi membranes are normally milky white. A brown solution indicates the presence of contaminating mitochondria. Consider the following.

- Reduce the volume or concentration of the homogenate used on the gradient in Step 6.ii.
- Make sure the sucrose concentration is accurate. Use a refractometer to determine the sucrose concentration.

Problem (Steps 26 and 28): The yield of Golgi membranes is low.

Solution: Consider the following.

- Make sure the sucrose concentration is accurate. Use a refractometer to determine the sucrose concentration.
- Reduce the volume or concentration of the homogenate used on the gradient in Step 6.ii.
- Limit sample proteolysis by keeping all of the solutions at 4°C, performing every step of the procedure at 4°C, and adding protease inhibitors to the solutions.
- Carry out all steps as quickly as possible. The entire procedure, from killing the rats to freezing the Golgi membranes, should be completed in 3–3.5 h.

DISCUSSION

The protocol described here is derived from several earlier methods (Fleischer et al. 1969; Leelavathi et al. 1970; Hino et al. 1978; Hui et al. 1998; Wang et al. 2006) for obtaining highly purified Golgi stacks from rat liver and for determining their yield. The use of a sieve rather than a motor-driven Teflon glass homogenizer gives better preservation of the stacked structure of the Golgi membranes, because shear forces are lower with this method (Wang et al. 2006; Wang 2008; Tang et al. 2010). This protocol typically yields Golgi membranes that are purified 80- to 100-fold over the homogenate. The majority (60%–70%) retain their stacked nature as confirmed by electron microscopy, and the preparations contain very little lysosomal or endoplasmic reticulum contamination as assessed by assay of β-*N*-acetylhexosaminidase (Landegren 1984) or rotenone-insensitive NADH-cytochrome *c* reductase (Sottocasa et al. 1967).

Golgi membranes purified using the above protocol can be used in a variety of cell biological and biochemical purposes, especially in cell-free assays reconstituting Golgi-associated membrane and cytoskeleton dynamics. These include the intra-Golgi transport assay (including vesicle budding and fusion processes) (Balch et al. 1984), the mitotic disassembly and reassembly assays (Misteli and Warren 1995; Rabouille et al. 1995; Tang et al. 2010), and assays examining the binding to and motility of the Golgi apparatus on the cytoskeleton including microtubules and actin filaments, as well as the cytoskeletal dynamics on Golgi membranes (Fullerton et al. 1998; Chen et al. 2005). Purified Golgi membranes have also been used in several quantitative proteomics analyses that identified thousands of candidate proteins whose roles in Golgi organization and functions are yet to be determined (Bell et al. 2001; Gilchrist et al. 2006; Chen et al. 2010, 2012).

RECIPES

GalT Assay Mixture

200 µL sodium cacodylate (0.4 M, pH 6.6)

6 µL β-mercaptoethanol

200 µL ovomucoid (175 mg/mL, prepared in H_2O and filtered through a 0.45-µm nitrocellulose filter)

40 μL UDP-galactose (10 mM)

40 μL Triton X-100 (10%)

20 μL ATP (0.2 M, pH 6.5–7.0)

40 μL $MnCl_2$ (2 M)

10 μL [^{3}H]UDP-galactose (specific activity of 30–50 Ci/mmol; NEN Research Products, NET758)

1044 μL H_2O

Prepare a fresh batch of the assay mixture before use. The final concentration of UDP-galactose in the assay mixture is 0.25 mM.

Gradient Buffers A–E for Golgi Isolation

	Buffer (sucrose concentration)				
	A (0 M)	B (0.25 M)	C (0.5 M)	D (0.86 M)	E (1.3 M)
Potassium phosphate buffer (0.5 M, pH 6.7)	10 mL	10 mL	20 mL	20 mL	10 mL
Sucrose (2 M)	0 mL	6.25 mL	25 mL	43 mL	32.5 mL
$MgCl_2$ (2 M)	0.125 mL	0.125 mL	0.250 mL	0.250 mL	0.125 mL
H_2O (ice-cold)	39.9 mL	33.6 mL	54.8 mL	36.8 mL	7.4 mL
Total volume	50 mL	50 mL	100 mL	100 mL	50 mL
Sucrose concentration	0% (w/w)	8.6% (w/w)	16.0% (w/w)	26.4% (w/w)	38.6% (w/w)
Refractive index	1.3330	1.3456	1.3574	1.3747	1.3973

Pre-cool the H_2O overnight at 4°C to ensure that all the buffers are ice-cold. On the next day, prepare buffers A–E by combining the reagents shown in the table. To buffers C and D, add one complete EDTA-free protease inhibitor cocktail tablet (Roche 1873580) per 50 mL of solution and pepstatin A to a final concentration of 5 μM (use a 5 mM stock of pepstatin A prepared in DMSO). (For washing the liver, it is recommended to prepare 500 mL of buffer C without protease inhibitors, which are expensive.) Be as accurate as possible when mixing various components and check the refractive index of each buffer using a refractometer. The final refractive index should be adjusted to within +0.5% sucrose (about 0.001 in refractive index) for buffers C and D in particular.

PTA/HCl (1% Phosphotungstic Acid/0.5 M HCl)

Dissolve 5 g of phosphotungstic acid in 400 mL of H_2O. Add 22 mL of concentrated HCl and bring the final volume to 500 mL with H_2O. Store at 4°C.

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Potassium Phosphate Buffer (0.5 M, pH 6.7)

Make up 500-mL solutions of 0.5 M anhydrous K_2HPO_4 (43.55 g) and 0.5 M anhydrous KH_2PO_4 (34.02 g). To 400 mL of the latter, gradually add the former until the pH reaches 6.7. Store at 4°C.

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