OptiPrep™ Application Sheet S20

Fractionation of smooth and rough endoplasmic reticulum and separation from Golgi (and other organelles)

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- To access other Application Sheets referred to in the text return to the Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- For a simplified system for separating ER and Golgi see Section 6
- An **OptiPrep™ Mini-Review (MS07)** "Analysis of membrane trafficking in mammalian tissues and cells: fractionation of ER, Golgi, TGN, PM and endosomes" provides a bibliography of all published papers reporting the use of OptiPrep™ for analysis of these membranes: to access return to the initial list of Folders and select "**Mini-Reviews**
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1. Background

The self-generated gradient system described in **Application Sheet S18** provides excellent resolution of the smooth and rough endoplasmic reticulum (SER and RER), but there is no clear separation of Golgi membranes from the lighter SER vesicles. In this Application Sheet the gradient has been modified to take account of the requirement for a gradient that can achieve simultaneous resolution of Golgi, SER and RER.

Such a gradient needs to be reasonably shallow over its entire density range. Although it is possible to achieve such a gradient profile by increasing the centrifugation time (to approx 3 h) using the 20% iodixanol starting concentration described in **Application Sheet S18**, Plonné et al [1] preferred an alternative approach of using a biphasic iodixanol starting concentration (equal volumes of 15% and 20% iodixanol). Such a technique has been previously shown to provide shallow gradients over a quite wide density range while maintaining either relatively short centrifugation times or low RCFs [2]. It is particularly important in the use of density gradients to analyze secretion and endocytosis to keep the centrifugation time and any concomitant proteolysis to a minimum.

If the microsomal fraction is only included in the high density layer (20% iodixanol), the Golgi and smooth ER will float out of the load zone into the gradient formed within the 15% iodixanol layer. Any soluble proteins in the crude microsomes on the other hand will tend to sediment through the 20% layer. If it is important to resolve soluble and membrane-bound proteins, such a system might be preferable to one in which the crude microsomes are distributed throughout the starting solution. The protocol described in this Application Sheet was designed for rat liver or for isolated rat hepatocytes [1], but might be extended (with or without modification) to other tissue or cell types.

2. Solutions required (see Section 7, Notes 1 and 2)

A. OptiPrep™
B. Isolation medium: 0.25 M sucrose, 10 mM Hepes-NaOH, pH 7.8
C. Diluent: 0.25 M sucrose, 60 mM Hepes-NaOH, pH 7.4
D. Working Solution of 50% (w/v) iodixanol (ρ = 1.272 g/ml): 5 vol. of solution A + 1 vol. of solution C

Keep the following stock solution at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 2.0 ml of Tris stock; adjust to pH 7.4 with 1 M HCl and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 50 ml water; add
10 ml of Tris stock; adjust to pH 7.4 with 1 M HCl and make up to 100 ml.

3. Ultracentrifuge rotor requirements

A vertical or near-vertical rotor (tube size approx 11 ml) capable of producing >300,000g_{av}, such as the Beckman VTi 65.1 or NVT65 or Sorvall 65V13 (**see Section 7, Notes 3 and 4**)

4. Protocol (for rat liver)

Carry out all operations at 0-4°C.

1. Mince the liver with scissors and then homogenize in Solution B (4 ml/g liver) using 30 strokes of the pestle of a loose-fitting Dounce homogenizer (**see Section 7, Notes 5 and 6**)

2. Centrifuge the homogenate at 10,000 *g* for 20 min to pellet most of the larger organelles (**see Section 7, Note 7**).

3. Centrifuge the 10,000 *g* supernatant at 100,000 *g* for 40 min and resuspend the microsomal pellet in Solution B (5.0 ml per 2 g liver), using 20 strokes of the pestle of the Dounce homogenizer.

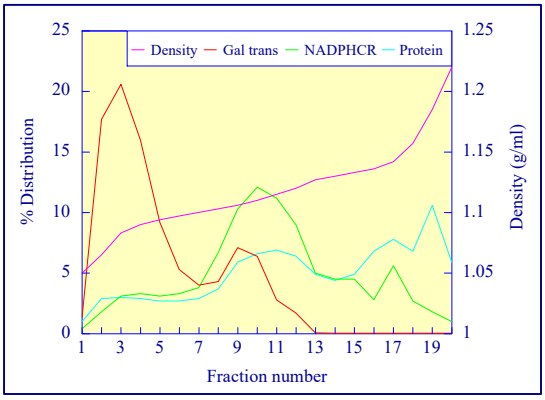
4. Mix 3 vol of the microsome suspension with 2 vol of Solution D (**see Section 7, Note 8**).

5. Transfer 4.5 ml to a vertical rotor tube and underlayer with 1.8 ml of 30% (w/v) iodixanol, made from 3 vol. of Solution D + 2 vol. Solution B (**see Section 7, Note 9**).

6. Layer approx 4.5 ml of 15% (w/v) iodixanol (1.5 vol. of Solution D + 3.5 vol. of Solution B) on top to fill the tube.

7. Centrifuge at 350,000 g_{av} for 2 h at speed (**see Section 7, Note 10**).

8. Collect the gradient in 20x0.5 ml fractions by upward displacement with a dense unloading solution, by tube puncture or aspiration from the meniscus (**see Section 7, Note 11**).



5. Analysis

A typical distribution of Golgi and ER markers is shown Figure 1. The top six fractions contain almost exclusively Golgi membranes, while the smooth ER bands in the mid-region of the gradient. RNA (not shown) increases from tube 16, indicating that the rough ER is located towards the bottom of the gradient. **See Section 7, Note 12** for more analytical information.

Figure 1 Distribution of Golgi and ER markers in gradient. Gal trans = galactosyl transferase, NADPHCR = NADPH-cytochrome c reductase

6. Other separations

If separation of smooth and rough ER is not a requisite, then Golgi and ER may be separated in a simplified system. Yamaguchi et al [3] adjusted the total microsomes to 12.5% (w/v) iodixanol and centrifuged in a near-vertical rotor (Beckman NVT65.2) at 365,000g for 2.5 h. The ER banded close to the bottom of the gradient and the Golgi biphasically in the middle and at the top of the gradient. The single density starting format described in **Application Sheet S18** has also been used for analysis of the ER and Golgi from intestinal cells and from brain [4, 5]. The method described in this Application Sheet has been used very successfully for a variety of cell types including astrocytes [6], neuroglioma cells [7] Chlamydia [8] and COS cells [9]. A recent review has compared some of the methods required for proteomic studies of ER [10].

7. Notes

1. It is important for the success of this protocol that EDTA is not included in isolation media.
2. Protease inhibitors (PMSF, leupeptin, antipain, aprotinin etc) may be included in any or all of the media at the operator's discretion. Strategies for preparing working solutions for mammalian tissues are given in Application Sheet S01.
3. Smaller volume rotors such as the NVT65.2 (5 ml tubes) will require more or less the same centrifugation conditions. It is possible to use a fixed-angle rotor but it needs to be a low volume, high-performance rotor to be able to form the appropriate density gradient profile in 2 h. The gradient forming capacity of such a rotor will need confirming before use. For more information on the formation of self-generated gradients see **Application Sheet S04.**
4. The tubes of choice are Optiseal™ tubes, which are only available for Beckman rotors. Since they are sealed with a central plastic plus rather then heat- or crimp-sealed, they are easy to use and most importantly, gradients within them can be unloaded by any of the standard techniques available to open-topped tubes for swinging-bucket rotors.
5. If isolated hepatocytes are used, allow them to "recover" in a culture medium (e.g. DMEM) gassed with 95% O_2/5% CO_2 for 30 min at 37°C before pelleting the cells at 800*g* for 2 min at 4°C. Wash the cells in ice-cold PBS and then allow them to swell in 10 mM Hepes-NaOH, pH 7.8 for 5-15 min on ice. Adjust the cell suspension to 0.25 M sucrose and then homogenize in the Dounce homogenizer using 30 strokes of the pestle [1].
6. For more information about homogenization of tissues and cells see, respectively, **Application Sheets S05 and S06.**
7. An optional 1000*g*/10 min step may be inserted prior to the 3000*g* centrifugation. This may prevent large amounts of rapidly sedimenting nuclei and debris trapping a lot of smaller particles. For more information on differential centrifugation **see Application Sheet S07**.
8. With smaller scale preparations it may be satisfactory to add Solution D directly to the 10,000g supernatant.
9. The small volume of 30% iodixanol cushion is included to prevent free ribosomes and protein reaching the wall of the tube.
10. Use a slow acceleration and deceleration program to and from 2000 rpm.
11. For more information on harvesting gradients **see Application Sheet S08.**
12. Ozawa et al [11] have used the method for rat C6 glioma cells; immunoblotting of gradient fractions with anti-TGN38 and anti-calnexin confirmed the enzyme distribution shown in Figure 1. Moreover, immunoblotting of rat hepatocyte fractions with anti-TGN38 and anti-GS28 (probes for the *trans* and *cis* Golgi domains) indicates that the *trans* domain is concentrated in fractions 1-2, while the *cis* domain is more evenly distributed across fractions 1-4 [12]. This suggests that there may be an opportunity for Golgi subfractionation using this system. If the top half of the gradient is made shallower by increasing the centrifugation time to 2.5-3 h higher resolution of these Golgi sub-domains may be obtained. This self-generated gradient technology was also used by Massarweh et al [13] in a study the involvement of the ER in the hydrolysis of oligosaccharide diphosphodolichol during protein *N*-glycosylation

8. References

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