OptiPrep™ Application Sheet S21

Analysis of ER, plasma membrane, endosomes, Golgi, ERGIC, TGN and other organelles from mammalian cells and tissues in continuous iodixanol gradients (1.5-3 h spin)

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The **Axis-Shield Mini-Review (MS05)** "Analysis of membrane trafficking and signaling" 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**". **The references are divided into cell or tissue type and highlight the analytical content**.
- 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.
- Section 7 of this Application Sheet is a short review of some of the variations in the methodology according to cell or tissue type and indicates the type of membranes that were analyzed.

1. Background

The protocol described in this Application Sheet is based on methods first published by Yang et al [1] and Zhang et al [2]. Yang et al [1] used a linear 0-26% (w/v) iodixanol gradient to study the localization of UBC6 ubiquitin-containing protein in COS-7 cells. By using the gradient to separate endoplasmic reticulum (ER) and Golgi, they established that the transmembrane domain of a carboxyl-terminal anchored protein predisposes it to locate to the ER, while modulation of this domain resulted in re-targeting of the protein to the Golgi. Zhang et al [2] used a 1-20% (w/v) iodixanol gradient also to separate the ER and Golgi from transfected CHO and human embryonic kidney (HEK293) cells. The authors showed that the full-length presenilins (PS1 and PS2) were located in the ER while N- and C-terminal fragments were distributed to the Golgi membranes.

Although the ER and Golgi were the membranes of interest in these studies, the gradient may provide simultaneous purification of the plasma membrane (PM). The density of the three types of membrane generally increases in the order PM<Golgi<ER; some exceptions to this have however been observed. These gradients may also identify other membrane compartments such as early and late endosomes, *cis*-Golgi, *trans*-Golgi network (TGN) and occasionally lysosomes and mitochondria.

- The relative banding patterns of membranes in the gradient often depends on the type of cell, the homogenization procedure and the precise gradient and centrifugation conditions.

2. Solutions required (see Section 6, Notes 1 and 2)

A. OptiPrep™

B. Homogenization medium (HM): 0.25 M sucrose, 1 mM EDTA 10 mM Hepes-NaOH, pH 7.4

C. Diluent: 0.25 M sucrose, 6 mM EDTA, 60 mM Hepes-NaOH, pH 7.4

D. Working Solution (WS) of 50% (w/v) iodixanol (ρ = 1.272 g/ml): 5 vol. of solution A + 1 vol. of solution C

Keep the following stock solutions at 4°C:
500 mM Hepes (free acid): 11.9 g per 100 ml water.
100 mM EDTA (Na_2•$2H_2O$): 3.72 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 2 ml of EDTA stock and 4 ml of Hepes stocks; adjust to pH 7.4 with 1 M NaOH and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 50 ml water; add 6 ml of EDTA stock and 12 ml of Hepes stock; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements (see Section 6, Note 3)

Any swinging-bucket rotor capable of approx 200,000-300,000 *g* with tube volumes of 5 ml (e.g. Beckman SW 55Ti or SorvallAH650) or 14 ml (e.g. Beckman SW 41 or Sorvall TH641)

4. Protocol

Carry out all operations at 0-4°C.

1. Wash the cells twice in phosphate-buffered saline to remove the culture medium, and then once in Solution B before resuspending in this solution (see Section 6, Note 4).

2. Suspend the cells in a small volume of Solution B (0.5-5.0 ml) and disrupt them by Dounce homogenization, repeated passages through a fine syringe needle or a ball-bearing homogenizer (see Section 6, Note 5).

3. Centrifuge the homogenate at 2000 *g* for 10 min and harvest the supernatant (see Section 6, Note 6).

4. **Optional step** Centrifuge the supernatant at 100,000 *g* for 40 min and then resuspend the pellet in 1-2 ml of Solution B (see Section 6, Note 7).

5. Prepare solutions of 2% and 25% (w/v) iodixanol solution by Solutions B and D 24:1 and 1:1 (v/v) respectively (see Section 6, Note 8).

6. Prepare 12-13 ml gradients in tubes for the swinging-bucket rotor from equal volumes of the 2% and 25% iodixanol solutions using a two-chamber gradient maker or a Gradient Master (see Section 6, Note 9).

7. Layer the vesicle suspension on top of the gradient and centrifuge at 200,000 *g* for 2-3 h (see Section 6, Notes 10 and 11).

8. Collect the gradient in 0.5 ml fractions (see Section 6, Note 12).

5. Analysis (see Section 7.5)

A typical distribution of plasma membrane (biotinylated cell surface proteins), Golgi (galactosyl transferase) and ER (ribophorin I) markers is shown Figure 1. Plasma membrane bands at the very top of the gradient, the Golgi is broadly banded in the top third and the ER in the bottom third of the gradient.

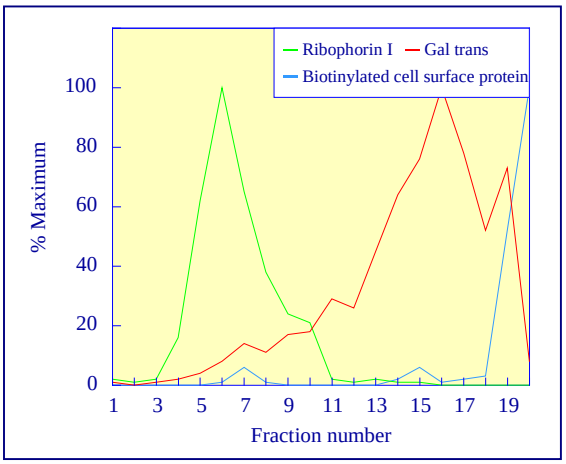

Figure 1: Distribution of Golgi, ER and plasma membrane markers from COS-7 cells in a 0-26% iodixanol gradient (115 min at 200,000g_{av}). Gal trans = galactosyl transferase. Figure adapted from ref 1 with kind permission of the authors and the American Society for Biochemistry and Molecular Biology

6. Notes

1. Protease inhibitors may be included in Solutions B and C at the operator's discretion. Solutions B and C may contain alternative buffers (e.g. Tris, Tricine or triethanolamine. Strategies for preparing working solutions for mammalian tissues/cells are given in **Application Sheet S01.**
2. Although traditionally a buffered isoosmotic solution of sucrose containing EDTA has been used as the HM for organelle fractionation, there has been a trend to use more ionic media, containing NaCl or KCl (or both), particularly for cultured cells. See Section 7.1 for some of the media that have been used.
3. Other swinging-bucket rotors or even vertical rotors may be used. Larger volume swinging-bucket rotors may require longer centrifugation times but vertical rotors will need shorter times. Most of the gradients have been run in 5 or 14 ml tubes.

4. Washing the cells with saline may be carried out at room temperature rather than at 4°C if preferred. Yang et al [1] used a modified homogenization buffer containing 0.21 M sucrose, 0.75 mM KCl, 19 mM NaCl, 1 mM EDTA to wash the cells prior to homogenization. See Section 7.1 for some variations to this theme.
5. The homogenization protocol will need to be tailored to the cell type. Dounce homogenization or 12-15 passages through a 25-gauge syringe needle [1] or sometimes a combination of both [2] are common. The ball-bearing homogenizer ("cell cracker") is now widely regarded as one of the most effective and reproducible of devices. See Section 7.2 for more information.
6. The pellet may be resuspended in Solution B; the centrifugation repeated and the two supernatants combined, if necessary. This strategy recovers membrane vesicles trapped in the first pellet.
7. The advantage of using a low-speed supernatant for the gradient input is that the procedure is quicker and that vesicles in the supernatant are not further exposed to the shearing forces that are required to resuspend a 100,000*g* pellet. On the other hand if it is important to remove any soluble cytosolic proteins, or if the volume of low-speed supernatant is inconveniently large, then preparing a 100,000*g* pellet may be a useful step. See Section 7.3 for more information.
8. A 2% iodixanol solution (rather than Solution B) has been chosen to allow easier layering of the sample. The concentrations of iodixanol used to form the gradient are variable; they may be customized to the operator's requirements. If the main interest is the lower density fractions (e.g. Golgi, PM or endosomes) then a 2-18% iodixanol gradient may be more useful. If the main interest is in denser compartments then a 15%-40% gradient may be required.
9. If neither of these gradient-making devices is available then a continuous gradient can be prepared by diffusion of a discontinuous gradient. For more information on gradient construction see **Application Sheet S03**.
10. There is evidence that flotation of particles can provide better resolution than sedimentation. To use this approach the gradient should be modulated to 2-22% (w/v) iodixanol and the vesicle suspension adjusted to 25% (w/v) iodixanol before being layered beneath the gradient using a syringe and metal cannula (i.d. 0.9 mm). Some examples are given in Sections 7.3.
11. The centrifugation time and/or RCF (*g*-force) may also be modulated; smaller volume gradients may require shorter times (e.g. 1.5 h) and/or lower RCFs (100-150,000*g*). - see Section 7.4 for more information.
12. Collect the gradient by tube puncture, upward displacement or aspiration from the meniscus. For more information on harvesting gradients see **Application Sheet S08**.

7. Literature review

7.1. Homogenization media

Organic osmotic balancers such as sucrose, mannitol and sorbitol were introduced for their compatibility towards functional studies on subcellular membranes; moreover these low ionic strength HMs and gradients permit the direct use of fractions for SDS-PAGE. Although 0.25 M sucrose buffered with either Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and containing 1 mM EDTA is still a widely used HM, supplementation with inorganic salts, is becoming increasingly common and can reduce ionic interactions, aggregation between membranes and combat any raised viscosity of the homogenate due to cytoskeletal proteins. Some media that omit sucrose entirely use either NaCl or KCl or both as the principal osmotic balancer(s). The composition of the HM should also be compatible with any subsequent analytical process. The inclusion of divalent cations can guard against nuclear breakage; stabilize membranes generally, but may lead to aggregation. There is no obvious advantage of one organic buffer over another, but generally, Hepes or Tricine are considered to be more "biological particle-friendly" than is Tris.

Table 1 summarizes some of the other HMs that have been reported in the literature. Other examples are given in **Application Sheets S05 (tissues) and S06 (cells)**. Usually a solution containing the same reagents is used to dilute the OptiPrep™, there are however a few instances where this is not the case.

Table 1: Homogenization media

Cell/tissue type	Homogenization medium (HM) composition	Ref #
Brain tissue (mouse)	40 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 20 mM Hepes-KOH, pH 7.3[1]	3
	0.32 M sucrose, 10 mM Hepes-KOH, pH 7.4	4
CHO	0.25 M sucrose, 0.2 mM $MgCl_2$, 5 mM KCl, 10 mM Tris-HCl, pH 7.4	5
COS	Medium A: 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4;	6
Embryo kidney	Medium B: 128 mM NaCl, 5 mM KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4[2]	7
Embryo kidney	0.75% (w/v) NaCl, 3 mM KCl, 0.3 mM EDTA, 5 mM Tris-HCl, pH 7.4[3]	8
Embryonic stem (mouse)	0.25 M sucrose, 2 mM EGTA, 20 mM Tris-HCl, pH 7.4	9
Liver (rat)	0.85% (w/v) NaCl, 10 mM Tris-HCl. pH 7.4	10
Neuroblastoma	Medium A: 0.25 M sucrose, 1 mM EDTA, 10 mM triethanolamine, pH 7.4;	11
	Medium B: 128 mM NaCl, 5 mM KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4[2]	
Pheochromocytoma (PC12)	0.25 M sorbitol, 1 mM EDTA, 10 mM Hepes-KOH, pH 7.4	12

1. Solution for homogenization only; gradients prepared in buffered 0.25 M sucrose, 1 mM EDTA
2. Cells washed in Medium A; homogenized in 85:15 (v/v) Medium A:B
3. Solution used for gradient only, tissues homogenized in buffered 0.25 M sucrose, 1 mM EDTA

7.2. Cell or tissue homogenization

There are certainly no rigid guidelines regarding the homogenization procedure; ideally the procedure should be as gentle and reproducible as possible, the aim being to cause at least 95% cell disruption without damage to the major organelles, particularly the nuclei and lysosomes. The type and severity of the homogenization process will have consequences for the integrity of the organelles and the size of the vesicles produced from tubular structures in the cytoplasm. Therefore the pattern of membrane banding in any subsequent gradient may not be easily predicted. Some hints on homogenization are given in **Application Sheets S05 (tissues) and S06 (cells).**

7.3. Sample preparation for gradient

Interposing the pelleting of particles from the PNS risks some loss of the smallest vesicles; the subsequent resuspension may cause disruption of organelles such as lysosomes and the process adds considerably to the preparation time. Nuclear pelleting may be carried out at 500-3000*g* for 5-10 min; the higher RCFs (*g*-forces) resulting in removal of some of the mitochondria. To recover any vesicles trapped in the pellet (more serious at the higher RCFs), the pellet is sometimes resuspended in HM, recentrifuged and the two supernatants combined. A possible disadvantage of this practice is that unless the resuspension of the pellet is carried out very gently, the nuclei may be damaged, with consequent leakage of DNA, which may lead to almost irreversible aggregation of the subcellular membranes.

Suspension of a 100,000*g* pellet in a dense solution of iodixanol, or adjustment of the PNS to a high density allows the sample to be loaded beneath the gradient. Cytosolic proteins are retained in the sample zone and are less likely the membranes that float into the gradient. Some examples of these alternative sample handlings are given in Table 2.

Table 2: Sample application to gradient[1]

Cell/tissue	Homogenate treatment	Comments	Ref #
Brain tissue	3000*g*→SN→9000*g*→SN→100,000*g*→pellet[2 3]	Pellet in 23% iodixanol (bottom-loaded)	3
CHO	3000*g*/10 min→SN→80,000*g*/1 h→pellet[3]	Nuclei and most of mitochondria removed	2
Epithelial	PNS→100,000*g*/1 h→pellet		13
Embryo kidney	PNS→100,000*g*/1 h→pellet		14
Neuroblastoma	1,450*g*/10 min→SN→15,000*g*/15 min→pellet	Light mitochondrial pellet applied to grad.	11
Pheochromocyt-oma cells (PC12)	PNS layered over 2% (2 ml) and 50% (0.5 ml) iodixanol, centrifuged at 100,000*g*/30 min[4]	Membranes at 2%/50% interface adjusted to 28% iodixanol (bottom loaded)	12

1. Unless indicated any pellet is resuspended in HM and applied to the top of the gradient, PNS = post-nuclear supernatant, SN = supernatant
2. Times not indicated
3. Centrifugation at 3000*g* rather than 1000*g* will remove most of the mitochondria and further centrifugation at 9000*g* will remove all mitochondria and some lysosomes and peroxisomes
4. The initial discontinuous gradient will effectively remove cytosolic proteins.

7.4. Centrifugation conditions

As far as is known the optimal centrifugation time and RCF have not been thoroughly investigated. Commonly used regimes are approx 280,000*g* for 2 h and 100-200,000*g* for 3 h. Short times at a relatively low RCF (e.g. 150,000*g* for 1.5 h) have generally only been used with 5 ml gradients. The separations are described as buoyant density therefore as long as the vesicles have had sufficient time to reach their banding density, the actual time and RCF are probably not critical. But if sedimentation velocity plays some part in the resolution, time and RCF will be more important and the sample volume must be no more than 10% of that of the gradient.

It should be pointed out that to get true equilibrium density banding centrifugation for at least 12 h at RCFs below 100,000*g* is required and there is evidence that such a practice can produce enhanced resolution – **see Application Sheet S22**.

7.5. Membrane analysis

Using the standard iodixanol gradient, resolution of the ER and *cis*-medial Golgi may be less than ideal; by adopting a 10-24% iodixanol gradient Drummer et al [8] were able to obtain a complete separation of calnexin and giantin (resident proteins for these two membranes respectively) from HEK cells. A 5-25% iodixanol gradient on the other hand provided more complete resolution of the lighter TGN from *cis*-Golgi but the latter banded very closely to the ER from embryo stem cells [9].

In two examples, from human primary fibroblasts [15] and from neuroblastoma cells [11], the Golgi banded at a higher density than the ER. In the case of the neuroblastoma cells, the gradient input was slightly unusual, inasmuch as it comprised a heavy+light mitochondrial fraction, i.e. it would be enriched in mitochondria, lysosomes, peroxisomes and maybe Golgi tubules and impoverished in vesicular microsomes. Whether this has any bearing on the relative densities of the Golgi and ER is not clear.

The plasma membrane from most cultured cells tends to be well defined in iodixanol gradients and almost invariably less dense than the Golgi and ER, but the PM from tissues is more heterogeneous. Thus when Newby et al [14] used the same procedure for both kidney and for mouse embryo kidney cells, in both cases the ER and Golgi were well separated but only in the case of the cultured cells was the PM also distinct and separate from the other two membranes. Aside from the unpredictable nature of the banding of plasma membrane from tissues however, there is evidence from liver [10,16], brain and spleen [16] that the density of early endosomes, Golgi (or TGN) and ER increases reproducibly in that order, as with most cultured cells. Late endosomes tend to overlap the ER.

Other organelles such as mitochondria tend to be denser than the ER. The density of lysosomes, is more variable, again it is usually denser than the ER but can, as in the case of pheochromocytoma (PC12) cells, band between the early endosomes and Golgi [12].

- Neural tissue poses additional requirements on the gradient; synaptic vesicles in particular may need to be identified. Araki et al [4] reported that, in mouse brain neurons, there was a clear distinction in the banding of ER, synaptic vesicles and Golgi (in order of decreasing density) in iodixanol gradients.

8. References

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