OptiPrep™ Application Sheet S24

Analysis of ER, plasma membrane, endosomes, Golgi, ERGIC and TGN from mammalian cells and tissues by sedimentation in discontinuous gradients

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- 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**".
- 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 5 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 strategy of using a multi-step discontinuous iodixanol gradient was first published by Xia et al [1] in 1998; subsequently many similar methods have been published and the number of layers varies from five to nine. Diffusion of iodixanol down the concentration gradient will occur across the interfaces during the centrifugation, but for relatively short time periods such as 1.5-3 h the gradients will certainly not become continuous. Moreover, the diffusion will be partly counterbalanced by sedimentation of the iodixanol molecules in the opposite direction; the higher the RCF (g-force) the more likely this is to occur. The retention of the discontinuous nature of the gradient also depends on the interfacial surface area and the linear distance between the interfaces. So to reproduce a particular published fractionation pattern it is probably good practice to use the recommended rotor, rather than adapt the method to a larger or smaller volume gradient.

- The separations are probably based on buoyant density but sedimentation velocity may make some contribution. Compare the shorter times and generally lower RCFs (g-forces) used in the protocol described in **Application Sheet S25**; in which the resolution is likely to be principally by sedimentation velocity.

Although layering each iodixanol solution can be rather tedious, by changing the increment in volume and/or density between adjacent layers, the gradients can reproducibly be made concave or convex or to contain irregular shallow or steep regions. In this manner, the gradients may provide better linear separation of some particles. Nevertheless there are many examples where excellent resolution is achieved with discontinuous gradients of uniform density increment and volume.

These discontinuous iodixanol gradients have been used to separate not only the major membrane compartments, plasma membrane (PM), Golgi and endoplasmic reticulum (ER), but also some of the important sub-compartments such as *cis*-Golgi, *trans*-Golgi network (TGN) and the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). The density of these compartments generally increases in the order PM<Golgi<ER with the *trans*-Golgi network (TGN) being less dense than the *cis*-Golgi and ERGIC less dense than the ER. In some instances early and late endosomes, lysosomes and mitochondria have also been identified.

- The precise banding patterns of membranes in the gradient may depend on the type of cell, the homogenization medium, the homogenization procedure, the type of gradient and the centrifugation conditions (see Section 5.7).
- The system described by Xia et al [1] has been used by a number of workers and it forms the basis of the protocol described in this Application Sheet.

2. Solutions required (**see Section 5.1**)

A. OptiPrep™

B. Homogenization medium: 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 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 2.0 ml of EDTA stock and 4 ml of Hepes stock; 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 5.2**)

Any swinging-bucket rotor capable of approx 200,000-300,000 *g* with tube volumes of approx. 13 ml (e.g. Beckman SW 41 or Sorvall TH641)

4. Protocol

Carry out all operations (except the phosphate-buffered saline washes in step 1) at 0-4°C.

1. Wash the cells twice in phosphate-buffered saline to remove the culture medium, and then once in the homogenization medium before resuspending in this medium.

2. Suspend the cells in a small volume of homogenization medium (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 5.3**)

3. Centrifuge the homogenate at 1500 *g* for 10 min. The pellet may be resuspended in homogenization medium; the centrifugation repeated and the two supernatants combined, if necessary (**see Section 5.4.1**)

4. Centrifuge the supernatant(s) at 65,000 *g* for 1 h and then resuspend the pellet in 1-2 ml of Solution B (**see Section 5.4.2**).

5. Prepare 10 ml each of 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, and 30% (w/v) iodixanol solution by mixing the appropriate volumes of Solutions B and D (**see Section 5.5.1**).

6. In tubes for the swinging-bucket rotor: layer 1 ml of 2.5%, 2 ml each of 5%, 7.5% and 10%, 0.5 ml of 12.5%, 2 ml of 15%, and 0.5 ml each of 17.5%, 20% and 30% (**see Sections 5.5.1 and 5.5.2**).

7. Layer the vesicle suspension (0.8-1.0 ml) on top of the gradient and centrifuge at 200,000 g_{av} for 2.5 h; allow the rotor to decelerate from 2000 rpm without the brake (**see Sections 5.4.2 and 5.6**).

8. Collect the gradient in 0.5 ml fractions either by tube puncture or upward displacement. For more information on harvesting gradients **see Application Sheet S08**.

9. Analyze the fractions as appropriate (**for examples of analyses from the published literature see 5.7**)

5. Technical review

5.1. Homogenization media and gradient solutions

The homogenization medium often has to be tailored to the tissue or cell type and it is not known if the composition of the HM is relevant to the separation. Organic osmotic balancers such as sucrose, mannitol and sorbitol were introduced for their compatibility in functional studies on subcellular membranes; moreover these low ionic strength HMs and gradient solutions 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. Table 1 summarizes some of the other HMs that have been used. Other examples are given in **Application Sheets S05 (tissues) and S06 (cells).**

Protease inhibitors may be included in Solutions B and C at the operator's discretion. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.

Table 1: Homogenization media

Cell/tissue type	Homogenization medium (HM) composition	Ref #
Brain (rat)	0.25 M sucrose, 5 mM Hepes-KOH, pH 7.4	2
Embryonic stem	130 mM KCl, 25 mM NaCl, 1 mM EGTA, 25 mM Tris-HCl, pH 7.4	3,4
Fibroblasts		
BHK	0.25 M sucrose, 130 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.4	5
Mouse embryo	0.25 M sucrose, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.4	6
Glomerular epithelial	0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM Tris-HCl, pH 7.0	7

5.2. Ultracentrifuge rotors

Many of these separations have been performed in 13 ml tubes for a Beckman SW41 type rotor at 200,000g for 2-3 h. Other swinging-bucket rotors or even vertical rotors may be used. Larger volume swinging-bucket rotors may require longer centrifugation times but smaller volume rotors and vertical rotors will need shorter times. Gradients and sample volume should be scaled up or down proportionately as required. Note however that the progressive change in gradient density profile (due to diffusion and sedimentation of the iodixanol molecules) may also be modulated in other rotors and affect the final resolution.

5.3. Homogenization

The homogenization protocol should be tailored to the cell (or tissue) type. Potter-Elevhjem homogenization for tissues and Dounce homogenization for cells used to be the standard procedures. For cells use of 12-15 passages through a 27- or 25-gauge syringe needle, sometimes preceded by Dounce homogenization, is more common. The ball-bearing homogenizer ("cell cracker") is now widely regarded as one of the most effective and reproducible of devices. Although the Polytron homogenizer is normally restricted to tissues, Iwata et al [8] successfully used this device for neuroblastoma cells.

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).**

5.4. Differential centrifugation

5.4.1. Removal of nuclei

Nuclear pelleting may be carried out at 500-3000g for 5-10 min; the higher RCFs (g-forces) resulting in removal of some of the mitochondria, which can facilitate subsequent layering of the sample on the gradient. 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.

5.4.2. Preparation of sample for gradient loading

Almost without exception, papers reporting the use of the method of Xia et al [1] describe the preparation of 65,000g (or sometimes 100,000g) pellet from the PNS and application of this to the gradient rather than the PNS itself. However if the volume of the 1500g supernatant (step 3) is sufficiently small it might be applied directly to the top of the gradient (in Step 7). Interposing the pelleting of particles from the PNS risks loss of the smallest vesicles and the subsequent resuspension may cause disruption of organelles such as lysosomes. On the other hand if it is important to remove the soluble cytosolic proteins, or if the volume of low-speed supernatant is inconveniently large, then preparing a 65,000g pellet is an essential step, although it does add significantly to the preparation time. Note that direct application of a PNS to a discontinuous gradient was used for hamster embryo kidney cells [9] and embryonic stem cells [3,4]

Suspension of a high-speed 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 to contaminate the membranes that float into the gradient. There is also some evidence that improved resolution can be obtained with this strategy, as the gradient is not disturbed by rapidly sedimenting dense and/or large particles. This approach was used for mouse embryo fibroblasts [6] and glomerular epithelial cells [7].

5.5. Density gradients

5.5.1. Alternative formats

Some variations in gradient format are given in Table 2. Those gradients comprising larger numbers of layers with smaller incremental densities are potentially more discriminating. Gradients of, for example 10-30% (w/v) iodixanol are more suited to fractionation of denser components such as ER and mitochondria than those of for example 1-25% (w/v) iodixanol. The latter are more suited to resolving lighter components such as Golgi and PM.

Table 2: Gradient format and centrifugation conditions, summary of published variations.

Cell/tissue type	Gradient: %(w/v) iodixanol [volumes][1]	Centrifugation	Ref #
Brain	10, 15, 20, 25, 30 [all 0.6 ml]	100,000*g*/1 h	2
Embryonic stem (BD8)	5, 7.5, 10, 12.5, 15, 20, 25,30 [all 1 ml]	126,000*g*/3 h	3
		126,000*g* 1.5 h	4
Fibroblast			
BHK	15, 17.5, 20, 25, [all 1 ml], 40 [0.5 ml]	100,000*g*/1 h	5
Mouse embryo	1, 4, 7, 10, 13, 16, 19, 22 [all 1.5 ml], 25% [1.0 ml-P]	200,000*g*/3 h	6
Glomerular epithelial	2.5, 10, 17.5, 20, 25 [all 0.8 ml], 30% [0.25 ml-P]	100,000*g*/3 h	7
Hamster embryo kidney	10, 15, 20, 25, 30	100,000*g*/3 h	9

1. P denotes layer containing pelleted membrane fraction

5.5.2. Construction

Discontinuous gradients are most easily prepared by underlayering (i.e. low density first) using a syringe (1-2 ml) and a long metal cannula); overlayering small volumes is more difficult using either a syringe or Pasteur pipette. An alternative for overlayering is to use a small volume (low-pulsating) peristaltic pump; first to take up the required volume of solution into the attached tubing and second to expel it slowly on to a denser layer in the centrifuge tube. For more information on gradient construction **see Application Sheet S03** If necessary, adjust all volumes proportionately so that (after sample application) tubes are properly filled according to the manufacturer's instructions.

5.6. Centrifugation conditions

As far as is known the optimal centrifugation time and RCF have not been thoroughly investigated. Commonly used regimes are in the range 100-200,000g for 2-3 h. Some variations are given in Table 2. Short times at a relatively low RCF (e.g. 100,000g for 1 h) have generally only been used with smaller volume (<5 ml) gradients. It should be pointed out that to get true equilibrium density banding centrifugation for at least 12 h at RCFs below 100,000g is required and there is evidence that such a practice can produce enhanced resolution – **see Application Sheet S22**.

5.7. Analysis

In one of the few reports of a direct comparison between the use of iodixanol and sucrose gradients [7], the former were found to give markedly better resolution of PM, ER and mitochondria in a much reduced time (3 h against 16 h). There may also be analytical reasons for the choice of iodixanol over sucrose. Iodixanol gradients were chosen by Campbell et al [10] because their isoosmotic nature (in contradistinction to the hyperosmotic nature of sucrose gradients) preserved vesicles structure and function and allowed the study of the *de novo* synthesis of the amyloid β protein in *in vitro* incubations of isolated membrane fractions.

These 2.5-30% iodixanol gradients are ideal for resolving ER and Golgi (see Figure 1) and are probably applicable to most cultured cells; although the density of PM<Golgi<ER generally increases in that order, the fine detail of the fractionation varies from cell type. The Golgi (as measured by galactosyl transferase from CHO cells appears biphasic [11], while the gradient seems to be able to resolve the TGN and *cis*-Golgi from neuroglioma cells, with the TGN being the denser of the two compartments [12]. On a 5-30% iodixanol gradient (see Table 2) the *cis*-Golgi from BD8 blastocyst-derived embryonic stem cells was more centrally located; in cells expressing PS1, this enabled some PS2 to be localized to a less dense non-ER, non-Golgi compartment.

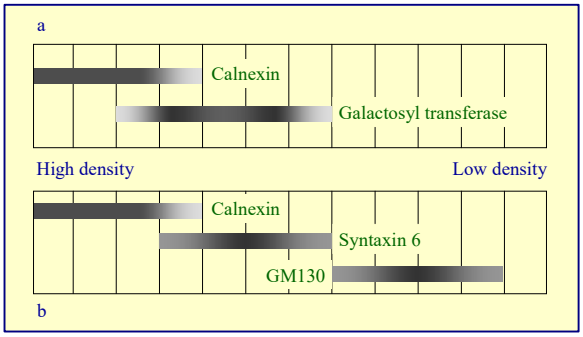

Figure 1 Panel a: Fractionation of CHO cells, approximate disposition of ER (calnexin) and Golgi (galactosyl transferase) in gradient. Data adapted from ref 11. Panel b: Fractionation of H4 neuroglioma cells, approximate disposition of ER, syntaxin 6 (TGN) and GM130 (*cis*-Golgi). Data adapted from ref 12. For more information see text.

Bottom-loaded 1-25% iodixanol gradients (see Table 2) were able to resolve ER, Golgi and PM from mouse embryo fibroblasts (Figure 2, Panel a) with essentially no overlap of markers at all [6]. The possible variation in detailed banding position between different types of cell is emphasized in the observation that the Golgi from wild-type cells was skewed to a slightly higher density than that from cells of a cav-1 knockout mouse. Gradients covering the range 10-30% iodixanol (see Table 2) are also able to resolve denser organelles such as mitochondria (Figure 2, Panel b) [2] or both mitochondria and lysosomes [9]. Although the plasma membrane from cultured cells is invariably the lightest membrane compartment, that from organized tissues may be denser if it is associated with an extensive cytoskeleton. None of these gradients are particularly successful in resolving recycling [9] or early [13] endosomes from Golgi/TGN or ER membranes respectively. Generally endosomes are better resolved in gradients centrifuged for longer times **see Application Sheet S22**.

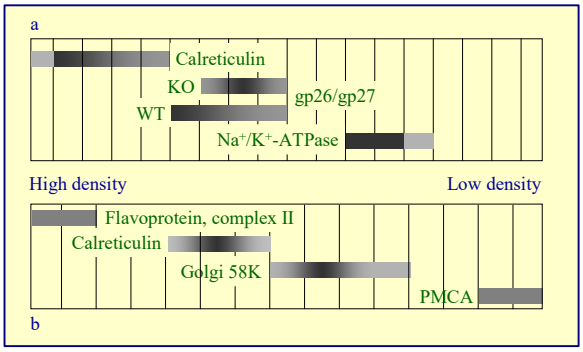

Figure 2 Panel a: Fractionation of mouse embryo fibroblasts, approx. disposition of ER (calreticulin), Golgi (gp26/gp27) and PM (Na^+/K^+-ATPase) in 1-25% iodixanol gradient; Golgi from wild-type (WT) and cav-1 knockout (KO) cells shown. Data adapted from ref 6. Panel b: Fractionation of rat brain, approx. disposition of mitochondria (flavoprotein), ER, Golgi and PM (PMCA) in 10-30% iodixanol gradient. Data adapted from ref 2. For more information see text.

6. References

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