OptiPrep™ Application Sheet S23

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

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- An **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

During the extended period of centrifugation, used in the protocol described in this Application Sheet, there will occur diffusion of iodixanol across the interfaces formed in the discontinuous gradient, but opposing this there will be some sedimentation of the iodixanol molecules. In tall narrow tubes (such as in a Beckman SW41Ti) there may be at least some retention of the discontinuous nature of the gradient, even after an overnight centrifugation. This will be particularly true of gradients of just three or four layers, but as far as is known however, the density profile of such gradients have not been checked after centrifugation. Flotation gradients such can be highly discriminating as they avoid the disturbance to the gradient and interfacial aggregation that may be created in the sedimentation format by rapidly sedimenting dense particles. It is also impossible to overload the gradient.

This discontinuous gradient in which the membranes reach their banding density by flotation from a dense sample was first used for PC12 cells [1]. In this method the post-nuclear supernatant (PNS) produced by low-speed centrifugation of the homogenate was used as the gradient input, hence to avoid the use of multiple gradients, the homogenate was restricted to 1 ml (the PNS requires dilution with an equal volume of 50% iodixanol for bottom-loading). The gradient was used primarily to identify plasma membrane and early endosomes.

The method was subsequently adapted: a 16,000*g*/30 min pellet, produced from a PNS, was used as the gradient input rather than the PNS itself, thus in this format the volume of homogenate is not critical. In this format the gradient was used to fractionate both PC12 and murine cortical neurons [2-4] and it is this format that is described in this OptiPrep™ Application Sheet.

2. Solutions required (see Section 5, Note 1)

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 20 mM Tricine-NaOH, pH 7.8 (see Section 5, Note 2)

C. Diluent: 0.25 M sucrose, 12 mM $MgCl_2$, 6 mM EDTA, 120 mM Tricine-NaOH, pH 7.8

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 Tricine (free acid): 8.95 g per 100 ml water.
100 mM EDTA (Na_2•$2H_2O$): 3.72 g per 100 ml water
100 mM $MgCl_2$•$6H_2O$: 2.03 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml of water; add 2.0 ml of EDTA stock, 4 ml of $MgCl_2$ stock and 8 ml of Tricine stock; adjust to pH 7.8 with 1 M KOH and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 40 ml of water; add 6 ml of EDTA stock, 12 ml of $MgCl_2$ stock, 24 ml of Tricine stock; adjust to pH 7.8 with 1 M KOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements

Any swinging-bucket rotor capable of approx 200,000-300,000g with tube volumes of 13 ml, such as Beckman SW 41 or Sorvall TH641 (see Section 5, Note 3)

4. Protocol

Carry out all operations at 0-4°C.

1. Suspend the cells or tissues in Solution B and homogenize using a Dounce homogenizer. Cells may require repeated passage through a 25G or 27G needle instead of (or sometimes in addition to) Dounce homogenization, (see Section 5, Note 4).

2. Centrifuge the homogenate at 800-1000 *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, Note 5).

3. Centrifuge the supernatant(s) at 16,000 *g* for 30 min (see Section 5, Note 6).

4. Resuspend the pellet in 1.5 ml (total volume) of Solution B and mix with an equal volume of Solution D.

5. Prepare 10 ml each of 5%, 10%, 15%, and 20% (w/v) iodixanol solution by mixing the appropriate volumes of Solutions B and D.

6. In approx. 13 ml tubes for the swinging-bucket rotor form a discontinuous gradient from 2.5 ml each of the four gradient solutions and the sample, by the underlayering technique using a syringe and metal cannula. If necessary top up the tube with 5% iodixanol to the correct filling. For more information on making discontinuous gradients see **Application Sheet S03**.

7. Centrifuge at 88,000 *g* for 18 h (see Section 5, Note 7).

8. If the material within the gradient is visible as a series of well-defined bands collect them using a syringe and metal cannula. Otherwise unload the gradient in 0.5-1.0 ml fractions either by tube puncture, aspiration from the meniscus or upward displacement. For more information about unloading gradients see **Application Sheet S08**.

5. Notes

1. Protease inhibitors may be included in any or all of the media at the operator's discretion. The solutions used for preparing density solutions may contain alternative buffers such as Tris, Hepes or triethanolamine.
2. This diluent, used for the preparation of the 50% iodixanol working solution (Solution D) ensures that the concentrations of EDTA and buffer remain constant throughout the gradient, which will be more or less isoosmotic. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.
3. Other swinging-bucket rotors of larger or smaller tube capacities may be used; scale all volumes up or down proportionately.
4. The homogenization protocol will need to be tailored to the cell or tissue type. For cells the ball-bearing homogenizer ("cell cracker") provides a very reliable alternative, see **Application Sheet S06**. For tissues see **Application Sheet S05**. It is not known what effect different homogenization media or protocols will have on the separation.
5. Washing the pellet in the manner described can release a significant amount of smaller material adventitiously trapped in the nuclear pellet, but the procedure does run the risk of causing nuclear disruption.
6. To apply the PNS to the gradient, omit this step and mix the PNS with an equal volume of Solution D.
7. Larger volume rotors (such as the Beckman SW28 or SW28.1) have similar tube lengths and the centrifugation conditions should not require modification. For smaller volume rotors (e.g. the Beckman SW55Ti) the centrifugation may be carried at 133,000g for 5 h [2].

6. Analysis

An example of the resolution that is achievable with the long-spin discontinuous iodixanol gradient is shown in Figure 1. Although the gradient was unloaded in a series of equal volume fractions, from the top of the gradient [2], major bands of material were found at the position of the original interfaces and the composition of these is shown in the figure. Although early endosomes were found across the position of the original 10% iodixanol layer, most of these subcellular membranes were found at the top interface. Golgi membranes (monitored by a *cis*-Golgi marker) were more or less evenly distributed in the two fractions shown in the figure, while all the ER was concentrated below the 20% iodixanol layer.

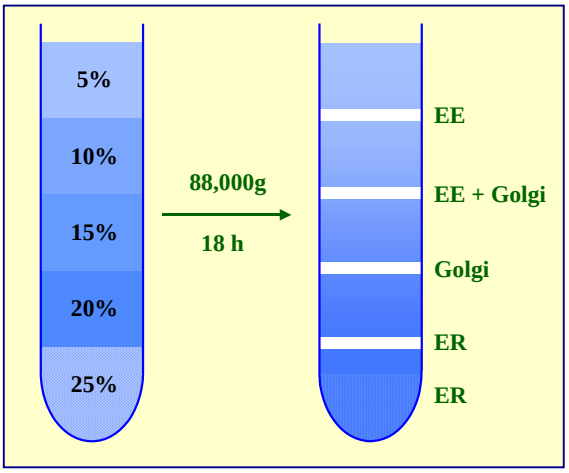

Figure 1: Fractionation of a cortical neuron light mitochondrial fraction in a discontinuous iodixanol gradient; EE = early endosomes, ER = endoplasmic reticulum; data adapted from ref 2.

As an example of the variation from tissue to tissue and cell to cell, Wu et al [1] found that major plasma membrane band from PC12 cells (identified by the EGFR) resided predominantly at the position of the top interface with a minor band at the second interface, while early endosomes banded at the bottom interface. Aside from the use of a PNS fraction rather than a 16,000*g* pellet, the only difference was the use of PC12 cells rather than cortical neurons.

7. Technical review

The strategy described in this OptiPrep™ Application Sheet has been used for studying nerve growth factor signaling in PC12 cells [1, 5 and 6] and neurotophin receptor signaling and transport in both cortical neurons [2-4] and PC12 cells [3]. Other gradient formats are summarized in Table 1.

Table 1: Bottom-loaded discontinuous gradients

Cell/tissue	% Iodixanol[1] (volume) and centrifugation/time	Membranes analyzed	Ref #
Astrocytoma	30% (0.75 ml), 25%, 17.5%, 10%, 2.5% (all 2.5 ml); 160,000*g*/ 3.5 h	PM, Golgi	7
COS-7	25%, 20.5%, 17%, 13.5%, 10%, 6.5%, 3% (total 13 ml); 50,000*g*/8 h	PM, ER	8
HeLa	32%, 24%, 20% (total approx. 5 ml); 90,000 *g*/2 h	Endosomes	9
Hippocampus	30% (0.75 ml), 25%, 17.5%, 10%, 2.5% (all 2.5 ml); 100,000*g*/ 2.5 h	IRAP/VAMP2/GLUT4 containing vesicles	10
MCF-7	32%, 24%, 20% (total approx. 5 ml); 90,000 *g*/2 h	Endosomes	11-13
Neuroblastoma	30% (1 ml), 25% (2 ml), 10% (1 ml); 250,000 *g*/3 h	APP containing vesicles	14
Sciatic nerve	23%, 15%, 10%, 5% (all 1 ml); 150,000*g*/1.5 h	ER, Golgi, light membr.	15

1. The microsomal membrane containing fraction is always in the densest solution
2. PM = plasma membrane, ER = endoplasmic reticulum, membr. = membranes, APP = Alzheimer precursor protein

In a less commonly used format the sample was median-loaded [16]. A 4000*g*/10 min supernatant from a myocardial homogenate (in 0.1 M sucrose, 10 mM EDTA, 46 mM KCl, 5 mM NaN_3, 100 mM Tris-HCl, pH 7.4); after adjusting to 5% (w/v) iodixanol; 3.0 ml was layered between 0.5 ml each of 15% iodixanol and the homogenization medium and centrifuged at 80,000 *g* for 16 h. In the five visible layers, the plasma membrane was restricted to the lowest density (Figure 2), while the ER, as determined by SERCA Ca^{2+} pump, banded in the two densest fractions, only one of which also contained the glucose-transporter GLUT4 [5]. The method was used in studies on the translocation of GLUT4 to the plasma membrane during the raised status of glucose oxidation in heart failure [16] and nitric oxide inhibition of myocardial glucose transport [17].

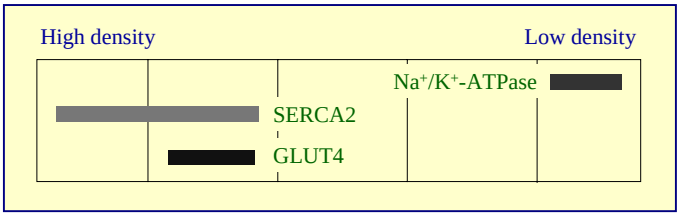

Figure 2: Fractionation of myocardial PNS on a 0%, 5%, 15% iodixanol gradient, adapted from ref 5, for more information see text

8. References

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