OptiPrep™ Application Sheet S22

Analysis of ER, plasma membrane, endosomes, Golgi, ERGIC and TGN from mammalian cells and tissues in continuous gradients (16-18 h spin)

- 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 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

Investigations into the processing of macromolecules, membrane trafficking and cell signaling often use density gradients, in association with confocal microscopy, to analyze these events. Such analyses usually involve detection of compartments such as endoplasmic reticulum (ER), Golgi, *trans*-Golgi network (TGN), ER-Golgi Intermediate Compartment (ERGIC), endosomes and the plasma membrane (PM). This Application Sheet describes "long-spin" protocols that have successfully resolved some of these compartments. There are many examples reported in the literature in which advantage of the low viscosity of iodixanol gradients (compared to those of sucrose) has been taken to carry out separations in 1.5-3 h, usually at 200,000-300,000g. Such short spin times can be important if the molecules and processes under study seriously degrade over time. The methodology is described in **Application Sheet S21**. There is evidence however that to obtain true equilibrium density banding it is preferable to carry out the centrifugation at lower RCFs (g-forces) for longer times and that under such conditions resolution is improved compared to the "short-spin" gradients.

- Some separations using intermediate centrifugation times of 5-6 h are also reported here

The following protocol is based on a method described by Woods et al [1] for the localization of the adaptor protein paxillin, in mouse 3T3 fibroblasts; it uses a continuous 10-40% iodixanol gradient and a long centrifugation time (18 h); a rather similar gradient and centrifugation conditions were used by Puglielli et al [2] for the fractionation of CHO cell membranes.

- The precise banding patterns of membranes in the gradient may depend on the type of cell, the homogenization medium, the homogenization procedure and the density range of the gradient

2. Solutions required (see Section 6, Notes 1-3)

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 140 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0

C. Diluent: 0.25 M sucrose, 140 mM NaCl, 3 mM EDTA, 60 mM Tris-HCl, pH 8.0

D. Working solution of 40% (w/v) iodixanol (ρ = 1.224 g/ml): 4 vol. of solution A + 2 vol. of solution C

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
100 mM EDTA ($Na_2 \bullet 2H_2O$): 3.72 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 4 ml, 2 ml and 28 ml respectively of Tris, EDTA and NaCl stocks; adjust to pH 8.0 with 1 M HCl and make up to 200 ml.

Solution C: Dissolve 8.5 g sucrose in 30 ml water; add 6 ml, 3 ml and 14 ml respectively of Tris, EDTA and NaCl stocks; adjust to pH 8.0 with 1 M HCl and make up to 100 ml.

3. Ultracentrifuge rotor requirements

Any swinging-bucket rotor for an ultracentrifuge capable of 100,000g with a tube capacity of approx 14 ml tubes, e.g. Beckman SW41Ti or Sorvall TH641 (**see Section 6, Note 4**)

4. Protocol

The protocol is described for a cultured cell monolayer. Step 1 may be carried out at room temperature, carry out all subsequent steps at 4°C.

1. Wash the cell monolayer twice with phosphate-buffered saline and once with Solution B (without the EDTA)

2. Scrape the cell monolayer into Solution B and homogenize the cells using no more than 20 strokes of the pestle of a Dounce homogenizer (**see Section 6, Note 5**).

3. Pellet debris and nuclei by centrifugation for 5 min at 700-800 *g*

4. Decant and retain the post-nuclear supernatant (PNS).

5. Prepare the two gradient solutions of 10% and 40% (w/v) iodixanol by diluting Solution D with Solution B.

6. Use either a standard two-chamber gradient maker or a Gradient Master™ to prepare 10-12 ml continuous 10-40% (w/v) iodixanol gradients from equal volumes of he two iodixanol solution in tubes for the suitable swinging bucket rotor (**see Section 6, Notes 6 and 7**).

7. Load the PNS (1-3 ml) on to each gradient (**see Section 6, Note 8**).

8. Centrifuge the gradients at 48,000 *g* for 18 h.

9. Unload the gradients in approx 0.5 ml fractions by upward displacement, aspiration from the meniscus or by tube puncture (**see Section 6, Note 9**) and analyze the fractions for appropriate membrane markers.

5. Analysis

The top half of the gradient resolves endosomes (approx 1.055-1.070 g/ml) and clathrin-coated vesicles (approx 1.08-1.09 g/ml). These densities correlate very closely with those observed in self-generated iodixanol gradients for these two endocytic compartments from rat liver (described in **Application Sheet S43**) The bottom half of the gradient resolves two fractions of ER, a light one (approx 1.110 g/ml) and a dense one (approx 1.145 g/ml). Paxillin (not shown) co-purifies with the dense ER [1] and confocal microscopy studies indicate that this dense ER is perinuclear.

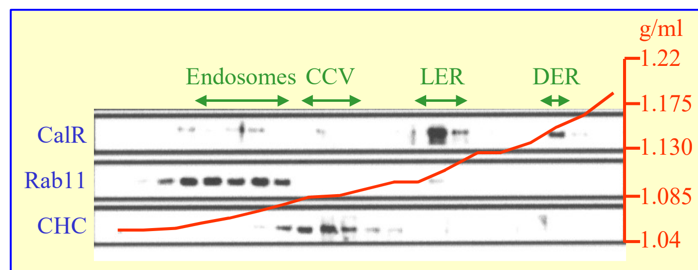

Figure 1 Fractionation of PNS from 3T3 cells on a continuous iodixanol gradient: fractions analyzed for CalR (calreticulin), Rab 11 and CHC (clathrin heavy chain) and density (red line).
CCV = clathrin coated vesicles, LER = light endoplasmic reticulum, DER = dense endoplasmic reticulum. For more information see text. Reproduced from ref 1 with kind permission of the authors and the American Society for Biochemistry and Molecular Biology

Puglielli et al [2] used a similar iodixanol gradient (8-34%) and centrifugation at a slightly higher RCF (100,000g) for the same time to study the processing of the amyloid β peptide From CHO cells stably transfected with PS1 the authors obtained a very satisfactory resolution (see Figure 2) of early endosomes (EEA-1 as marker), Golgi (syntaxin6 as marker) and ER (calnexin as marker). **For more information on analysis see Section 7.5**.

6. Notes

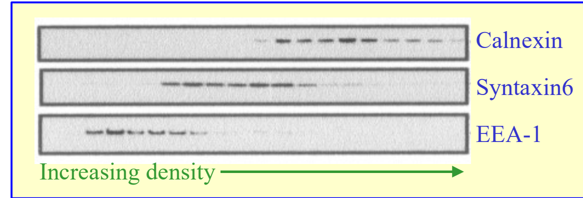

Figure 2 Fractionation of a PNS from CHO cells fractionated on a continuous iodixanol gradient: distribution of ER (calnexin), Golgi (syntaxin6) and early endosome (EEA-1) markers. Reproduced from ref 2 with kind permission of the authors and Macmillan Magazines Ltd.

1. Although traditionally a simple 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. The solutions used for preparing density solutions may contain alternative buffers such as Tris, Tricine or triethanolamine. **See Section 7.1 for some of the media that have been used**.
2. Protease inhibitors may be included in Solutions B and C at the operator's discretion.
3. The diluent (Solution C), used for the preparation of the 40% iodixanol working solution (Solution D) ensures that the concentrations of EDTA and buffer remain constant throughout the gradient. If it is considered the NaCl should also be constant throughout the gradient, then this should be present in Solution C at 3x the given concentration. Under these conditions however, the gradient solutions will be hyperosmotic. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01**.
4. The method should be adaptable to larger or smaller volume swinging-bucket rotors; increase or decrease the volumes of sample and gradient proportionately.
5. The homogenization protocol will need to be tailored to the cell type. Dounce homogenization or passages through a 25-gauge syringe needle or sometimes a combination of both 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. 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**.
7. An alternative approach is a discontinuous gradient prepared from multiple layers, the density increment between layers being very small. Such a gradient can be regarded essentially as continuous and linear. An example is provided by Lee et al [3] who used 1 ml each of 5.0, 6.5, 8.5, 10.5, 12.5, 14.5, 16.5, 18.5 and 20% (w/v) iodixanol.
8. There is evidence that flotation of particles can provide better resolution than sedimentation. To use this approach the gradient should be modulated to 10-35% (w/v) iodixanol and the vesicle suspension adjusted to 40% (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 Section 7.3.
9. For more information on harvesting gradients **see Application Sheet S08**.

7. Technical 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, such as the 140 mM NaCl used in this protocol, 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)**. 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 (see Table 1).

Table 1: Homogenization media

Cell/tissue type	Homogenization medium (HM) composition	Ref #
Adenocarcinoma	78 mM KCl, 4 mM $MgCl_2$, 8.32 mM $CaCl_2$, 10 mM EGTA, 50 mM Hepes-KOH, pH 7.0[1]	4
Brain (mouse)	0.25 M sucrose, 1mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, pH 7.4	3
Epithelial	120 mM NaCl, 20 mM KCl, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5	5
Liver	0.5 M sucrose, 5 mM $MgCl_2$, 5 mM mercaptoethanol, 50 mM K-phosphate, pH 6.5	6
Neuroglioma	78 mM KCl, 4 mM $MgCl_2$, 8.32 mM $CaCl_2$, 10 mM EGTA, 50 mM Hepes-KOH, pH 7.0	7
Neuronal	0.25 M sucrose, 1 mM EDTA, 2 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0	8
Pheochromocytoma	0.25 M sucrose, 78 mM KCl, 4 mM $MgCl_2$, 8.32 mM $CaCl_2$, 10 mM EGTA, 50 mM Hepes-KOH, pH 7.0[2]	9

1. Solution used for dilution of OptiPrep™: 0.75% NaCl, 2 mM KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0
2. Solution used for dilution of OptiPrep™ contained 42 mM sucrose

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

Probably the most common approach is the simplest one: to remove the nuclei from the homogenate and apply a total post-nuclear supernatant (PNS) to the top of the gradient. The big advantage of using a low-speed supernatant is that little of the total vesicle fraction or the smaller and less dense organelles will be lost; moreover the particles separated in the gradient will not have experienced the serious aggregation that can occur during pelleting and it saves a considerable amount of time. Nuclear pelleting is normally carried out at 500-1000g for 5-10 min. To recover any vesicles trapped in the pellet, the latter is sometimes resuspended in the 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. If the RCF used for this preliminary "clarification" is raised to 3000g, a variable proportion of the mitochondria will also be removed.

If the PNS is inconveniently large for application to the gradient, then an alternative approach is to centrifuge this supernatant at approx. 80,000-150,000 g for 45-60 min and to resuspend the pellet in HM before applying to the gradient. This strategy also removes some of the cytosolic proteins; these can sediment and diffuse from a PNS into the gradient during the subsequent centrifugation. The extent of this movement into the gradient depends of course on the molecular mass of the proteins, centrifugation time and RCF. A variation in this strategy, which avoids contamination from cytosolic proteins, is to suspend the 80,000-150,000g pellet in a solution of iodixanol so that it can be loaded beneath the gradient rather than on top, so the membranes float rather than sediment to their banding positions. Some examples of these alternative sample handlings are given in Table 2.

Table 2: Sample application to gradient[1]

Cell/tissue	Procedure	Comments	Ref #
Brain (mouse)	PNS adjusted to 25% (w/v) iodixanol	Loaded under a 5-20% iodixanol gradient	3
CHO	PNS→100,000g/1 h→pellet		10
Embryo kidney	PNS→100,000g/1 h→pellet		10
Epithelial	PNS→20,000g/20 min→SN	Microsomal SN loaded on top of gradient	5
Fibroblasts	PNS adjusted to 30% (w/v) iodixanol	Loaded under a 10-25% iodixanol gradient	11
HeLa	PNS→100,000g/30 min→pellet		12

1. Unless indicated any pellet is resuspended in HM and applied to the top of the gradient, PNS = post-nuclear supernatant, SN = supernatant

7.4. Centrifugation conditions

The most commonly used regime is 100,000g for 18 h, but the advantage of using a lower RCF of 48,000g is that any tendency for the iodixanol molecules to sediment, and so make the linear gradient slightly steeper close to the bottom of the tube, is minimized. A compromise regime that uses a higher RCF (200,000g) for a shorter time (6 h) has also been reported [12]

7.5. Membrane analysis

As a general rule the density of membrane compartments separated on these long-spin iodixanol gradients increases in the order PM<early endosomes<TGN<*cis*-medial Golgi<ERGIC<ER<late endosomes<lysosomes. There are however exceptions to this, in pheochromocytoma (PC12) cells for example the early endosomes banded just below the middle of a 5-20% iodixanol gradient; they were however predictably less dense than the late endosomes [9]. Their position may simply reflect the lower density range of the gradient and/or the hyperosmotic nature of the homogenization medium. In shallow flotation gradient lysosomes from mouse brain banded between the early endosomes and Golgi [3].

The method described above has also been used more recently by Woods et al [13], who confirmed the banding of endosomes and ER shown in Figure 1 and showed in addition that the plasma membrane banded at the very top of the gradient, while the kinase PKD1 showed no correlation with any of these three major membrane compartments, banding instead between the endosomes and ER. The β3 integrin on the other hand co-purified with all three of the major compartments from a wild-type cell, but appeared additionally in the PKD1-containing zone from cells expressing catalytically inactive PKD1. Often the "tight" banding of membranes in iodixanol gradients permits the definitive identification of a particular compartment, even though its separation from a neighbouring compartment is minimal. For example [12] although the early endosomes from HeLa cells in a 0-28% iodixanol gradient were only slightly less dense than the Golgi, the definition was nevertheless very clear.

These gradients are also useful for at least partial resolution of sub-compartments of the Golgi and ERGIC. Panel a of Figure 3 indicates the banding of four protein markers within a 10-25% (w/v) iodixanol: ribophorin II (an ER resident protein), mannosidase II (an enzyme characteristic of the medial-*trans* Golgi) and 14-3-3 (a *trans*-Golgi marker) from an adenocarcinoma post-nuclear supernatant [4]. The fraction marked with a green arrow was identified as ERGIC. Panel b of Figure 3 similarly shows the banding of markers from a human neuroglioma cell post-nuclear supernatant; the median fractions containing β-COP and Rab6 identified this region as being enriched in membranes associated with Golgi-to-ER retrograde transport and the presence of immature APP suggested that this was either ERGIC or early Golgi [7]. These two examples are chosen not only to display the fine resolution achievable with these gradients, but also to highlight that although the gradient and centrifugation conditions were almost identical and the relative densities of the various membrane compartments were very similar, significant differences in detailed banding can be observed, which is almost certainly related to the use of difference cell types.

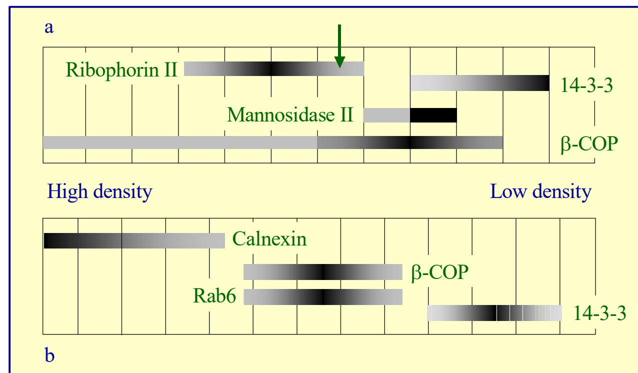

Figure 3 a: Fractionation of a lung adenocarcinoma cell post-nuclear supernatant, banding of membrane markers in a 10-25% (w/v) iodixanol gradient – data adapted from ref 4, see text.
b: Fractionation of a human neuroglioma cell post-nuclear supernatant, banding of membrane markers in a 10-28% (w/v) iodixanol gradient – data adapted from ref 7, see text.

8. References

1. Woods, A.J., Roberts, M.S., Choudhary, J., Barry, S.T., Mazaki, Y., Sabe, H., Morley, S.J., Critchley, D.R. and Norman, J.C. (2002) *Paxillin associates with poly(A)-binding protein 1 at the dense endoplasmic reticulum and the leading edge of migrating cells* J. Biol. Chem., **277**, 6428-6437
2. Puglielli, L., Konopka, G., Pack-Chung, E., MacKenzie Ingano, L .A., Berezovska, O., Hyman, B.T., Chang, T. Y., Tanzi, R.E. and Kovacs, D.M. (2001) *Acyl-coenzyme A: cholesterol acyltransferase modulates the generation of the amyloid β-peptide* Nature Cell Biol., **3**, 905-912
3. Lee, M-S., Kao, S-C., Lemere, C.A., Xia, W., Tseng, H-C., Zhou, Y., Neve, R., Ahlijanian, M.K. and Tsai, L-H. (2003) *APP processing is regulated by cytoplasmic phosphorylation* J. Cell Biol., **163**, 83-95
4. Hood, J.L., Brooks, W.H. and Roszman, T.L. (2004) *Differential compartmentalization of the calpain/calpastatin network with the endoplasmic reticulum and Golgi apparatus* J. Biol. Chem., **279**, 43126-43135
5. Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B.E. and Somlo, S. (2002) *Polycystin-2 is an intracellular calcium release channel* Nature Cell Biol., **4**, 191-197
6. Trombetta, E.S. and Helenius, A. (1999) *Glycoprotein reglucosylation and nucleotide sugar utilization in the secretory pathway: identification of a nucleoside diphosphatase in the endoplasmic reticulum* EMBO J., **18**, 3282-3292
7. Tekirian, T.L., Merriam, D.E., Marshansky, V., Miller, J., Crowley, A.C., Chan, H., Ausiello, D., Brown, D., Buxbaum, J.D., Xia, W. and Wasco, W. (2001) *Subcellular localization of presenilin 2 endoproteolytic C-terminal fragments* Mol. Brain Res., **96**, 14-20
8. Kim, A.H., Yano, H., Cho, H., Meyer, D., Monks, B., Margolis, B., Birnbaum, M.J. and Chao, M.V. (2002) *Akt1 regulates a JNK scaffold during excitotoxic apoptosis* Neuron, **35**, 697-709
9. Li, Y., Chin, L-S., Levey, A.I. and Li, L. (2002) *Huntingtin-associated protein 1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate and functions in endosomal trafficking* J. Biol. Chem., **277**, 28212-28221
10. Augustin, R., Riley, J. and Moley, K.H. (2005) *GLUT8 contains a [DE]XXXL[LI] sorting motif and localizes to a late endosomal/lysosomal compartment* Traffic, **6**, 1196-1212
11. Molinari, M., Eriksson, K.K., Calanca, V., Galli, C., Cresswell, P., Michalak, M. and Helenius, A. (2004) *Contrasting functions of calreticulin and calnexin in glycoprotein folding and ER quality control* Mol. Cell, **13**, 125-135
12. Hirose, H., Arasaki, K., Dohmae, N., Takio, K., Hatsuzawa, K., Nagahama, M., Tani, K., Yamamoto, A., Tohyama, M. and Tagaya, M. (2004) *Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi* EMBO J., **23**, 1267-1278
13. Woods, A.J., White, D.P., Caswell, P.T. and Norman, J.C. (2004) *PKD1/PKCμ promotes αvβ3 integrin recycling and delivery to nascent focal adhesions* EMBO J., **23**, 2531-2543

Application Sheet S22; 7th edition, January 2018

Alere Technologies AS

Axis-Shield Density Gradient Media
is a brand of Alere Technologies AS