Analysis of membranes from *Drosophila*

- 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.
- This Application Sheet contains the following: Section 1 - Fractionation of ER, Golgi and PM; Section 2 - Lipid rafts; Section 3 – Other analyses.
- At the end of Sections 1 and 2 there are important technical reviews.
- Section 4 summarizes some of the more recent papers.

1. Fractionation of endoplasmic reticulum (ER), Golgi and plasma membrane (PM)

1a. Background

Although iodixanol gradients have been used widely since 1996 for the fractionation of these subcellular membrane compartments from mammalian cells, their application to the membranes from *Drosophila* has only been realized relatively recently (mainly since 2004). The methodology described in this OptiPrep™ Application Sheet employs a pre-formed 10%, 20%, 30% (w/v) iodixanol discontinuous gradient in a near-vertical rotor [1]. A crude membrane fraction was incorporated into each iodixanol solution and in this respect the method is similar to that devised by Yeaman et al [2] for MDCK cells. A more or less linear continuous gradient will from, partly by self-generation in the centrifugal field and partly by diffusion. Membranes are separated on the basis of buoyant density. It is quite likely that other iodixanol gradients, developed for mammalian cells would also be applicable, but this can only be verified by experimentation.

1b. Solutions required (see Section 1e-1)

A. OptiPrep™

B. Wash Solution 1: 0.7% (w/v) NaCl, 0.03% (w/v) Triton X-100

C. Homogenization Medium (HM): 0.25 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5

D. KCl: 2M KCl

E. Sucrose Cushion: 0.5 M sucrose, 0.5 mM EDTA, 50 mM KCl, 15 mM Tris-HCl, pH 7.0

F. Wash Solution 2: 5 mM EDTA, 50 mM KCl, 10 mM Tris-HCl, pH 7.5

G. Membrane Suspension Buffer: 0.25 M sucrose, 50 mM KCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5

H. Optiprep™ Dilution Buffer: 300 mM KCl, 30 mM EDTA, 60 mM Tris-HCl, pH 7.5

I. Optiprep™ Working Solution (50% iodixanol): Dilute 5 vol. OptiPrep™ with 1 vol. of Solution H

Protease inhibitors may be included in Solutions B, C and E-H at the operator's discretion.

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
2 M KCl: 14.9 g per 100 ml water

Solution C: Dissolve 17 g sucrose in 100 ml water; add 10 ml and 2 ml respectively of EDTA and Tris stock solutions; adjust to pH 7.5 with HCl and make up to 200 ml.

Solution E: Dissolve 17 g sucrose in 50 ml water; add 2.5 ml, 0.5 ml and 1.5 ml respectively of KCl, EDTA and Tris stock solutions; adjust to pH 7.0 with HCl and make up to 100 ml.

Solution F: To 50 ml of water add 2.5 ml, 5 ml and 1 ml respectively of KCl, EDTA and Tris stock solutions; adjust to pH 7.5 with HCl and make up to 100 ml.

Solution G: Dissolve 8.5 g sucrose in 50 ml water; add 2.5 ml, 5 ml and 1 ml respectively of KCl, EDTA and Tris stock solutions; adjust to pH 7.5 with HCl and make up to 100 ml.

Solution H: Mix 15 ml, 30 ml and 6 ml respectively of KCl, EDTA and Tris stock solutions; adjust to pH 7.5 with HCl and make up to 100 ml.

1c. Ultracentrifuge rotor requirements (see Section 1e-2)

Swinging-bucket rotor (e.g. Beckman SW55Ti or SW41Ti) and a near-vertical rotor (e.g. Beckman NVT90 or NVT65.2)

1d. Protocol (adapted from refs 1 and 3)
Carry out all operations at 0-4°C.

Option A: Membranes in the 3000 *g* supernatant (from Step 4) may simply be pelleted and resuspended before iodixanol gradient centrifugation (Step 5)
Option B: Membranes may be separated from cytosolic proteins and small contaminants on a sucrose density barrier (Steps 6-8) before iodixanol gradient centrifugation.

1. Wash dechorionated 1-3 h embryos twice in Solution B and once in Solution C.

2. Suspend embryos in 10 vol. of Solution C and homogenize firstly in a loose-fitting Dounce (glass-glass) homogenizer (Wheaton type B) and then a tight-fitting one (Wheaton type A). Monitor the homogenization by light microscopy.

3. Add Solution D to the homogenate (0.1 vol. + 3.9 vol. respectively) to adjust the KCl concentration to 50 mM (see Section 1e-1).

4. Centrifuge the homogenate at 3000 *g* for 10 min to remove debris and larger organelles.

5. **Option A**: Pellet the membranes from the 3000 *g* supernatant at 100,000 *g* for 1 h (total membrane fraction) **OR** at 20,000 *g* for 30 min to isolate a Golgi-rich fraction (see Section 1e-3).

6. **Option B**: Layer the 3000 *g* supernatant on a cushion of Solution E and centrifuge in a swinging-bucket rotor at 100,000 *g* for 1 h (see Section 1e-3).

7. **Option B**: Using a syringe and metal cannula (i.d. 0.8-1.0 mm) aspirate as much of the supernatant as possible without disturbing the pellet.

8. **Option B**: Resuspend the pellet in the residual solution; mix with 5 vol. of Solution F and re-centrifuge at 100,000 *g* for 1 h.

9. Using a syringe and metal cannula aspirate the supernatant from Step 5 or Step 8 and resuspend the pellet in approx. 1 ml of Solution G.

10. Prepare three gradient solutions (approx. 2 ml each) from the following volume ratios of Solution I, the membrane suspension and Solution G: 3:2:0, 2:2:1 and 1:2:2 (see Section 1e-4).

11. Layer 1.8 ml of each gradient solution in Optiseal™ tubes for the near-vertical rotor by, under- or over-layering (see Section 1e-5).

12. Centrifuge at 340,000 g_{av} for 3 h using a slow acceleration program.

13. Allow the centrifuge to decelerate to rest from 2000 rpm without the brake or use a slow deceleration program.

14. Collect the gradient in 0.25 ml fractions by tube puncture, upward displacement or aspiration from the meniscus. For more information on harvesting gradients from sealed tubes see **Application Sheet S08**.

- For information on analysis of the gradient fractions and a short review of some of the other papers see Section 1e-6.

1e. Technical Notes and Review

1e-1. Homogenization media

The homogenization medium (HM) often has been specifically tailored to fractionation of *Drosophila* membranes. In the example given solutions are buffered with Tris but Hepes, Tricine or triethanolamine (at the same concentration) may be used if preferred. It is unlikely that the type of buffer significantly influences the homogenization or fractionation, although with mammalian cells triethanolamine does seem to offer some particular advantages in homogenization efficiency.

The preparation of a Working Solution (Solution I) as described, ensures that the concentrations of KCl, EDTA and Tris buffer are constant throughout the gradient, while the sucrose and iodixanol act as osmotic balancers to maintain an approx. constant osmolality. If this is deemed unimportant the gradient solutions may be prepared directly from OptiPrep™, but if this option is chosen then the concentrations of KCl, EDTA and buffer will decrease with increasing solution density.

Beronja et al [1] adjusted the KCl concentration in the homogenate (50 mM) as described in the protocol, but Papoulas et al [3] adjusted it to 100 mM. If the KCl concentration is adjusted to 100 mM KCl, then all subsequent solutions used (Solutions E-H) should be similarly adjusted.

Tan et al [4] used 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 10 mM HEPES, pH 7.4 as an homogenization medium in their proteomic studies and in some cases the osmotic balancer is NaCl and not sucrose, e.g. 150 mM NaCl, 0.2 mM EGTA, 100 mM Tris, pH 7.4 [5]

1e-2. Ultracentrifuge rotors

Choose whichever swinging-bucket rotor is most suitable for the amount of material available (see Step 6). The iodixanol gradient centrifugation is carried out in a near-vertical rotor. The gradient is formed partly by self-generation, partly by diffusion. Other rotors with different sedimentation path lengths may be suitable but the optimal centrifugation conditions will require investigation. Near-vertical rotors are preferred over vertical ones because any very dense particles will form a well defined pellet close to the bottom of the tube (as in a fixed-angle rotor), while in a vertical rotor any dense material will pellet along the entire length of the wall of the tube and may contaminate fractions during unloading. The use of Beckman Optiseal™ tubes is recommended because of the ease of use and the ability to use a variety of options for gradient unloading (see Step 14); for other tubes such as heat-sealed tubes, tube puncture is the only safe and reliable option. For more information self-generated gradients **see Application Sheet S04**.

1e-3. Preliminary purification

For the analysis of the major membrane fractions (plasma membrane, Golgi and ER) the 3000 *g* supernatant may be simply centrifuged at 100,000 *g* to pellet the microsomes [3] or partially purified by sedimentation through a sucrose cushion [1]. The latter will more effectively remove soluble proteins, which will remain principally in the sample zone. The 100,000 *g* step was replaced by Papoulas et al [3] by a 20,000 *g* step if the aim was to isolate primarily the Golgi for use *in vitro* incubations.

1e-4. Gradient variations

Adolfson et al [6] adopted a simpler iodixanol gradient format; the post-nuclear extract was adjusted to 26% (w/v) iodixanol to form a self-generated gradient at 300,000 g_{av} in a near-vertical rotor, while Niimura et al [7] used a discontinuous 2.5-30% (w/v) iodixanol gradient.

1e-5 Gradient layering

Although underlayering with a syringe and metal cannula is the recommended method for making discontinuous gradients, overlayering maybe more convenient since the tubes need to be filled exactly to the bottom of the neck. For more information on gradient construction **see Application Sheet S03**. If necessary, adjust all volumes proportionately so that tubes (after sample application) are properly filled according to the manufacturer's instructions.

1e-6. Method review

In the gradient system described in this OptiPrep™ Application Sheet, the Golgi, ER and PM banded approximately as shown in Figure 1. The Golgi was identified by the Lava lamp protein, the ER by BiP and the PM by α-spectrin, which was also present in the Golgi region [3]. Unlike most mammalian cells the Golgi was denser than the ER; but like mammalian cells, the PM banded close to the top of the gradient. Dynein also co-banded with the Golgi but was also present in some denser fractions. These gradients were used in characterization of the Sec6 component of the exocyst complex [1], the dynein based motility of Golgi membranes [3] and to confirm the association of dLgl and dFmr1 with the Golgi [8]. The method has also been used in proteomic studies [9] and to identify an ER location for the Seele protein [10].

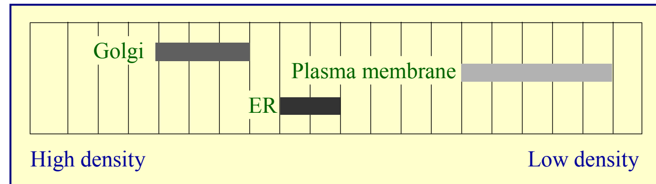

Figure 1. Distribution of *Drosophila* membranes in iodixanol gradient; for more information see text. Data adapted from ref 3

In the 2.5-30% discontinuous iodixanol gradients used by Niimura et al [7] the ER banded at a higher density than the Golgi. In the self-generated gradients reported by Adolfsen et al [6], synaptic vesicles banded close to the top of the gradient and clearly discriminated low-density vesicles containing Synaptotagmin 1 from denser ones containing Synaptotagmins 4 and 7. Paneels et al [11] used a 5%, 30%, 40% iodixanol flotation gradient to band the plasma membrane at the 5%/30% interface (see Section B).

A 10-40% (w/v) iodixanol gradient (250,000 *g*, for 3 h) separates the plasma membrane from the cytoskeleton [12]. A simple three-layer gradient in which a 1000 *g* supernatant in 40% iodixanol is layered beneath layers of 5% and 30% iodixanol [5,13] centrifuged at 100,000 *g* for 3 h results in the plasma membrane banding below the 5% layer. Tan et al [4] underlaid a 2000 *g*/5 min supernatant with 6% and 8% (w/v) iodixanol and centrifuged at 100,000 *g* for 90 min to concentrate the membranes at the interface of the two iodixanol solutions. The recovered membranes were adjusted to 12.5% iodixanol and centrifuged in a Beckman VTi65.1 vertical rotor for 1 h. The method resolved PM, Golgi, ER and mitochondria.

♦ For some more recent publications see Section 4

2. Isolation of lipid rafts (as detergent-resistant membranes)

2a. Background

Rietveld et al [14] were the first to report isolation of lipid rafts from *Drosophila* using flotation in iodixanol gradients. A crude PM fraction was first produced from a post-nuclear supernatant of the homogenate (adjusted to 1.4 M sucrose) by flotation through a layer of 1.22 M sucrose. Hoehne et al [15] used a similar approach. Paneels et al [11] and Eroglu et al [16] adapted this plasma membrane isolation method to iodixanol. The crude low-density membrane fraction was then extracted with Triton X-100 and the lipid rafts isolated by flotation through a discontinuous iodixanol gradient. Zhai et al [17] prepared lipid rafts from both *Drosophila* and from the *Drosophila* S2 cell line directly from a 5000*g* supernatant of the homogenate, without a preliminary preparation of a crude PM fraction.

♦ The method below is adapted from refs 11, 14 and 16. Some of the variants are described in the Technical Notes and Review Section (Section 2e).

2b. Solutions required (see Box on next page and Section 2e-1)

A. OptiPrep™
B. Wash Solution 1: 0.9% (w/v) NaCl, 0.1% (w/v) Triton X-100
C. Wash Solution 2: 0.9% (w/v) NaCl
D. Homogenization Medium (HM): 0.3 M sucrose, 150 mM NaCl, 0.2 mM EGTA, 100 mM Tris-HCl, pH 7.5
E. TNE: 150 mM NaCl, 0.2 mM EGTA, 100 mM Tris-HCl, pH 7.5
F. Optiprep™ Dilution Buffer: 150 mM NaCl, 1.2 mM EGTA, 100 mM Tris-HCl, pH 7.5

G. Optiprep™ Working Solution (50% iodixanol): Dilute 5 vol. OptiPrep™ with 1 vol. of Solution F

Keep the following stock solutions at 4°C:
1 M Tris (free base): 12.1 g per 100 ml water
1 M NaCl: 5.84 g per 100 ml water
100 mM EGTA (free acid): 3.80 g per 100 ml water (pH 11-12)

Solution D: Dissolve 20.5 g sucrose in 100 ml water; add 30 ml, 20 ml and 0.4 ml respectively of NaCl, Tris and EGTA stock solutions; adjust to pH 7.5 with HCl and make up to 200 ml.

Solution E: To 100 ml of water add 30 ml, 20 ml and 0.4 ml respectively of NaCl, Tris and EGTA stock solutions; adjust to pH 7.5 with HCl and make up to 200 ml.

Solution F: To 50 ml water add 15 ml, 10 ml and 1.2 ml respectively of NaCl, Tris and EGTA stock solutions; adjust to pH 7.5 with HCl and make up to 100 ml.

2c. Ultracentrifuge rotor requirements (see Section 2e-2)

Swinging-bucket rotors: approx. 27 ml tubes (e.g. Beckman SW28) and approx. 5 ml tubes (e.g. Beckman SW55)

2d. Protocol

Carry out all operations, except step 1, at 0-4°C.

1. Wash the dechorionated embryos twice in Solution B, three times in Solution C.
2. Wash the embryos twice in Solution D.
3. Suspend washed embryos in 10 vol. of Solution D and homogenize firstly in a loose-fitting Dounce (glass-glass) homogenizer (Wheaton type B) and then a tight-fitting one (Wheaton type A). Monitor the homogenization by light microscopy (see Section 2e-3).
4. Centrifuge the homogenate at approx. 3000 *g* for 10 min to remove nuclei and debris.
5. Adjust the iodixanol concentration of the 3000 *g* supernatant to 40% (w/v) iodixanol by mixing 1 vol. with 4 vol. of Solution G (see Section 2e-4).
6. Prepare solutions of 30% and 5% (w/v) iodixanol from Solution G and Solution D (volume ratios of 3:2 and 1:9 respectively).
7. Distribute the 3000 *g* supernatant (in 40% iodixanol) equally amongst tubes for the 27 ml swinging-bucket rotor and layer 10 ml and 5 ml respectively of the 30% and 5% iodixanol solutions on top to fill the tube (see Section 2e-5).
8. Centrifuge at 100,000 *g* for 3 h.
9. Collect the plasma membrane enriched fraction from the 5%/30% iodixanol interface and dilute with 3 vol. of Solution E.
10. Pellet the membranes at 50,000 *g* for 30 min.
11. Aspirate the supernatant; resuspend the pellet in Solution E and repeat Step 10.
12. Resuspend the pellet in 0.5 ml of Solution E and mix with an equal volume of Solution E containing 2% (w/v) Triton X-100 (or other chosen detergent at twice the required concentration).
13. Keep at 4°C for 30 min to solubilize the detergent-sensitive membranes.
14. During the solubilization prepare solutions of 21%, 15% and 6%(w/v) iodixanol by diluting Solution G with Solution E at volume ratios of 2.1:2.9, 1.5:3.5 and 0.6:4.4 respectively. Note that there are important published variations in the density of the gradient solutions (see Section 2e-6).
15. Mix the suspension from Step 13 with an equal volume of Solution G.
16. In tubes for the approx. 5 ml swinging-bucket rotor, over layer 2 ml of the sample with 1 ml each of the 21%, 15% and 5% (w/v) iodixanol solutions (see Section 2e-5).

17. Centrifuge at approx 150,000 g_{av} for 5-6 h (see Section 2e-7).

18. Allow the rotor to decelerate without the brake below 2000 rpm or use a controlled deceleration program.

19. The lipid rafts band as a visible layer at the top interface. Harvest this layer or collect the gradient in 0.25-0.5 ml fractions by tube puncture, upward displacement or aspiration from the meniscus. For more information on harvesting gradients see **Application Sheet S08**.

2e. Technical Notes and Review

2e-1. Homogenization media

The homogenization medium (HM) often has been specifically tailored to fractionation of *Drosophila* membranes. In the example given solutions are buffered with Tris but Hepes, Tricine or triethanolamine (at the same concentration) may be used if preferred. It is unlikely that the type of buffer significantly influences the homogenization or fractionation, although with mammalian cells triethanolamine does seem to offer some particular advantages in homogenization efficiency.

HM variations include 30 mM NaCl, 5 mM EDTA, 20 mM HEPES, pH 7.5, 1% TX-100 for *Drosophila* photoreceptors [18], 150 mM NaCl, 20 mM EGTA, 100 mM Tris-HCl pH 7.5, 1% TX-100 for a *Drosophila* neuronal cell line [19] and *Drosophila* heads [20].

The preparation of a Working Solution (Solution G) as described, ensures that the concentration of EGTA is constant throughout the gradients. If this is deemed unimportant the gradient solutions may be prepared directly from OptiPrep™. An advantage of this approach is that the final volume of dense membrane suspension (Steps 5 and 15 of the protocol) is smaller.

Protease inhibitors may be included in Solutions D-F at the operator's discretion.

2e-2. Ultracentrifuge rotors

For smaller amounts of starting material a rotor such as a 14 ml rotor (e.g. Beckman SW41) may be substituted for the SW28.

2e-3. Homogenization

Other means of homogenization have been reported; Rietveld et al [14] used a Potter-Elvehjem (glass-Teflon) homogenizer before the double Dounce homogenization. Zhai et al [17] homogenized the embryos directly in a detergent-containing buffer by passing the suspension 20x through the fine needle (27G) of a syringe, thus obviating the first plasma membrane gradient.

2e-4. Adjustment of density of 3000*g* supernatant

If OptiPrep™ is used to adjust the density then mix 2 vol. of OptiPrep™ with 1 vol. of supernatant. Although reducing the volume, the EGTA concentration will also be reduced.

2e-5. Layering the gradient

Although underlayering with a syringe and metal cannula is the recommended method for making discontinuous gradients, overlayering should be acceptable in view of the large difference in density between the solutions. For more information on gradient construction **see Application Sheet S03**. If necessary, adjust all volumes proportionately so that tubes (after sample application) are properly filled according to the manufacturer's instructions.

2e-6. Lipid raft gradient

There is considerable scope for variation in the exact format of the iodixanol gradient: (1) sample in 40% iodixanol, overlaid with 1.2 ml of 30% iodixanol and 0.2 ml of 0% iodixanol [11,18]; (2) sample in 40% iodixanol, overlaid with 1ml each of 30% iodixanol, 20%, 5% and 0% iodixanol [15] and (3) sample in 40% iodixanol, overlaid with 0.9 ml of 30% iodixanol and 0.3 ml of 5% iodixanol [16,19,20]. The latter was carried out in the small volume Beckman TLS55 rotor. Zhai et al [17] also used this format for *Drosophila* S2 cells in the much larger volume Beckman SW60 rotor.

It is also worth noting that in most cases [11,14,15] Triton X-100 (or other detergent) was only included in the dense sample layer, but Zhai et al [17] included 1% Triton X-100 in all the gradient solutions.

2e-7. Centrifugation

The centrifugation conditions vary from laboratory to laboratory; shorter times at higher *g*-forces (e.g. 2 h at 280,000 *g*) may be used or lower *g*-forces for longer times.

3. Other analyses

3a. Cytosolic and membrane proteins

A very simple method for resolving the cytosolic from membrane proteins involves homogenization of the *Drosophila* heads in 0.25 M sucrose, 10 mM KOAc, 2 mM $Mg(OAc)_2$, 5 mM DTT, 30 mM HEPES, pH 7.4; clarifying the lysate three times at 1000 *g* for 5 min, then adjusting the supernatant to 30% (w/v) iodixanol and centrifuging at 350,000 *g* for 1 h. All of the membranes float to the top and the soluble proteins sediment [21].

3b. Early endosomes

A 3000 *g* supernatant of an embryo lysate in 100 mM KCl, 0.25 M sucrose, 5 mM EDTA, 10 mM Tris, pH 7.5 was layered over a 2.5-30% (w/v) iodixanol gradient, centrifuged at 37,000 *g*, for 1 h. Early endosomes were clearly identified by Rab5 antibodies [22].

3c. Rhabdomere membranes

The resolution of rhabdomere membranes from *Drosophila* eyes is dependent on the severity of the homogenization (reciprocating shaker in the presence of silica beads or an Ultra-Turrax macerator). The smaller membrane fragments produced by the latter permitted the resolution of Rh-1 and *Hs*SERT containing subpopulations. For more information please see ref 23.

3d. Nuclei

A simple cushion of Optiprep™ (1000 *g* for 10 min) was used to band nuclei (repeated twice), principally to remove cytoplasmic components but the method would also remove smaller particles without the damage that may be caused by repeated pelleting [24]. Ye et al [25] also used just a single round of this cushion method. Alternatively the crude nuclear fraction may be suspended in 25% (w/v) (OptiPrep™ diluted as usual, with 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-Cl, pH 7.8) and the nuclei pelleted by centrifugation at 10,000 × *g* for 10 min. After removal of the supernatant the process was repeated [26].

4. Short review of other recent publications

Endoplasmic reticulum. Iodixanol gradients were able to monitor a banding density shift of Rab7 +ve vesicles during photoreceptor cell degeneration [27]. The method described in ref 9 was used by Sekine et al [28] to establish the ER location of the nucleotide sugar transporter Meigo. Kruppa et al [29] used a discontinuous gradient of 0-30% (v/v) OptiPrep™ underlayered by a 3000 *g* supernatant (adjusted to 35% v/v OptiPrep™) in studies on the β-amyloid peptide. A microsomal fraction from *Drosophila* brain tissue, loaded on to a discontinuous gradient of 40%, 35%, 30%, 25%, 20%, 10%, 5% and 2.5 % (w/v) iodixanol, centrifuged 340,000 g for 3 hr showed considerable functional diversity: in particular HSC3 distribution in $HTorA^{\Delta E}$-expressing brains was different from $HTorA^{WT}$-expressing brains [30].

Endoplasmic reticulum and Golgi. Wan et al [31] used a median loaded discontinuous gradient in which the microsomal sample was adjusted to 20% (w/v) iodixanol and sandwiched between 20% and 15% (w/v) iodixanol solutions. After centrifugation for 3h at 150,000 *g,* Golgi and ER were separated across the original sample layer. This is an ideal way of separating the two membranes,

Endosomes and endoplasmic reticulum were well separated on a 5-20% (w/v) iodixanol gradient centrifuged at 90,000 *g* for 18 h [32] in a study of miRNA, in particular the association of a particular type of miRNAinduced silencing complex with these membranes.

Mitochondria were isolated in a discontinuous iodixanol gradient covering a similar density range to that used for mammalian cells [33]

Exovesicles banded in a 10%, 25%, 35%, 45% (w/v) iodixanol gradient centrifuged for 16–18 h at 120,000 *g*, were shown to contain Hedgehog proteins; these membranes banded around 35% (w/v) iodixanol [34].

5. References

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