Isolation of plasma membrane from cardiac muscle

- 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.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 5)

1. Background

Using iodixanol gradients, isolation of a plasma membrane (PM) fraction is often achieved simultaneously with the purification of other subcellular membranes. Most of these methods are suitable for work with cultured cells and soft tissues such as liver but tissues such as muscle often require a customized method. Nevertheless some of the other iodixanol gradients may be adaptable to muscle tissue; for other plasma membrane methods return to the Index.

Research into cardiac muscle function often involves the complex intracellular processes by which the GLUT4 glucose transport protein is translocated to and from the plasma membrane (sarcolemma). Thus in fractionations of cardiac muscle, a prime requirement is to be able resolve the plasma membrane from GLUT-4 transport vesicles and other membrane compartments. The overall strategy of this protocol is thus similar to that using a cultured cell line or other tissue but the details of the technique are rather different.

The PM is usually the least dense of all the subcellular membranes and this Application Sheet highlights a novel exploitation of this property [1-3]. In this protocol, a crude plasma membrane suspension is adjusted to a density (1.049 g/ml, equivalent to 5% w/v iodixanol) just greater than that of the plasma membrane of cardiac muscle, thus during the centrifugation the plasma membrane will float to the top the liquid. By layering the sample upon a small cushion of 15% (w/v) iodixanol (1.103 g/ml) and centrifuging for 16 h, a shallow gradient will form due to diffusion of iodixanol and also to some sedimentation of iodixanol molecules close to the bottom of the tube. This will therefore allow not only isolation of the plasma membrane, but also partial fractionation of some of the slightly denser membrane compartments.

The particular format described in this protocol also allows the sample to be contained within a relatively large volume; this also minimizes interactions between particles in the gradient.

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Homogenization medium (HM): 0.1 M sucrose, 10 mM EDTA, 46 mM KCl, 5 mM NaN_3, 100 mM Tris-HCl, pH 7.4

C. OptiPrep™ Diluent: 0.25 M sucrose, 6 mM EDTA, 60 mM Tris-HCl, 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

E. WS diluent: 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4

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 KCl: 7.45 g per 100 ml water
100 mM NaN_3: 0.65 g per 100 ml water

Solution B: Dissolve 6.84 g sucrose in 100 ml water; add 20 ml, 20 ml, 9.2 ml and 10 ml respectively of Tris, EDTA, KCl and azide stock solutions; adjust to pH 7.4 with 1 M HCl and make up to 200 ml.

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

Solution E: as Solution C but use 1 ml of the stocks

3. Ultracentrifuge rotor requirements (**see Section 5.2**)

Any swinging-bucket rotor capable of 100,000-200,000g with tube volumes of approx. 14 ml (e.g. Beckman SW 41 or Sorvall TH641)

4. Protocol (adapted from refs 1 and 2]

Carry out all operations at 0-4°C.

1. Place 5 g of frozen tissue in saline and strip off epicardium and endocardium.

2. Place the tissue in Solution B (0.2 g/ml) and mince finely to produce 2 mm^3 fragments.

3. Homogenize the tissue using 30 strokes of the pestle of a loose-fitting Dounce homogenizer (Wheaton Type B) followed by the same number of strokes in a tight-fitting Dounce homogenizer (Wheaton Type A).

4. Centrifuge the homogenate at 4000 *g* for 10 min and harvest the supernatant (**see Section 5.3**).

5. Adjust the 4000 *g* supernatant to 5% (w/v) iodixanol by mixing 9 vol. with 1 vol. of Solution D (**see Section 5.1**).

6. Prepare a solution 15% (w/v) iodixanol solution by mixing 1.5 vol. and 3.5 vol., respectively, of Solutions D and E (**see Section 5.1**).

7. To tubes for the swinging-bucket rotor transfer 10 ml of 4000 *g* supernatant in 5% iodixanol; using a syringe and metal cannula underlay with 1.5 ml of the 15% iodixanol and then overlay with approx 1 ml of Solution E (**see Section 5.4**).

8. Centrifuge at 80,000 *g* for 16 h (**see Section 5.5**).

9. Collect the plasma membrane fraction from the top of the gradient or harvest the gradient in 0.5 ml fractions either by tube puncture, aspiration from the meniscus or upward displacement. For more information on harvesting gradients **see Section 5.6 and Application Sheet S08**.

♦ **See Section 5.7 for other method strategies**

5. Technical Notes and Review

5.1. Homogenization media and gradient solutions

The homogenization medium has been tailored to the tissue type but whether other media can be been used without any effect on the fractionation is not known. An alternative medium containing 0.1 M sucrose, 5 mM $MgCl_2$, 100 mM KCl, 10 mM EDTA, 10 mM azide and 50 mM Tris-HCl, pH 7.4 had no discernible influence on the subsequent behaviour of the plasma membrane [4].

The inclusion of divalent cations can guard against nuclear breakage and stabilize membranes generally, but may lead to aggregation. Azide was added as a protease inhibitor [1] but alternative or additional inhibitors may be included in Solution B at the operator's discretion. Protease inhibitors may also be included in Solutions C and E.

If it is considered desirable that the concentrations of EDTA and KCl in the gradient should be identical with those of the homogenization medium then Solution C should contain 0.1 M sucrose, 60 mM EDTA and 276 mM KCl and the WS diluent (Solution E) should be replaced with Solution B. Concomitantly increasing the sucrose concentration to 0.6 M however should not be adopted as this will considerably raise the osmolality. The validity of these solutions will however require testing. Strategies for preparing working solutions for mammalian tissues and cells are given in **Application Sheet S01.**

5.2. Ultracentrifuge rotors

The method was originally developed for the 4 ml tubes of a Beckman SW60 swinging-bucket rotor using 3 ml of sample. Larger volume swinging-bucket rotors may require longer centrifugation times but smaller volume rotors may need shorter times. All volumes 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. Low speed centrifugation of the homogenate

Nuclear pelleting is routinely carried out at 500-4000 *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.

5.4. Density gradients

For more information on gradient construction **see Application Sheet S03**. If necessary, adjust all volumes proportionately so that tubes (after layering Solution D) are properly filled according to the manufacturer's instructions.

5.5. Centrifugation conditions

The 80,000 *g* recommended in ref 1, has subsequently been reduced to 65,000 *g* in ref 2.

5.6. Analysis

Five visible layers were observed in the gradient after centrifugation, the plasma membrane (Na^+-K^+-ATPase) was restricted to the lowest density (Figure 1), while the ER, as determined by SERCA2 Ca^{2+} pump, banded in the two densest fractions, only one of which (the lighter one) also contained the glucose-transporter GLUT4 [1]. 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 [1], nitric oxide regulation of the myocardium in hyperhomocysteinemia [2]; nitric oxide inhibition of myocardial glucose transport [3] aquaporin expression [5] and for studying the roles for SUR subunits in KATP channel membrane targeting and regulation [6].

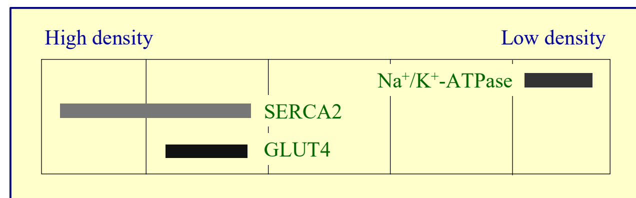

Figure 1: Fractionation of myocardial PNS on a 0%, 5%, 15% iodixanol gradient, adapted from ref 1, for more information see text

5.7 Other methodological modifications

Hong et al [7] increased the *g*-force and time centrifugation time to 200,000 *g* for 2.5 h and observed seven distinct zones of material. Subsequent modifications have included an increase in the density of the lower layer to 18% (w/v) iodixanol and a reduction in the centrifugation time to 1 h [8].

- ♦ A review of the technology for studying cardiac muscle biochemistry can be found in ref 9.

6. References

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7. Acknowledgements

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