OptiPrep™ Application Sheet S48

Analysis of membrane trafficking and intracellular signaling in self-generated gradients

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- The protocols in this Application Sheet were developed principally as a simple and reproducible means of fractionation low-density microsomes for analysis of the complex endosome-TGN transport cycle and trafficking of the GLUT4 glucose transporter
- **To access other Application Sheets** referred to in the text: return to the **2020SMemapp** file and select the appropriate S-number.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 5)

1. Background

It is often difficult to devise suitable and reliable gradients which are able to resolve the multiple smooth membrane compartments which are involved in (a) the trafficking of macromolecules between the *trans*-Golgi network (TGN), plasma membrane and parts of the endocytic system and (b) the complex patterns of intracellular signaling which control many cellular processes. Sucrose gradients have been partially successful in dissecting out these smooth membranes but their high osmolality tends to compromise the resolving power of both equilibrium density and sedimentation velocity gradients.

Self-generated isoosmotic iodixanol gradients offer a superior strategy for the study of the inter-relationships of these complex smooth membrane compartments. A major advantage of the use of these gradients is that centrifugation conditions can be chosen which produce suitably shallow gradients reproducibly. Moreover the density range of the shallowest part of the gradient can be adjusted by varying either the starting concentration of iodixanol or the centrifugation time or both. Similar pre-formed continuous (or discontinuous) gradients can be difficult to prepare. Adjusting the sample to a uniform concentration of iodixanol and centrifuging it in a vertical or near-vertical rotor is not only a simple way of achieving the right sort of gradient, the lack of any interfaces which may cause accumulation and aggregation of particles is avoided. Some of the density profiles that can be obtained in this manner are described in **Application Sheet S04**.

This protocol, developed for analyzing the translocation of GLUT4 containing vesicles between the TGN and the plasma membrane by Hashiramoto and James [1], has been taken up by a number of research groups working in similar areas [2-4] and adapted to studies of other membranes [2]. It incorporates the strategy of using a low-density microsome fraction as the gradient input, commonly used in GLUT 4 studies that may have a wider application to other investigations. This avoids the inclusion of any of the denser organelles such as lysosomes, peroxisomes and rough endoplasmic reticulum in the gradient; an advantage denied the vast preponderance of methods that use a post-nuclear supernatant.

2. Solutions required (see Section 5.1)

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 1 mM EDTA, 20 mM Hepes-NaOH, pH 7.4

C. Diluent: 0.25 M sucrose, 6 mM EDTA, 120 mM Hepes-NaOH, pH 7.4 (optional)

D. Working Solution (50%, w/v iodixanol): Mix 5 vol. of Solution A with 1 vol. of Solution C (optional)

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 ml of EDTA stock and 8 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 24 ml of Hepes stock; adjust to pH 7.4 with 1 M NaOH and make up to 100 ml.

3. Ultracentrifuge rotor requirements

Fixed angle rotors (e.g. Beckman 80Ti or 70.1Ti or Sorvall T865.1 or T-1270) for sedimentation of LDM fraction

Near vertical rotor with a tube capacity 4-5 ml (Beckman NVT65.2) or a vertical rotor (e.g. Beckman VTi65.2 or VTi90) with Optiseal™ tubes for the self-generated gradient (**see Section 5.2**)

4. Protocol (adapted from refs 1 and 4)

Following any metabolic labeling steps performed at 37°C; carry out all subsequent operations at 0-4°C.

1. Wash the cells two or three times in Solution B to remove any culture medium (it is important to remove all traces of any culture/incubation medium prior to homogenization).

2. Suspend the cells in Solution B and homogenize either in a Dounce homogenizer, by repeated passages through a fine syringe needle (22-gauge) or in a "cell cracker" (**see Section 5.3**).

3. Centrifuge the homogenate at 1000 *g* for 10 min to pellet the nuclei and cell debris (**see Section 5.4**).

4. Centrifuge the 1000*g* supernatant at 27,000 g_{max} in a fixed-angle rotor for 15 min to pellet all of the major organelles and most of the high-density microsomes (see Section 5.4).

5. Centrifuge the 27,000 *g* supernatant at 235,000 g_{max} to pellet the low-density microsomes (LDM).

6. Resuspend the LDM in Solution B and mix well with Solution D or OptiPrep™ to give the appropriate iodixanol concentration, e.g. 14% or 30 w/v (**see Sections 5.1 and 5.5**).

7. Transfer 4-5 ml to a sealed tube for a near-vertical or vertical rotor and centrifuge at 265,000 g_{av} for 4 h (see Section 5.5).

8. Allow the rotor to decelerate using a controlled deceleration program or turn off the brake at 2000 rpm.

9. Collect the gradients from Beckman Optiseal™ tubes by tube puncture, upward displacement with a dense liquid or aspiration from the meniscus (see Section 5.2). **For more information on harvesting gradients see Application Sheet S08**.

♦ A brief summary of the analytical capabilities of this gradient system is given in Section 5.6

5. Technical Notes and 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. All of the published methods using the type of protocol described in this OptiPrep™ Application Sheet have employed a 0.25 M sucrose buffered with either Tris, Hepes, Tricine or triethanolamine (at 10-20 mM concentration) and often, but not always, containing 1 mM EDTA.

Supplementation of the HM 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. **Other examples are given in Application Sheets S05 (tissues) and S06 (cells)**.

The optional production of a working solution of iodixanol containing the same concentrations of EDTA and buffer as the homogenization medium allows these concentrations to be maintained in the LDM suspension when its density is raised (see Step 6). If this is not regarded as important the density can be raised by mixing with OptiPrep™ (as used in the original method).

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**.

5.2 Ultracentrifuge rotors and tubes

A near-vertical rotor of slightly smaller tube volume such as the TLN100 (3 ml) will also be very suitable. Larger volume vertical and near-vertical rotors can also be used for self-generated gradients but the optimal centrifugation conditions of those with longer sedimentation path lengths may require investigation. 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 9); for other tubes such as heat-sealed tubes, tube puncture is the only safe and reliable option.

5.3 Homogenization

The homogenization protocol should be tailored to the cell (or tissue) type. Potter-Elevhjem or Dounce homogenization for tissues and Dounce homogenization for cells used to be the standard procedures. For cells however use of 12-20 passages through a syringe needle (the Gauge Number (G) varies from 21 to 25) sometimes preceded by Dounce homogenization, has become very common. The ball-bearing homogenizer ("cell cracker") is now widely regarded as one of the most effective and reproducible of devices.

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 tips on homogenization are given in **Application Sheets S05 (tissues) and S06 (cells).**

5.4 Preparation of the LDM fraction

The 1000*g* centrifugation may be omitted but removal of nuclei and cell debris at a low speed may enhance the recovery of smaller less dense particles later on. If the source material is a tissue (e.g. skeletal muscle) or primary cells from a tissue, the LDM is normally prepared by centrifugation of a 10,000*g* supernatant through a 0.4/1.5M sucrose gradient (see ref 1 for more details).

5.5 Self-generated gradient formation

By modulating the starting concentration of the iodixanol and the time of centrifugation it is possible to enhance the resolution of vesicles of different densities; 14% or 30% (w/v) iodixanol and 1 or 4 h are the two commonly used starting concentrations and centrifugation times respectively, but these should be modified in the light of experience. For more information on the influence of these parameters on the density profile **see Application Sheet S04**.

If a vertical rotor is used, it may be necessary to include a small cushion of 20% iodixanol to prevent any dense contaminants from reaching the wall of the tube. In a near-vertical rotor this poses no problem.

5.6 Analysis

Immunoblotting of the gradient fractions with antibodies to GLUT4, TfR, sortilin, VAMP2 and Rab4 showed that the gradient formed from 14% iodixanol was capable of fine discrimination of the complex endosomal-TGN system [1]. In 4 h gradients it was possible to detect at least two populations of GLUT4 containing vesicles, coincident with TGN or endosomal markers.

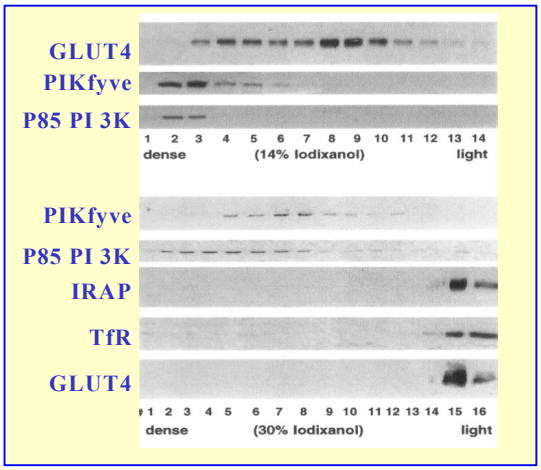

Figure 1 Distribution of two phosphoinositide kinases and markers for recycling endosomes, from 3T3-L1 adipocyte LDM, in self-generated gradients formed from 14% (top panels) and 30% (bottom panels) iodixanol at 265,000g for 4 h. Reproduced from ref 2 with kind permission of the authors and the American Society of Biochemistry and Molecular Biology.

The steep nature of the denser regions of this gradient however does not allow simultaneous resolution of denser vesicles. From this 14% iodixanol gradient, for example (top 3 panels of Figure 1), two populations of GLUT4 containing vesicles are evident but it is not entirely clear if the phosphoinositide kinases PIKfyve and P85 PI3K are entirely confined to a denser membrane compartment [2]. The gradient formed from 30% iodixanol on the other hand not only completely resolves the kinases from endosome-TGN markers (bottom five panels of Figure 1), it is also able to partially resolve vesicles containing the two types of kinase [2]. PhosphoSer-p40 was found to co-fractionate with PIKfyve, while the recycling protein IRAP was detected principally in fractions of lower density [6].

Using the 14% iodixanol starting concentration, Maier and Gould [3] reduced the centrifugation time to 1 h in the Beckman TLN100 rotor and were able to identify the denser fraction as the GLUT4 storage vesicle (GSV), whose GLUT4 responded hugely to short term insulin stimulation. The lower density fraction was coincident with TGN and endosome markers, which was also rich in the cysteine-string protein Csp-1 [4]. Moreover Syntaxin STX16 was targeted to the GSV, while STX6 targeted the TNG/endosomes [5]. Yeh et al [7] and Liu et al [8] also used the standard 14% iodixanol starting concentration and centrifugation times of 4 h and 2.5 h respectively in GLUT4 transport studies. Ma et al [9], used a broadly similar methodology to that of Liu et al [8], to demonstrate that insulin-generated oxidative stress directs GLUT4 to lysosomes.

Ikonomov al [10] employed the same self-generated gradient system [2] to show that the kinesin adapter, JLP, interacts with PIKfyve and that both proteins and their association are required in microtubule-based, but not in microtubule independent, endosome-to-TGN cargo transport. A self-generated gradient system used by Chen et al [11] to study exocyst regulation of vesicle delivery to the centrosome prior to cytokinesis in COS cells, in which they observed a co-banding of RalA with TfR and Rab11 but not with early endosomes, Golgi or cytosol markers has been adapted by Landry et al [12] to 293T cells to identify TGN, *cis*-Golgi and recycling endosomes (RE) in the gradient and to study RE trafficking to the Golgi. Xie et al [13] also used a self-generated gradient for investigating the insertion of GLUT4 into the plasma membrane.

Karunanithi et al [14] used the established 14% (w/v) iodixanol starting concentration to discover a G protein cascade that regulated GLUT4 trafficking. The same gradient formula also demonstrated that VAMP2 also has a major influence on GLUT4 trafficking [15].

♦ Sadler et al [16] have recently produced a detailed methodology for the use of iodixanol gradients in studies on GLUT4.

6. References

1. Hashiramoto, M. and James, D. E. (2000) *Characterization of insulin-responsive GLUT4 storage vesicles isolated from 3T3-L1 adipocytes* Mol. Cell. Biol., **20**, 416-427.
2. Shisheva, A., Rusin, B., Ikonomov, O. C., DeMarco, C. and Sbrissa, D. (2001) *Localization and insulin-regulated relocation of phosphoinositide 5-kinase PIKfyve in 3T3-L1 adipocytes* J. Biol. Chem., **276**, 11859-11869.
3. Maier, V. H. and Gould, G.W. (2000) *Long-term insulin treatment of 3T3-L1 adipocytes results in mis-targeting of GLUT4: implications for insulin-stimulated glucose transport* Diabetologia, **43**, 1273-1281.
4. Chamberlain, L. H., Graham, M. E., Kane, S., Jackson, J. L., Maier, V. H., Burgoyne, R. D. and Gould, G. W. (2001) *The synaptic vesicle protein, cysteine-string, is associated with the plasma membrane in 3T3-L1 adipocytes and interacts with syntaxin 4* J. Cell Sci., **114**, 445-455.
5. Perera, H. K. I., Clarke, M., Morris, N. J., Hong, W., Chamberlain, L. H. and Gould, G. W. (2003) *Syntaxin 6 regulates Glut4 trafficking in 3T3-L1 adipocytes* Mol. Biol. Cell, **14**, 2946-2958
6. Ikonomov, O.C., Sbrissa, D., Mlak, K., Deeb, R., Fligger, J., Soans, A., Finley, R.L. and Shisheva, A. (2003) *Active PIKfyve associates with and promotes the membrane attachment of the late endosome-to-trans-Golgi network transport factor Rab9 effector p40* J. Biol. Chem., **278**, 50863-50871
7. Yeh, T-Y.J., Sbodio, J.I., Tsun, Z-Y., Luo, B. and Chi, N-W. (2007) *Insulin-stimulated exocytosis of GLUT4 is enhanced by IRAP and its partner tankyrase* Biochem. J. **402**, 279-290
8. Liu, L-B., Omata, W., Kojima, I. and Shibata, H. (2007) *The SUMO conjugating enzyme Ubc9 is a regulator of GLUT4 turnover and targeting to the insulin-responsive storage compartment in 3T3-L1 adipocytes* Diabetes, **56**, 11977-1985
9. Ma, J., Nakagawa, Y., Kojima, I. and Shibata, H. (2014) *Prolonged insulin stimulation down-regulates GLUT4 through oxidative stress-mediated retromer inhibition by a protein kinase CK2-dependent mechanism in 3T3-L1* J. Biol. Chem., **289**, 133-142
10. Ikonomov, O.C., Fligger, J., Sbrissa, D., Dondapati, R., Mlak, K., Deeb, R. and Shisheva, A. (2009) *Kinesin adapter JLP links PIKfyve to microtubule-based endosome-to-trans-Golgi network traffic of furin* J. Biol. Chem., **284**, 3750–3761
11. Chen, X-W., Inoue, M., Hsu, S. and Saltiel, A.R. (2006) *RalA-exocyst-dependent recycling endosome trafficking is required for the completion of cytokinesis* J. Biol. Chem., **281**, 38609-38616
12. Landry, M-C., Sicotte, A., Champagne, C. and Lavoie, J.N. (2009) *Regulation of cell death by recycling endosomes and Golgi membrane dynamics via a pathway involving Src-family kinases, Cdc42 and Rab11a* Mol Biol. Cell, **20**, 4091-4106
13. Xie, X., Gong, Z., Mansuy-Aubert, V., Zhou, Q.L., Tatulian, S.A., Sehrt, D., Gnad, F., Brill, L.M. Motamedchaboki, K., Chen, Y., Czech, M.P., Mann, M., Krüger, M. and Jiang, Z.Y. (2011) *C2 domain-containing phosphoprotein CDP138 regulates GLUT4 insertion into the plasma membrane* Cell Metab., **14**, 378–389
14. Karunanithi, S., Xiong, T., Uhm, M., Leto, D., Sun, J., Chen, X-W., and Saltiel, A.R. (2014) *A Rab10:RalA G protein cascade regulates insulin-stimulated glucose uptake in adipocytes* Mol. Biol. Cell, **25**, 3059-3069
15. Sadler, J.B.A., Bryant, N.J. and Gould, G.W. (2015) *Characterization of VAMP isoforms in 3T3-L1 adipocytes: implications for GLUT4 trafficking* Mol. Biol. Cell, **26**, 530-536
16. Sadler, J.B.A., Lamb, C.A., Gould, G.W. and Bryant, N.J. (2016) *Iodixanol gradient centrifugation to separate components of the low-density membrane fraction from 3T3-L1 adipocytes* Cold Spring Harb. Protoc., doi:10.1101/pdb.prot083709

serumwerk bernburg

Serumwerk Bernburg AG • Hallesche Landstraße 105 b • 06406 Bernburg • Germany
phone: +49 3471 860 429 • diagnostic.serumwerk.com • diagnostic@serumwwerk.com • serumwerk.de