OptiPrep™ Application Sheet S56

Analysis of mammalian lysosomes (ER, endosomes and plasma membrane) in continuous gradients

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- An **OptiPrep™ Reference List (RS04) "Lysosomes – a bibliographical review"** provides a bibliography of all published papers reporting the use of OptiPrep™ for analysis of these organelles: to access return to the initial list of Folders and select "**Reference Lists**". 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 **2020SMemapp** file and select the appropriate S-number.
- See **Application Sheet S55** for information on the use of discontinuous gradients
- See **Application Sheet S55** also for cell and tissue homogenization**.**
- See **Application Sheet S16** for information on the use of self-generated gradients

1. Introduction

Continuous gradients may be prepared by allowing discontinuous gradients to diffuse; by using one of the standard two-chamber devices or a Gradient Master™. These methods are fully described in **Application Sheet S03.**

As an illustration of the effectiveness of continuous iodixanol gradients in resolving lysosomes, Figure 1 shows the separation of the major organelles from a rat liver light mitochondrial fraction (LMF). The LMF was adjusted to approx. 35% (w/v) iodixanol, layered beneath a 19-27% (w/v) iodixanol gradient and centrifuged 70,000 g_{av} for 2 h. The sharp reduction in density in the top fraction occurs because 1-2 ml of 0.25 M sucrose was layered on top of the gradient (after underlayering of the LMF) to fill the tube. The distribution of enzyme markers shows the efficacy of such gradients in resolving all of the principal organelles.

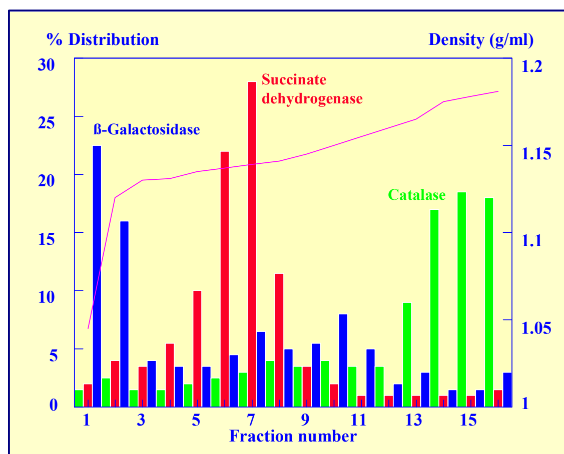

Figure 1 Fractionation of a rat liver LMF on a pre-formed bottom-loaded iodixanol gradient (19-27% w/v): enzyme distribution; mitochondria were detected using succinate dehydrogenase; lysosomes by ß-galactosidase and peroxisomes by catalase.

2. Solutions required

A. OptiPrep™
B. Homogenization medium: 0.25 M sucrose, 1 mM EDTA, 20 mM HEPES-NaOH, pH 7.4 (see Section 4)
C. OptiPrep™ diluent: 0.25 M sucrose, 2 mM EDTA, 40 mM HEPES-NaOH, pH 7.4
D. 30% (w/v) Iodixanol working solution: Mix equal volumes of Solutions A and C

Add protease inhibitors as required.

Keep the following stock solutions at 4°C:

500 mM HEPES (free acid): 11.9 g per 100 ml water

100 mM EDTA ($Na_2 \bullet 2H_2O$): 3.72 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 2.0 ml and 8.0 ml respectively of EDTA and HEPES stock solutions; adjust to pH 7.4 with 1 M NaOH and make up to 200 ml

Solution C: Dissolve 17 g sucrose in 100 ml water; add 4.0 ml and 16.0 ml respectively of EDTA and HEPES stock solutions; adjust to pH 7.4 with 1 M NaOH and make up to 200 ml

The solutions described here may be unsuitable to the cell or tissue type under study; they are what may be regarded as the "traditional ones" devised originally for rat liver but may be unsuitable for a

particular cell type or study. For brief summaries of some of the other solutions used in lysosome studies **see Application Sheets S55, S05 and S06; see also the publications described in Section 3.**

3. A review of the published methodology

Other tissues or cultured cells not listed below may require modification of the density gradient configuration.

3a. HeLa cells. An LMF was loaded on top a continuous gradient formed from layers of 4, 10, 16 and 24% (w/v) iodixanol and centrifuged at 20,000 *g* for 17 h [1]. There was clear distinction in the distribution of mitochondrial and lysosomal markers, although the separation of these two organelles might have been enhanced in a more shallow gradient spanning a higher density range. The optimal density range may also be cell or tissue specific. A 15-30% (w/v) iodixanol has also been used for HeLa cells [2]. See also Section 3c.

3b. Osteoclasts. The chosen density range may also be a function of the aim of the study and the type of cell. A post-nuclear supernatant (PNS) from osteoblast cells was loaded on to a 0-17% (w/v) iodixanol gradient and centrifuged at approx. 150,000 g_{av} for 100 min [3]. More unusually, this was carried out in a fixed-angle rotor, but so long as the acceleration to and from 2000 rpm is carried slowly and smoothly during the reorientation of the gradient in the centrifuge tube, there is no obvious reason why such a rotor cannot be used. The gradient completely separated the lighter plasma membrane from the denser lysosomes.

3c. Human skin fibroblsts. A 5-20% (w/v) iodixanol gradient, centrifuged for 20 h at 90,000 *g*, which was used for separation of ER and lysosomes from human skin fibroblasts [4]. These gradient and centrifugation conditions were originally described by Sugii et al [5] for studying endocytosis in CHO cell lines. Sugii et al [4] commented that Percoll gradients were unable to resolve plasma membrane from endosomal compartments, while in the iodixanol gradient the Na^+/K^+-ATPase was completely separated from EEA1, Rab9 and syntaxin 6 regions, each of which showed distinctive gradient banding patterns. This gradient has also been used for HeLa cells [6]

3d. Mouse brain. The homogenization medium contained 150 mM NaCl as the main osmotic balancer, in 1mm EDTA, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4, containing 1% Triton X-100. Inclusion of detergent in the medium is a brain-specific strategy to overcome the myelin problem. The same gradient and centrifugation conditions as in Section 3c. The gradient completely resolves the lysosomes+late endosomes from the plasma membrane [7].

3e. HEK cells. This informative paper displayed how important it is to adapt methods to a particular cell or analytical requirement. An original 7.5-25% (w/v) iodixanol gradient was modified to an 11-22% gradient to maximize the separation of lysosomes and ER for demonstrating the localization of mature and immature forms of α-N-acetylgalactosaminidase to lysosomes and ER respectively [8].

3f. Primary human fibroblasts. A 10-30% (w/v) iodixanol gradient with a 40% iodixanol cushion, top-loaded with an LMF and centrifuged in a vertical rotor at 55,000 g_{av} for 90 min completely separated the lysosomes, mitochondria and peroxisomes but the lighter ER overlapped the lysosomes [9]. It is difficult to assess whether a longer centrifugation time may have resolved the ER and mitochondria more effectively.

♦ Lysosomes from other cells that have isolated using similar continuous iodixanol gradients are MDCK cells [10], HepG2 cells [11] and also from he *Bombyx mori* silk gland [12].

4. References

1. Cardoso, C.M.P., Groth-Pedersen, L., Høyer-Hansen, M., Kirkegaard, T., Corcelle, E., Andersen, J.S., Jäättelä, M. and Nylandsted, J. (2009) *Depletion of kinesin 5B affects lysosomal distribution and stability and induces peri-nuclear accumulation of autophagosomes in cancer cells* PLoS One, **4**:e4424

2. Matsuda, S., Okada, N., Kodama, T., Honda, T. and Iida, T. (2012) A Cytotoxic Type III Secretion *Effector of Vibrio parahaemolyticus targets vacuolar* H^+*-ATPase subunit c and ruptures host cell lysosomes* PLoS Pathog., **8**: e1002803
3. Kariya, Y., Homma, M., Aoki, S., Chiba, A. and Suzuki, H. (2009) *Vps33a mediates RANKL storage in secretory lysosomes in osteoblastic cells* J. Bone Mineral Res., **24**, 1741-1752
4. Higaki, K., Li, L., Bahrudin, U., Okuzawa, S., Takamuram, A., Yamamoto, K., Adachi, K., Paraguison, R.C., Takai, T., Ikehata, H., Tominaga, L., Hisatome, I., Iida, M., Ogawa, S., Matsuda, J., Ninomiya, H., Sakakibara, Y., Ohno, K., Suzuki, Y. and Nanba, E. (2011) *Chemical chaperone therapy: chaperone effect on mutant enzyme and cellular pathophysiology in β-galactosidase deficiency* Hum. Mutat., **32**, 843–852
5. Sugii, S., Reid, P.C., Ohgami, N., Du, H. and Chang, T-Y. (2003) *Distinct endosomal compartments in early trafficking of low density lipoprotein-derived cholesterol* J. Biol. Chem., **278**, 27180-27189
6. Takamura, A., Higaki, K., Ninomiya, H., Takai, T., Matsuda, J., Iida, M., Ohno, K., Suzuki, Y. and Nanba, E. (2011) *Lysosomal accumulation of Trk protein in brain of* G_{M1}*-gangliosidosis mouse and its restoration by chemical chaperone* J. Neurochem., **118**, 399–406
7. Shi, J., Chou, B., Choi, J.L., Ta, A.L. and Pun, S.H. (2013) *Investigation of polyethylenimine/DNA polyplex transfection to cultured cells using radiolabeling and subcellular fractionation methods* Mol. Pharm., **10**, 2145-2156
8. Clark, N.E., Metcalf, M.C., Best, D., Fleet, G.W.J. and Garman, S.C. (2012) *Pharmacological chaperones for human α-N-acetylgalactosaminidase* Proc. Natl. Acad. Sci. USA, **109**, 17400-17405
9. Wiesinger, C., Kunze, M., Regelsberger, G., Forss-Petter, S. and Berger, J. (2013) *Impaired very long-chain Acyl-CoA β-oxidation in human X-linked adrenoleukodystrophy fibroblasts is a direct consequence of ABCD1 transporter dyfunction* J. Biol. Chem., **288**, 19269-19279
10. Van Itallie, C.M., Tietgens, A.J., LoGrande, K., Aponte, A., Gucek, M. and Anderson, J.M. (2012) *Phosphorylation of claudin-2 on serine 208 promotes membrane retention and reduces trafficking to lysosomes* Journal of Cell Science **125**, 4902–4912
11. Seggewiß, N., Paulmann, D. and Dotzauer, A. (2016) *Lysosomes serve as a platform for hepatitis A virus particle maturation and nonlytic release* Arch. Virol., **161**, 43–52
12. Shiba, H., Yabu, T., Sudayama, M., Mano, N., Arai, N., Nakanishi, T. and Hosono, K. (2016) *Sequential steps of macroautophagy and chaperone-mediated autophagy are involved in the irreversible process of posterior silk gland histolysis during metamorphosis of Bombyx mori* J. Exp. Biol., **219**, 1146-1151

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