OptiPrep™ Application Sheet S53

Isolation of mammalian lysosomes in discontinuous gradients

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- An **OptiPrep™ Mini-Review (MS06) "Lysosomes – a methodological and 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 "**Mini-Reviews**". 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 Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- See **Application Sheet S54** for information on the use of pre-formed continuous gradients
- See **Application Sheet S16 "Light mitochondrial fraction analysis"** for information on the use of self-generated gradients

1. Background

Wattiaux et al [1] were the first to describe the use of metrizamide for the purification of mammalian liver lysosomes. They were also the first to point out the huge advantage of using a gradient of much lower osmolality than the traditional sucrose gradient, which can only provide a reasonable separation of lysosomes and mitochondria if the density of the former is artificially reduced by Triton WR1339 [2]. It was also established that, by bottom loading of the density gradient, advantage could be taken of the greater sensitivity of mitochondria to hydrostatic pressure, thereby causing an increase in their density relative to that of the lysosomes [3].

In a discontinuous gradient of 30%, 26%, 24% and 19% (w/v) metrizamide (covering the density range of 1.16-1.105 g/ml), rat liver lysosomes float up from a light mitochondrial fraction, loaded in approx 35% metrizamide, to band at the 24%/19% interface after centrifugation at 95,000 *g* for 2 h [1,3]. The relative specific activity of β-galactosidase (over the homogenate) was reported as 80, while for a mitochondrial marker (cytochrome oxidase) the value was only 0.17 [3]. Metrizamide is no longer commercially available and Olsson et al [4] translated the flotation method to Nycodenz® and reported an even higher relative specific activity for *N*-acetyl-β-glucosaminidase of 108. The method has also been extended to HepG2 cells [5].

The procedure has now been adapted to the use of OptiPrep™ and a large number of published papers have predominantly reported the use of a discontinuous iodixanol gradient. In some cases the centrifugation is carried out for approx. 5 h, in others for 20 h and under these conditions the continuous gradient will become continuous, but not necessarily linear. Note that the density of lysosomes in iodixanol gradients is lower than that in either metrizamide or Nycodenz® gradients because of the reduced osmolality of iodixanol gradients.

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

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

Add protease inhibitors as required to Solutions B and D.

3. Ultracentrifuge rotor requirements

In this protocol, a swinging-bucket rotor with a tube capacity of approx 13 ml (e.g. Beckman SW 41Ti, Sorvall TH641 or equivalent) or 4-5 ml (e.g. Beckman SW60Ti, TH-660 or equivalent) is suitable. The use of a vertical rotor is a valid alternative; the short sedimentation path length not only reduces the centrifugation time, the reduced hydrostatic pressure also favours retention of organelle integrity. This alternative has not been used with iodixanol gradients as far as is known, but it is quite a common practice with Nycodenz® gradient purification of mitochondria.

4. Homogenization

For tissues: Mince the tissue very finely with scissors (or with a tissue chopper) and transfer to a Potter-Elvehjem (Teflon and glass) homogenizer with solution A (use 10 ml medium for every 2.5 g tissue). Homogenize using approx 6 strokes of the pestle (500-700 rpm).

For cells: Wash 1-3x10^8 cells in 5 ml of phosphate buffered saline and again with 5 ml of Solution A. Suspend the cells in 3 ml of Solution B and homogenize in a ball-bearing homogenizer using five passes.

Any suitable buffered isoosmotic solution may be used. The recommended version is a common one (see e.g. refs 6 and 7). But there are some significant variations (see Table 1). For more information about homogenization media for tissues and cells **see Application Sheets S05 and S06 respectively**.

Table 1: Homogenization media

Tissue/cell	Homogenization media	Ref. #
Mouse brain	0.32 M sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1mM $NaHCO_3$, 5 mM Tris, pH 7.5	8
	50 mM NaCl, 50 mM Tris-HCl, pH 8.0 1% Nonidet P40, 0.05% deoxycholate, 0.1% SDS	9
	130 mM KCl, 25 mM Tris-HCl, pH 7.4, 1 mM EGTA	10
Myeloid cells	210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 20 mM HEPES, pH 7.5	11
HeLa cells	0.25 M sucrose, 10 mM triethanolamine-10 mM acetic acid, pH 7.8	12

For the isolation of lysosomes from cultured cells, the ball bearing homogenizer is considered to offer the gentlest means of disruption. Most soft tissues can be homogenized in a Potter-Elvehjem homogenizer, but alternatives such as the Polytron homogenizer have been used. Some procedures for tissues and cells are described in **Application Sheets S05 and S06 respectively**. Always monitor the efficacy of the homogenization by phase contrast microscopy.

5. Preparation of the light mitochondrial fraction

1. Pellet the nuclei, cell debris and any unbroken cells by centrifuging at 800-1000 *g* for 10 min.

2. Decant or aspirate the supernatant and retain on ice.

3. Resuspend the pellet in Solution B using 2-3 gentle strokes of the pestle of a loose-fitting Dounce homogenizer. Resuspension of the pellet must be carried out under the mildest of conditions, to avoid damage to the delicate organelles.

4. Repeat the centrifugation and combine the supernatants.

5. Centrifuge the combined supernatants at 3,000 *g* for 10 min to pellet the heavy mitochondria. Aspirate the supernatant and keep on ice.

6. Resuspend the pellet in Solution B (see Step 3) and repeat the 3000 *g* centrifugation

7. Combine the two 3000 *g* supernatants and centrifuge at 17-20,000 *g* for 10 min to produce a light mitochondrial pellet.

8. Resuspend the light mitochondrial pellet in Solution B and repeat the 17-20,000 *g* centrifugation. This pellet is used for the gradient input.

A variety of centrifugation conditions have been used for this part of the procedure. In some cases a light mitochondrial fraction is used for the gradient input, which may be prepared in the mode described. There are wide variations in the protocol used to produce the gradient input. The first 800 *g* step [6] or the 3000 *g* step [8,9] may be omitted. Sometimes the entire 3000 *g* supernatant is applied to the gradient [7] or the entire 800-1000 *g* (post-nuclear) supernatant (PNS) is used [12-14] and in more rare examples the whole homogenate is used [15].

The advantage of using a PNS is that the pre-gradient procedure is accelerated and organelles are less likely to be lost due to the repeated centrifugation and re-suspension steps, on the other hand the presence of all the membrane-bound organelles and vesicles (except the nuclei) will severely test the resolving power of the gradient.

♦ **See Application Sheet S07 for more information on differential centrifugation**

6. Discontinuous gradient centrifugation

♦ Note that in some published methods the gradient is described in terms of % (w/v) iodixanol and sometimes as % (v/v) OptiPrep™; the two conventions give quite different densities: 20% (w/v) iodixanol is 1.127 g/ml (using a 0.25 M sucrose diluent), 20% (v/v) OptiPrep™ is equivalent to 12% (w/v) iodixanol and a density of 1.088 g/ml. For clarity all of the gradients are expressed as % (w/v) iodixanol in this Application Sheet.

The most commonly used discontinuous gradient format is 10%, 12%, 14%, 16%, 18% (w/v) iodixanol (equal volumes of each) prepared by diluting Solution D with Solution B. The crude lysosomal fraction is adjusted to 9% (w/v) and layered on top of the gradient. Tubes for the SW41 can accommodate 2 ml of each of the density gradient solutions and sample. For smaller volume rotors, all volumes should be scaled down proportionately. Gradients are normally centrifuged at 145,000 *g* for 2 h. Lysosomes band close to the top of the gradient. The method has been used for the following cell types: myeloid cells [11] neuroblastoma [12,13], renal cortex cells [14], pancreatic cancer cells [16], human lung carcinoma cells [17].

Other discontinuous gradients have been used to fractionate a tissue or cell PNS.

1. A gradient of 4,10,16 and 24% (w/v) iodixanol, with the sample loaded on top in the homogenization medium was used for HeLa cells [6] and 3T3 fibroblasts [7]. In this example, the gradients were centrifuged for 17 h at 20,000 *g*, during which time they will certainly become continuous.
2. A mouse blastocyst homogenate was fractionated on a 6, 9, 12,15, 18% (w/v) iodixanol gradient, centrifuged at 100,000 *g* for 16 h [15].
3. HeLa cell lysosomes were isolated on a 12, 14, 16, 18, 20% (w/v) iodixanol gradient, centrifuged at 150,000 *g* for 5 h [18]
4. In a 12.8, 16, 19, 22.5, 27% (w/v) iodixanol gradient, a lymphocyte light mitochondrial fraction was median loaded in the 19% iodixanol layer with centrifugation was at 150,000 *g* for 5 h [19,20]. Median loading has the advantage that the lysosomes will float to their banding density while most of the denser organelles will sediment. Very good resolution of lysosomes, mitochondria and dense membrane-bound granules was observed.
5. Renal cortex lysosomes have been purified from a PNS adjusted to 15% (w/v) iodixanol, loaded on to a 17, 20, 23, 27 and 30% (w/v) iodixanol (145,000 *g* for 2 h); the organelles banded at the 15-17% iodixanol interface [21]. The same gradient and centrifugation conditions have been used for HeLa cells [22]. Purification of lymphocyte lysosomes [23] used a slightly modified gradient of 17, 23, 25, 27, 29 and 30% (w/v) iodixanol. Broadly similar discontinuous gradients were used for a mammary gland PNS [24], microvascular endothelial cells [25], Caco-2 cells [25] and brain [9]. Note that several commercial companies including Sigma-Aldrich, Pierce Biotechnology and Thermo-Scientific produce kits for the purification of lysosomes using OptiPrep™, which recommend iodixanol gradients of the type described in this section. In all these gradients the lysosomes will band towards the top of the gradient.
6. A rather more elaborate system was devised by Khundadze et al [26] for brain material. Essentially a heavy mitochondrial supernatant was first fractionated into a heavy and light membrane fraction

on a discontinuous sucrose gradient; lysosomes were then enriched from the heavy fraction by flotation through a 25, 23, 21 and 19% (w/v) iodixanol gradient (centrifuged at 110,000 *g* for 2 h).

- For more information on the preparation of discontinuous gradients see **Application sheet S03**.
- Lysosomes from approximately 30 different types of cell or tissue have been purified in discontinuous iodixanol gradients; for further information see **OptiPrep™ Mini-Review MS06**.

7. References

1. Wattiaux, R., Wattiaux-De Coninck, S., Ronveaux-Dupal, M. P. and Dubois, F. (1978) *Isolation of rat liver lysosomes by isopycnic centrifugation in a metrizamide gradient* J. Cell Biol., **78**, 349-368
2. Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. de Duve, C. (1968) *The large scale separation of peroxisomes, mitochondria and lysosomes from livers of rats injected with Triton WR-1339* J. Cell Biol., **37**, 482-516.
3. Wattiaux, R. and Wattiaux-De Coninck, S. (1983) *Separation of cell organelles* In: Iodinated Density Gradient Media – a practical approach (ed. Rickwood, D.) IRL Press at Oxford University Press, Oxford, UK, pp 119-137
4. Olsson, G. M., Svensson, I., Zdoisek, J. M. and Brunk, U. T. (1989) *Lysosomal enzyme leakage during the hypoxanthine/xanthine oxidase reaction* Virchows Arch. B Cell Pathol., **56**, 385-391
5. Jadot, M., Andrianaivo. F., Dubois, F. and Wattiaux, R. (2001) *Effects of methylcyclodextrin on lysosomes* Eur. J. Biochem., **268**, 1392-1399
6. 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
7. Fehrenbacher, N., Bastholm, L., Kirkegaard-Sørenson, T., Rafn, B., Bøttzauw, T., Nielsen, C., Weber, E., Shirasawa, S., Kallunki, T. and Jäättelä (2008) *Sensitization to the lysosomal cell death pathway by oncogene-induced down-regulation of lysosome-Associated membrane proteins 1 and 2* Cancer Res., **68**, 6623-6633
8. Xiao, M-F., Xu, J-C., Tereshchenko, Y., Novak, D., Schachner, M. Kleene, R. (2009) *Neural cell adhesion molecule modulates dopaminergic signaling and behavior by regulating dopamine D2 receptor internalization* J. Neurosci., **29**, 14752-14763
9. Annunziata, I., Patterson, A., Helton, D., Hu, H., Moshiach, S., Gomero, E., Nixon, R. and d'Azzo, A. (2013) *Lysosomal NEU1 deficiency affects amyloid precursor protein levels and amyloid-β secretion via deregulated lysosomal exocytosis* Nat. Comm 4: 2734
10. Khundadze, M., Kollmann, K., Koch, N., Biskup, C., Nietzsche, S., Zimmer, G., Hennings, J.C., Huebner, A.K., Symmank, J., Jahic, A., Illina, E.I., Karle, K., Schöls, L., Kessels, M.., Braulke, T., Qualmann, B., Kurth, I., Beetz, C. and Hübner, C.A. (2013) *A hereditary spastic paraplegia mouse model supports a role of ZFYVE26/SPASTIZIN for the endolysosomal system* PloS Genet., **9**: e1003988
11. Oberle, C., Huai, J., Reinheckel, T., Tacke, M., Rassner, M., Ekert, P.G., Buellesbach, J. and Borner, C. (2010) *Lysosomal membrane permeabilization and Cathepsin release is a Bax/Bak-dependent, amplifying event of apoptosis in fibroblasts and monocytes* Cell Death Differ., **17**, 1167–1178
12. Sevlever, D., Jiang, P. and Yen, S-H.C. (2008) *Cathepsin D is the main lysosomal enzyme involved in the degradation of α-synuclein and generation of its carboxy-terminally truncated species* Biochemistry, **47**, 9678-9687
13. Wei, J., Fujita, M., Nakai, M., Waragai, M., Sekigawa, A., Sugama, S., Takenouchi, T., Masliah, E. and Hashimoto, M. (2009) *Protective role of endogenous gangliosides for lysosomal pathology in a cellular model of synucleinopathies* Am. J. Pathol., **174**, 1891–1909
14. 1556
15. Lee, J-H., Yu, W.H., Kumar, A., Lee, S., Mohan, P.S., Peterhoff, C.M., Wolfe, D.M., Martinez-Vicente, M., Massey, A.C., Sovak, G., Uchiyama, Y., Westaway, D., Cuervo, A.M. and Nixon, R.A. (2010) *Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations* Cell, **141**, 1146–1158
16. Udelnow, A., Kreyes, A., Ellinger, S., Landfester, K., Walther, P., Klapperstueck, T., Wohlrab, J., Henne-Bruns, D., Knippschild, U. and Würl, P. (2011) *Omeprazole inhibits proliferation and modulates autophagy in pancreatic cancer cells* PLoS One, **6**: e20143
17. Liu, L., Zhang, Z. and Xing, D. (2011) *Cell death via mitochondrial apoptotic pathway due to activation of Bax by lysosomal photodamage* Free Radic., Biol. Med., **51**, 53–68
18. Edelmann, B., Bertsch, U., Tchikov, V., Winoto-Morbach, S., Perrotta, C., Jakob, M., Adam-Klages, S., Kabelitz, D. and Schütze, S. (2011) *Caspase-8 and caspase-7 sequentially mediate roteolytic activation of acid sphingomyelinase in TNF-R1 receptosomes* EMBO J., **30**, 379–394
19. Schmidt, H., Gelhaus, C., Lucius, R., Nebendahl, M. Leippe, M. and Janssen, O. (2009) *Enrichment and analysis of secretory lysosomes from lymphocyte populations* BMC Immunol., **10**:41

20. Schmidt, H., Gelhaus, C., Nebendahl, M., Lettau, M., Lucius, R., Leippe, M., Kabelitz, D. and Janssen, O. (2011) *Effector granules in human T lymphocytes: proteomic evidence for two distinct species of cytotoxic effector vesicles* J. Proteome Res., **10**, 1603–1620
21. Dobrinskikh, E., Giral, H., Caldas, Y.A., Levi, M. and Doctor, R.B. (2010) *Shank2 redistributes with NaPilla during regulated endocytosis* Am. J. Physiol. Cell Physiol., **299**, C1324–C1334
22. Meerovich, I., Koshkaryev, A., Thekkedath, R. and Torchilin, V.P. (2011) *Screening and optimization of ligand conjugates for lysosomal targeting* Bioconjugate Chem., **22**, 2271–2282
23. Ruppert, S.M., Li, W., Zhang, G., Carlson, A.L., Limaye, A., Durum, S.K. and Khaled, A.R. (2012) *The major isoforms of Bim contribute to distinct biological activities that govern the processes of autophagy and apoptosis in interleukin-7 dependent lymphocytes* Biochim. Biophys. Acta, **1823**, 1877–1893
24. Arnandis, T., Ferrer-Vicens, I., García-Trevijano, E.R., Miralles, V.J., García, C., Torres, L., Viña, J.R. and Zaragozá, R. (2012) *Calpains mediate epithelial-cell death during mammary gland involution: mitochondria and lysosomal destabilization* Cell Death Differ., **19**, 1536–1548
25. Bielaszewska, M., Ruter, C., Kunsmann, L., Greune, L., Bauwens, A., Zhang, W., Kuczius, T., Kim, K.S., Mellmann, A., Schmidt, M.A. and Karch, H. (2013) *Enterohemorrhagic Escherichia coli hemolysin employs outer membrane vesicles to target mitochondria and cause endothelial and epithelial apoptosis* PloS Pathog., **9**: e1003797
26. Khundadze, M., Kollmann, K., Koch, N., Biskup, C., Nietzsche, S., Zimmer, G., Hennings, J.C., Huebner, A.K., Symmank, J., Jahic, A., Illina, E.I., Karle, K., Schöls, L., Kessels, M.., Braulke, T., Qualmann, B., Kurth, I., Beetz, C. and Hübner, C.A. (2013) *A hereditary spastic paraplegia mouse model supports a role of ZFYVE26/SPASTIZIN for the endolysosomal system* PloS Genet., **9**: e1003988

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