Excellence in Separations

OptiPrep™ Mini-Review MS06

Lysosomes – a methodological and bibliographical review

- **PART A SUMMARIZES ALL OF THE POSSIBLE METHODOLOGICAL OPTIONS FOR DIFFERENTIAL AND GRADIENT CENTRIFUGATION**
- **PART B IS A BIBLIOGRAPHICAL REVIEW LISTING ALL PUBLICATIONS REPORTING THE USE OF OPTIPREP™**

PART A: METHODOLOGICAL REVIEW

A-1. Homogenization

Tissues are generally homogenized (in a Potter-Elvehjem apparatus) in buffered 0.25 M sucrose usually, but not invariably, containing 1 mM EDTA. The buffer (Figure 1) is usually 10-20 mM Tris-HCl or Hepes-NaOH. For cultured cells the homogenization medium (HM) is rather more variable; with lymphoid cells for example the buffered sucrose medium contained 1 mM $MgCl_2$ [1] and the homogenizer may be a ball-bearing device [1], nitrogen cavitation vessel [2] or Dounce homogenizer [2]. It is generally accepted that for cultured cells the ball-bearing device provides the necessary gentle conditions suited to intact lysosome recovery.

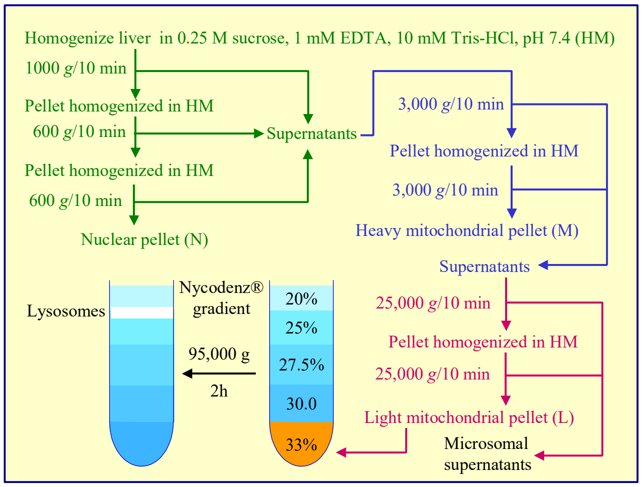

Figure 1. Flow chart describing purification of lysosomes by flotation through a discontinuous gradient of Nycodenz®: production of the nuclear pellet is in green text, the heavy mitochondrial pellet in blue text and the light mitochondrial pellet in magenta text

A-2. Differential centrifugation

The production of a light mitochondrial pellet (L) by differential centrifugation that is described in Figure 1 is based on the scheme originally published by de Duve et al [3], but there are many variations on this theme. The first phase (green in Figure 1) or second phase (blue in Figure 1) is sometimes omitted; sometimes both blue and magenta phases are omitted and the gradient is thus loaded with a post-nuclear supernatant. Additionally the *g*-forces and/or times for one or more of the phases may vary from those given in Figure 1. For example the L pellet from lymphoid cells has been recovered at 22,000 *g* for 60 min [1] or 17,000 *g* for 20 min [4]. Occasionally the differential centrifugation follows a rather different format: for example Klein et al [5-7] first centrifuged a liver homogenate at 10,700 *g* for 20 min; the pellet was resuspended in HM and centrifuged at 120 *g* for 10 min. This nuclear pellet was washed twice and the combined supernatants were centrifuged at 23,000 *g* for 10 min. This L pellet was washed twice before being resolved on a Nycodenz® gradient. The authors used the supernatant from the first centrifugation as a source of microsomes and cytosol and for comparative analytical purposes this approach has much to recommend it. In the more widely-used sequence of centrifugations shown in Figure 1 soluble and fragmented material derived from partial disruption of organelles during manipulation of the N, M and L pellets will eventually end up in the microsomal supernatant.

- The L pellet is further resolved by a variety of gradient strategies and these are reviewed in Section 3.

A-3. Discontinuous - bottom-loaded

In sucrose gradients the buoyant density banding positions of lysosomes and mitochondria are too close to permit a useful separation, consequently it became common to use a strategy first described by Leighton et al [8] in 1968 in which the density of rat liver lysosomes was artificially reduced by prior administration of Triton WR1339 to the animals. However a number of functional changes in the lysosomes are caused by the detergent. In the late nineteen-seventies Wattiaux's group at the University of Namur in Belgium investigated the alternative use of the first non-ionic iodinated density gradient medium - metrizamide – for the fractionation of subcellular organelles from the light mitochondrial fraction from rat liver [9,10]. These workers showed that the resolution of lysosomes from the denser mitochondria was much improved if the light mitochondrial fraction was layered in a dense solution beneath the gradient rather than layered on top of the gradient. For routine preparation of lysosomes from rat liver a bottom-loaded discontinuous gradient was recommended. More recently metrizamide, which is now commercially unavailable, was replaced by Nycodenz® [10-13] and it is this method that is summarized in Figure 1. In a typical experiment using rat liver the enrichment of a lysosomal marker such as *N*-acetyl-β-glucosaminidase in the 20%/25% Nycodenz® band is approx. 100-fold over the homogenate [10,11]. The method as has also been applied to HepG2 cells [14].

Organelles, particularly mitochondria, are very sensitive to hydrostatic pressure and there are many examples of mitochondrial purification methods using discontinuous gradients in which the crude organelle fraction is loaded in a median layer rather than bottom-loaded in order to reduce the hydrostatic pressure on the sample. This strategy was first used by Okado-Matsumoto and Fridovich [15]. It may be good practice to reduce the hydrostatic pressure in all flotation fractionations of the light mitochondrial pellet. Cabrita et al [16] used 40%, 30%, 25%, 23%, 20%, 15% and 10% (w/v) Nycodenz® (0.5, 1.0, 3.5, 2.0, 2.0, 2.0 and 1.0 ml respectively) with the light mitochondrial pellet in the 25% layer; the lysosomes from rat liver banded at the 15%-20% Nycodenz® interface. The centrifugation conditions were also very mild – 52,000 *g* for 90 min. Another means of reducing the hydrostatic pressure on the sample is to use a vertical rotor rather than a swinging-bucket rotor.

- Although no published papers have reported the use of OptiPrep™ in this flotation mode, there is no obvious reason why this would not be effective.

A-4. Discontinuous gradients – top-loaded

An L fraction from rat liver (in 2 ml of HM) layered over 24%, 27%, 28%, 33% and 40% (w/v) Nycodenz® (2 ml, 2 ml, 3 ml, 1 ml and 1 ml respectively) and centrifuged at 74,000 *g* for 3 h provided a very useful separation of mitochondria and lysosomes [5-7] as s described in Figure 2.

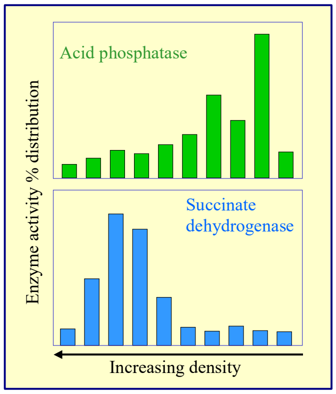

Figure 2: Separation of lysosomes and mitochondria from a rat liver L fraction in top-loaded discontinuous Nycodenz® gradient (adapted from ref 5).

Marshall et al [2] employed a novel discontinuous gradient in which the low-density layer contained 6% Percoll® and the two denser layers comprised 17% and 35% Nycodenz®. Moreover these workers highlighted two problems that are often overlooked; in particular, with cultured cells the homogenization strategy often needs to be tailored to the cell type and the organelles from different cell types may behave distinctively in gradients. Human breast carcinoma cells were homogenized using nitrogen cavitation, while human T-cell leukaemia cells were lysed in a Dounce homogenizer. Moreover while the lysosomes from breast carcinoma cells banded at the 17%-33% Nycodenz® interface, those from the leukaemia cells banded at the 6% Percoll®-17% Nycodenz® interface [2]. Optimization of the centrifugation conditions may also be needed; at 20,000 g breast carcinoma cell lysates required 20 min, leukaemia cells - 30 min. Whether this organelle density difference is a consequence of the cell type or the mode of homogenization, or both, is not known.

Iodixanol gradients have been widely used in the top-loaded discontinuous gradient mode, usually covering a slightly lower density range. Layers of 17%, 20%, 23%, 27% and 30% (w/v) or 8, 12, 16, 19, 22.5 and 27% (w/v) iodixanol are quite common. Sometimes the crude fraction is a total cell lysate, sometimes a post-nuclear supernatant (PNS) and sometimes a light mitochondrial fraction (see Section 2). Table 1 lists the cell types that have been analyzed in these gradients and summarizes the gradient format.

Table 1: Lysosome separations in top-loaded discontinuous iodixanol gradients

Cell/tissue	Gradient (%, w/v) iodixanol	Ref. #	Cell/tissue	Gradient (%, w/v) iodixanol	Ref. #
Blastocysts	10,15,20,25,30	17	Fibroblasts	6,10,16,24	21
Brain	8,12,16,19,22.5,27	18-20	Mononuclear	8,12,16,19,22.5,27	25
Carcinoma cells	6,10,16,24	21	Myeloid	8,12,16,19,22.5,27	26
	8,12,16,19,22.5,27	22	Neuroblastoma	15,17,20,23,27,30	27,28
	15,17,20,23,27,30	23,24	NK cells	8,12,16,19,22.5,27	29
			Renal cortex	15,17,20,23,27,30	30

In the 15%, 17%, 20%, 23%, 27%, 30% (w/v) iodixanol gradient format the sample (normally a light mitochondrial pellet) is usually in the 15% iodixanol layer. The centrifugation conditions vary rather widely: 50,000 *g* for 17 h [21]; 145,000 *g* for 2 h [27,28,30]; 150,000 *g* for 4 h [26,29]; 150,000 *g* for 5 h [22,25] and

100,000 *g* for 16 h [17]. The lysosomes are normally located in the top quarter of the gradient and the recoveries are very good; the lysosome fraction, recovered from the top of the gradient, contained over 80% of the total cathepsin D activity [27]. Lysosomes from osteoclasts have also been fractionated in a discontinuous gradient [31]. Over the longer periods of centrifugation the gradient will become essentially continuous.

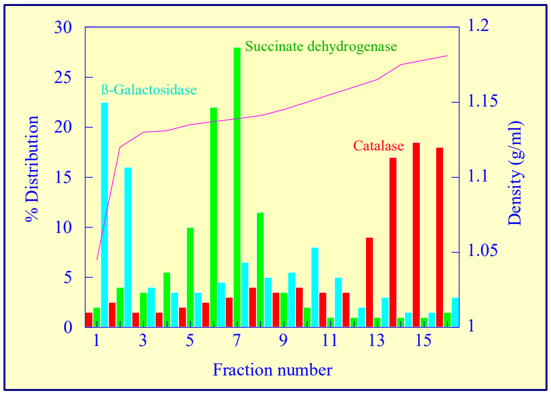

Figure 3 Separation of lysosomes, mitochondria and peroxisomes from a mouse liver L fraction in 30% iodixanol by flotation through a 19-27% iodixanol gradient, 70,000 *g* for 1.75 h

A-5. Continuous gradients

This methodology has been widely used with iodixanol. Graham et al [32] were the first to report this technology with mouse liver. A variety of density profiles were studied; an efficacious system comprises a 19-27% (w/v) iodixanol gradient, overlaid with HM and underlaid by the L fraction adjusted to 30% iodixanol. After 70,000 g_{av} for 1.5-2 h the distribution of markers is as shown in Figure 3, with the lysosomes at the top of the gradient. Separations similar to those shown in Figure 3 have been obtained with an L-fraction from human breast carcinoma cells [33] loaded in 35% (w/v) under a 10-30% iodixanol gradient (52,000 *g* for 1.5 h) or top-loaded in 5% iodixanol [34].

A light mitochondrial fraction from carcinoma cells has been fractionated on a 4-24% (w/v) iodixanol gradient; the *g*-force was only 20,000 *g* but the time was extended to 17 h [35], the lysosomes banded around 1.12 g/ml and the gradient was used in the localization of the KIF5B kinesin heavy chain protein. Higaki et al [36] fractionated a human skin fibroblast PNS on a continuous 5-20% (w/v) iodixanol graident (90,000 *g* for 20 h) that was first described by Sugii et al [37] in endocytosis studies.

A-6. Self-generated gradients

Graham et al [32] were the first to demonstrate the usefulness of self-generated gradients of iodixanol to fractionate the light mitochondrial fraction from mouse liver. Self-generated gradients are simple to set up and the lack of any interfaces between the sample and the gradient reduces particulate aggregation. The L fraction is adjusted to, for example 17.5% (w/v) iodixanol, and centrifuged in a suitable rotor, either vertical, near-vertical or low-angle fixed-angle rotor. In the example in Figure 4 a fixed-angle rotor (10 ml tube, 20° angle) was used at 270,000 g_{av}, for 3 h. A small tube volume (2 ml) Beckman TLV-100 vertical rotor allowed the centrifugation time to be reduced to 1.5 h for a light mitochondrial fraction from glioma cells [4].

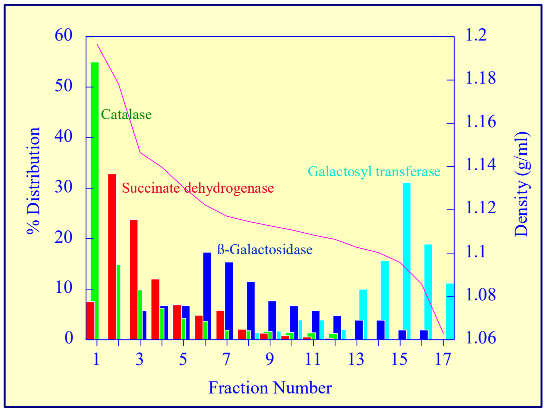

Figure 4 Fractionation of a mouse liver L pellet in a 17.5% iodixanol self-generated gradient: for more details see text

The strategy also appears very successful with promyeloid [38] and lymphoma cells [39]; in both these cases the crude organelle fraction was adjusted to 20% iodixanol and the lysosomes recovered from close to the top of the gradient. This 20% (w/v) iodixanol gradient may be less successful in resolving the lysosomes from any Golgi membranes in the fraction but this will also depend on the density profile of the gradient, and that depends not only on the rotor type but also on the *g*-force and the centrifugation time. Beckman VTi65.2 vertical rotor (350,000 *g* for 3 h) and a Beckman NVT90 near-vertical rotor (320,000 *g* for 3 h) have also been used for these gradient separations for retinal epithelial cells [40] and Caco-2 cells [41] respectively. In the latter case the starting concentration of iodixanol was 30% and consequently the lysosomes banded close to the top of the gradient.

A detailed description of the OptiPrep™ methodologies can be found on the relevant OptiPrep™ Applications flash-drive or on the following website: www.axis-shield-density-gradient-media.com (click on “Methodology”, then “Organelles and Subcellular Membranes”) and scroll down the Index.

- Application Sheet S53 describes the use of discontinuous gradients
- Application Sheets S15 and S54 describe the use of continuous gradients
- Application Sheet S16 describes the use of self-generated gradients
- Application Sheet S04 describes the construction of self-generated gradients

A-7. References

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PART B: BIBLIOGRAPHICAL REVIEW OF ALL PUBLICATIONS REPORTING THE USE OF OPTIPREP™ IN LYSOSOMAL STUDIES

- References are divided alphabetically according to tissue or cell source and listed alphabetically according to **first author**
- Part(s) of the titles are highlighted in blue to facilitate identification of particular research topic(s)

Blastocysts

Lee, J-H., Yu, W.H., Kumar, A., Lee, S., et al (2010) *Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations* Cell, **141**, 1146–1158

Lee, J-H., McBrayer, M.K., Wolfe, D.M., Haslett, L.J., Kumar, A., Sato, Y., Lie, P.P.Y., Mohan, P. et al (2015) *Presenilin 1 maintains lysosomal* Ca^{2+} *homeostasis via TRPML1 by regulating vATPase-mediated lysosome acidification* Cell Rep., **12**, 1430–1444

Bombyx mori silk gland

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

Brain

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Caenorhabditis elegans

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Carcinoma cells (includes HeLa, lymphoma and hepatoma cells)

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Glioblastoma cells (see "Neuroblastoma)

HEK cells

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Yin, J., Liu, X., He, Q., Zhou, L., Yuan, Z. and Zhao, S. (2016) *Vps35-dependent recycling of Trem2 regulates microglial function* Traffic, **17**, 1286–1296

HeLa cells, see “Carcinoma cells”

Kidney

König, J., Besoke, F., Stuetz, W., Malarski, A., Jahreis, G., Grune, T. and Höhn, A. (2016) *Quantification of age-related changes of a-tocopherol in lysosomal membranes in murine tissues and human fibroblasts* Biofactors, **42**, 307–315

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Lung

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Lymph node cells

Jin, R.U. and Mills, J.C. (2014) *RAB26 coordinates lysosome traffic and mitochondrial localization* J. Cell Sci., **127**, 1018–1032

Lymphocytic/lymphoblastic/lymphoma

Alexia, C., Poalas, K., Carvalho, G., Zemirli, N., et al (2013) *The endoplasmic reticulum acts as a platform for ubiquitylated components of nuclear factor kB signaling* Sci. Signal., **6(291)**, ra79

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Microglial cells

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Monocytes

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Myosarcoma

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Mini-Review MS06: 5th edition, October 2017

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