OptiPrep™ Application Sheet S42

Endocytosis analysis – a review of density gradient methods

1. Nycodenz® gradients

1a. Long-spin Nycodenz® continuous gradients

Some of the first reports on the use of Nycodenz® for the fractionation of endosomes were published in the mid-nineteen eighties by Howard Evans and his co-workers, working at the National Institute for Medical Research in London. They worked primarily on the endocytosis of a variety of ^{125}I-labelled asialo-glycoproteins by the perfused rat liver. After differential centrifugation of the homogenate, a supernatant from a light mitochondrial pellet was first applied to a continuous 15-43% (w/v) sucrose gradient (over layers of 43% and 70% sucrose) and centrifuged at 140,000 *g* for 3.5 h. The lightest fraction (1.095-1.117 g/ml) was re-centrifuged on a 13.8-27.6% (w/v) Nycodenz® gradient at 110,000 *g* for 18 h. Two very distinct, well-separated peaks of radioactivity were obtained at 1.090 and 1.115 g/ml. The denser material was identified as early endosomes and the lighter as late endosomes [1].

1b. Short-spin Nycodenz® velocity gradients

At about the same time, Trond Berg's group at the University of Oslo, working primarily with isolated hepatocytes, developed a variety of Nycodenz® gradients for the analysis of ligand internalization. The gradients were continuous 0-45% (w/v) Nycodenz®, centrifuged at 85,000 *g* for a variety of times, with the sample either top- or bottom-loaded. Two principal times were chosen, either 5 h for buoyant density analysis or 45 min for rate-zonal (sedimentation or flotation velocity) analysis. The top-loaded 45 min gradients showed that, with increasing internalization time (30 sec to 30 min), asialofetuin was associated with membrane compartments of increasing sedimentation velocity [2], which were all distinct from the bulk of the lysosomes (see Figure 1). If the centrifugation was carried out for 3 h, so that the membrane vesicles reached their banding density, then there was only a relatively small difference between the peak density at 1 and 15 min incubation times (1.10 and 1.11 g/ml respectively). Using the 45 min gradient format, it was later shown, using a polyethylene glycol-modified asialofetuin, that the peak around 1.05-1.06 g/ml was early endosomes and that at 1.09 g/ml comprised multi-vesicular bodies; the latter were quite distinct from lysosomes at 1.14 g/ml [3].

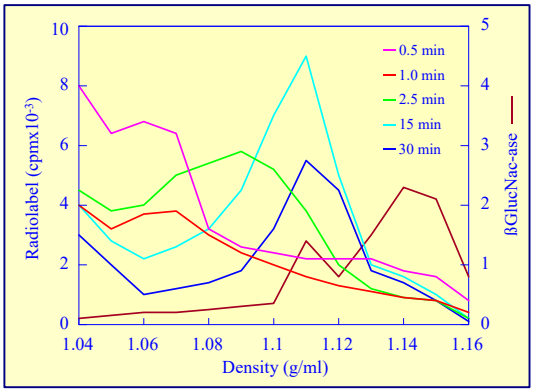

Figure 1: Distribution of ^{125}I-labelled ligand at different incubation times. Also shown is the banding position of lysosomes (GNAcase = ß-N-Acetylglucosaminidase). Figure adapted from ref 2.

1c. Hybrid Nycodenz®/polysucrose gradients

Branch et al [4] compared the efficacy of short-spin (1 h) continuous polysucrose and Nycodenz® gradients in the analysis of the membrane compartments in rat liver during the transcytosis of polymeric IgA and endocytosis of asialofetuin. In both instances a Beckman VTi50, vertical rotor was used at 206,000 *g*. The authors concluded that while polysucrose gradients were superior for resolving light (early) and dense (late) endosomes, Nycodenz® gradients provided far greater discrimination between late endosomes and lysosomes; moreover discrimination was achieved between lysosomes and very dense endosomes [5]. This led to the use of hybrid polysucrose-Nycodenz® gradients for the simultaneous isolation of early and late endosomes and lysosomes [6-9]; also, a simplified discontinuous gradient for the separation of lysosomes, very dense endosomes and other less dense endosomes was developed.

2. OptiPrep™

2a. Flotation velocity gradients

In 1997 the first paper reporting the use of an iodixanol gradient in endocytic studies was published by Orlandi [10]; the bottom-loaded 9-30% (w/v) iodixanol gradient centrifuged at 52,000 *g* for 90 min was similar to the Nycodenz® flotation velocity gradient (see Section 1b) of Kindberg et al [2]. It was used to analyze the internalization of cholera toxin by Caco-2 cells and over a period of 1 h the toxin peak showed a very clear shift from approx. 1.11 (0 min) to 1.12 (30 min) to 1.13 (60 min) g/ml.

2b. Overnight buoyant density gradients

A gradient system that has been widely used is that of Sheff et al [11]. In a study of the internalization of transferrin by MDCK cells transfected with the human Tfn receptor a shallow 5-20% (w/v) iodixanol gradient centrifuged at 100,000 *g* for 18 h was used to analyze a post-nuclear supernatant. Although many workers take advantage of the availability of high-performance rotors and the much lower viscosity of iodixanol gradients (compared to those of sucrose) to carry out buoyant density separations in 2-3 h, there is a widespread view that for the highest resolution of particles relatively low *g*-forces for long times are to be preferred. Sheff et al [11] were able to identify clearly a separation of early (EE) from recycling endosomes (RE), which were both well separated from the plasma membrane (PM) and lysosomes, which banded at lower densities (Figure 2).

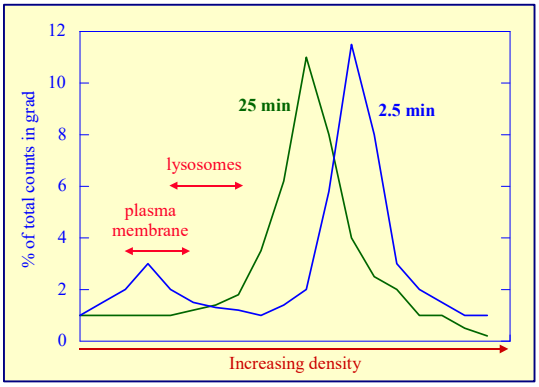

Figure 2 Distribution of transferrin in MDCK cell membrane fractions in long-spin iodixanol gradient: effect of chase time (from ref 11, with kind permission of the authors and The Rockefeller University Press).

It is important to note however that there may be significantly different patterns of banding in the case of other studies with other cell types. For example, in both HeLa [12] and COS-7 cells [13] EE banded at a lower density than RE, while the HeLa cell PM [12] banded close to the bottom of the gradient. LAMP-2 positive particles (late endosomes (LE) and lysosomes) from PC12 cells [14] and HeLa cells [15] banded at a higher density than the Rab5 positive EE. The banding patterns may also be influenced by practical variations in the handling of the cell homogenization.

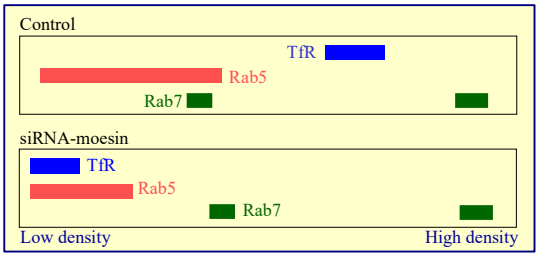

Figure 3 Distribution of markers in membranes from HeLa cells (long-spin iodixanol gradient): comparison of control and siRNA-moesin treated cells (adapted from ref 16)

In a study on the effect of moesin on receptor recycling in HeLa cells [16], this gradient system revealed two well separated bands of Rab7, the lighter one of which overlapped the lower density Rab5-containing vesicles (Figure 3). Barroso-González et al [16] compared control "scrambled" cells (nucleofected with scrambled oligonucleotides) and test cells nucleofected with siRNA moesin oligonucleotides. The TfR, which was located predominantly between the two Rab7 bands from control cells, shifted significantly into the low-density region in the siRNA moesin oligonucleotide-treated cells (Figure 3).

A very detailed analysis of the membranes from monkey kidney cells on an 8-25% (w/v) iodixanol gradient [17] identified (in increasing banding density) PM, lysosomes, endosomes + Golgi and two distinct bands of endoplasmic reticulum (ER). McKenzie et al [17] used the gradient to study the internalization of Shiga toxin (and its B subunit) and detected a single toxin-rich band in the Golgi + endosomes region; AlF_4^- treatment caused a pronounced dichotomy of the band. In an unrelated study Woods et al [18] also reported the resolution of two distinct fractions of ER from 3T3 cells, the denser of which was identified as perinuclear. Other uses of the long-spin continuous gradient are given in Table 1.

Table 1: Long-spin continuous gradient use

Cell type	Compartments analysed	Ref #
Neuroblastoma	EE (Rab5A), LE (Rab7); dopamine transporter	19
HepG2	EE subfractions (denser overlapping LE); transferrin labelling in denser EE	20
HeLa	Sorting/recycling endosomes, EE, LE, lysosomes; EE/Golgi cycling	21
HeLa	PM, LE; distribution of stomatin-like protein 1 and cholesterol transfer to LE	22

Idkowiak-Baldys et al [23] used a similar long-spin, low *g*-force strategy but started with a discontinuous 5%, 10%, 15% and 20% iodixanol gradient; although this will have become continuous during the centrifugation there may be small important differences in the final density profile when compared with a pre-formed continuous gradient. In HEK cells the PM and ER were concentrated at the light and dense ends respectively, while LAMP1-1 positive late endosomes banded about $2/3^{rds}$ down the gradient and three distinct Rab11 areas were observed (Figure 4). In phorbol-ester treated cells, protein kinase C, which was broadly distributed in control cells, became markedly and distinctively concentrated in the lightest of the Rab11 bands.

- In contrast to iodixanol gradients, Percoll® gradients cannot be centrifuged for long time periods at the *g*-forces required for effective membrane fractionation without most of colloidal silica pelleting; thus its use in endocytosis studies is very restrictive. In studies on the trafficking of LDL cholesterol Percoll® gradients were unable to resolve PM and EE from CHO cells [24]; long-spin iodixanol gradients were able to exhibit distinctive banding patterns not only for PM and EE, but also LE and Golgi.

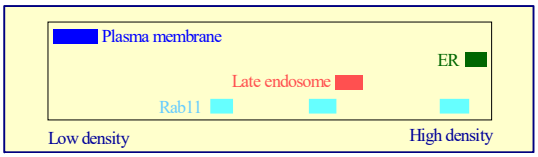

Figure 4: Distribution of HEK cells membrane compartments and Rab11 in iodixanol gradient: adapted from ref 23

2c. Short-spin continuous density gradients

Gradients covering more or less the same density as those described in 2b, when centrifuged for only 3 h at 100-130,000 *g* can provide excellent separation of EE from PM and Golgi from HeLa and HEK cells [25,26]. In the case of human HT1080 (human sarcoma) cells two LE fractions were identified; moreover chloroquine treatment of the cells induced a pronounced shift of Rab9 to the denser LE [27].

2d. Double-gradient strategy

An initial sedimentation velocity gradient followed by a second buoyant density gradient enabled Lin et al [28] to achieve very fine fractionation of light endosomes fractions from PC12 cells, in their studies on the internalization of nerve growth factor. To obtain a membrane vesicle fraction the cells were initially permeabilized by a single passage through a ball-bearing homogenizer. Semi-intact cells and larger membrane particles were removed by low-speed centrifugation. The supernatant was first layered on top of a 0-30% (w/v) iodixanol gradient and centrifuged at 133,000 *g* for 1.5 h. Fractions from this gradient were adjusted to 32.5% iodixanol by mixing with OptiPrep™; layered under a second 0-30% iodixanol gradient and centrifuged at the same speed for 18 h. The second fraction from the velocity gradient was resolved into two completely distinct non-overlapping sub-fractions; the lighter contained the neurotrophin receptor pTrkA plus its associated proteins APPL1 and GIPC1; the denser only contained APPL1. More recently [29] the same double-gradient strategy was able to resolve three distinct subclasses of endosomes: (1) those bearing activated receptor tyrosine kinases, (2) those containing p75NTR (tumor necrosis receptor superfamily) and (3) those exhibiting PAC1 (a G-protein-coupled receptor).

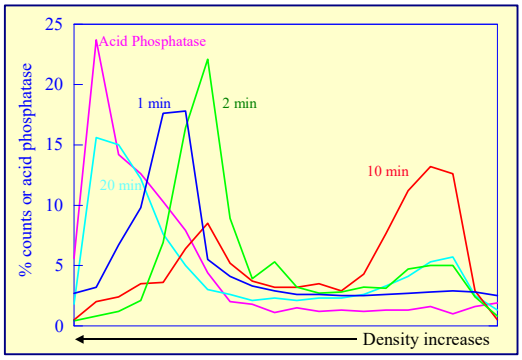

Figure 5 Distribution of labelled ligand (1 min pulse) from rat liver, at various chase times, in a self-generated iodixanol gradient.

2e. Self-generated gradients

The use of self-generated gradients was first reported by Billington et al [30] for analysis of the internalization of asialoglycoproteins by the perfused rat liver. One of its attractions is its simplicity and reproducibility of gradient formation. After homogenization of the liver, a 3000 *g* supernatant was simply adjusted to 12.5% (w/v) iodixanol, underlaid with 1 ml of 20% iodixanol and centrifuged in a vertical or near-vertical rotor at 350,000 g_{av} for 1.5 h.

The results of a 1 min pulse of labelled ligand after various chase times are shown in Figure 5. The data is interpreted as follows: (1 min chase) the ligand is found initially in a dense clathrin-coated vesicle; (2 min) uncoating of the vesicle reduces its density; (10 min) the ligand has been transferred to a low-density endosome and (20 min) the ligand appears in a lysosomal or pre-lysosomal compartment.

Sometimes the gradient is generated not from an iodixanol solution of uniform density but from a discontinuous iodixanol gradient in of 10%, 20% and 30% (w/v) iodixanol (with the sample in the latter). Centrifugation for 3 h will produce a more or less linear gradient (that produced from a single concentration will tend to be shallower towards the top and steeper towards the bottom). This tri-layered gradient has an additional feature; all of the cytosolic proteins remain at the bottom of the gradient. It was used to investigate the distribution of Ra1A (a GTPase which interacts with the exocyst complex) that resides in recycling endosomes. The Ra1A in COS cells overlapped only the denser parts of the TfR and Rab11 region, but not with EEA1, syntaxin 6 or the cytosolic Akt. Indeed the best coincidence was observed with another recycling endosome marker – Rab4, which was clearly associated with a denser particle than Rab11 [31]. This gradient format has

also been used for studying the changes induced by the adenovirus early region 4 open reading frame 4 protein in 293T cells [32].

- **See Reference List RS-12** for a complete bibliography of all the published papers that have reported gradients prepared from OptiPrep™ for the analysis of all aspects of endocytosis and trafficking within the endosomal system. To access return to the initial list of Folders and select "**Reference Lists**".

- There are several relevant OptiPrep™ Application Sheets that provide detailed protocols:
  - **Cultured cells – buoyant density: Application Sheet S46**
  - **Rat liver/hepatocytes – lysosome/late-endosome events: Application Sheet S54**
  - **Rat liver/hepatocytes – sedimentation velocity gradients: Application Sheet S44**
  - **Clathrin-coated vesicles/endosomes/lysosomes (self-generated gradient): Application Sheet S45**

  - **To access other Application Sheets** referred to in the text: return to the **2020SMemapp** file and select the appropriate S-number.

- It is not known if all or any of the Nycodenz®-based methods can be transposed directly to iodixanol. Nycodenz® and iodixanol solutions of the same % (w/v) have approximately the same density but the former have a higher osmolality (see Application Sheets for more information).

3. References

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