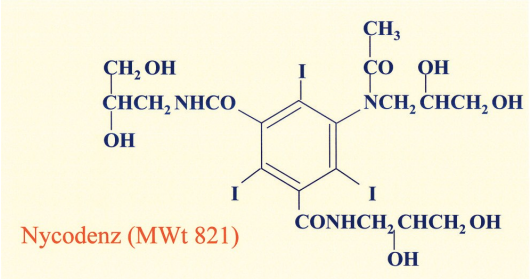
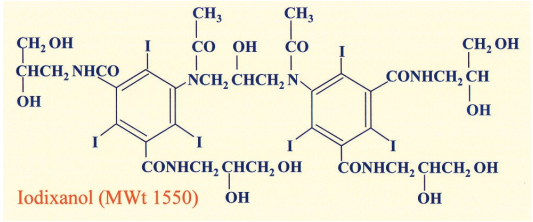
DENSITY GRADIENT MEDIA

OptiPrep™ and Nycodenz®
Analysis of endocytic compartment

Nycodenz® is supplied as a powder, freely soluble in water and solutions up to 80% (w/v) with a density of 1.426 g/ml can be prepared.

OptiPrep™ is a sterile endotoxin tested solution of 60% iodixanol in water with a density of 1.32 g/ml.



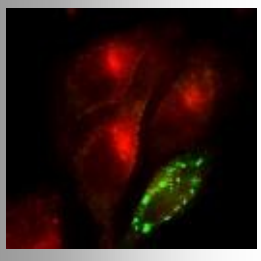


These compounds were developed as X-ray contrast media and have therefore been subjected to rigorous clinical testing. They are both non-ionic, non-toxic to cells and metabolically inert.

Actual endotoxin levels in each batch are usually measured at <0.13 EU/ml.

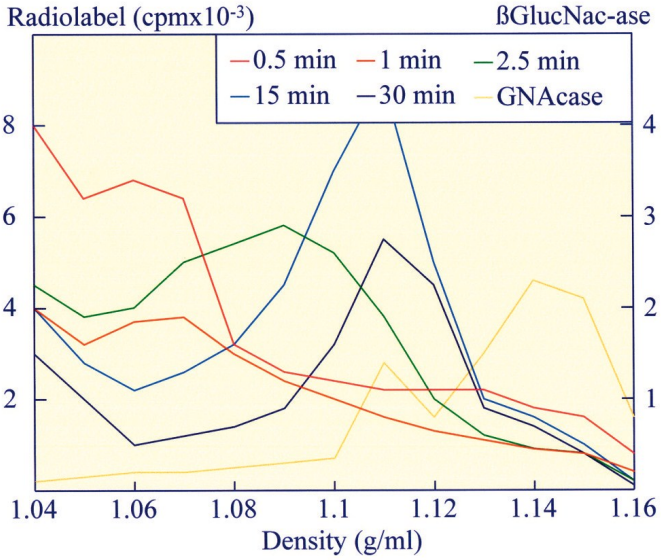
Although endosomal compartments may be analysed during a variety of studies into membrane trafficking and cell signalling, this leaflet is primarily concerned with the analysis of the endocytic process itself and the identification of clathrin-coated vesicles, early, late and recycling endosomes, the pre-lysosomal compartment and lysosomes.

Resolution may be carried out on either the basis of sedimentation velocity (rate-zonal) or equilibrium density. Both Nycodenz® and OptiPrep™ have been used but there is no reason why a strategy using one medium should not be used with the other, although the inability of Nycodenz® to give isoosmotic solutions above ρ = 1.15 g/ml may influence the relative efficacies of the two media.



Fractionation of early and late endosomes using sedimentation velocity

A postnuclear supernatant is loaded on to a 0-35% Nycodenz® (or iodixanol) gradient and centrifuged at 85,000g for 45 min. The figure shows the distribution of ^{125}I-asialofetuin on Nycodenz® gradients after internalisation during incubation of rat liver hepatocytes for different times. After 1 min, only a small amount of the ligand has been internalized and is associated with slowly sedimenting particles. With increasing times, more ligand becomes internalized and the distribution of radiolabel demonstrates that the endocytic structures containing the ligand increase their sedimentation rate. Nycodenz® gradients can identify pre-lysosomal and lysosomal compartments.

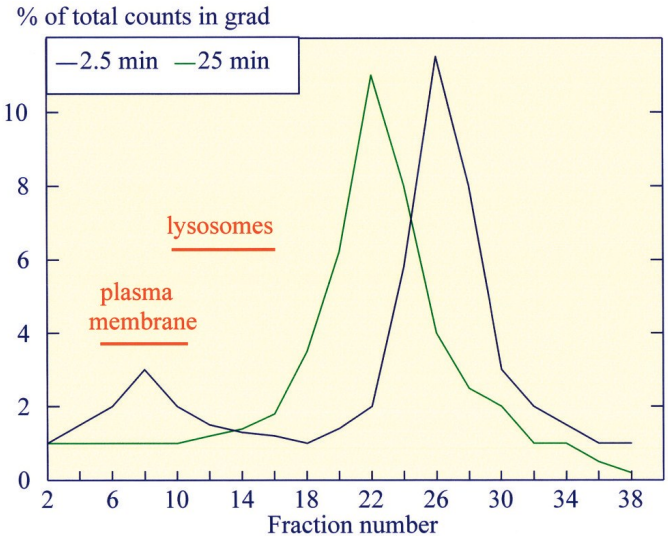


The centrifugation conditions for sedimentation velocity separations are variable. Early endosomes, plasma membrane and transport vesicles from rat kidney cells have been analyzed on 12.5-27.5 (w/v) iodixanol gradients at 140,000g for 1.5 h.

For a detailed protocol and references see S42 on the OptiPrep™ Applications CD or online at www.axis-shield-density-gradient-media.com

Fractionation of early and recycling endosomes in a continuous gradient

Continuous gradients prepared from OptiPrep™ can separate early and recycling endosomes (see figure): a postnuclear supernatant is layered on to a 5-20% or 10-20% iodixanol gradient and centrifuged at 90,000g_{av} for 18 h in a 13-14 ml swinging-bucket rotor. The gradient is also able to resolve plasma membrane and lysosomes. Long centrifugation times ensure that all the vesicles in a top-loaded sample reach their buoyant density, but it is important not to use significantly higher RCFs since the sedimentation of iodixanol will distort the pre-formed gradient, particularly at the bottom of the tube.



Additional resolution may be obtained by an initial sedimentation velocity fractionation followed by a second equilibrium density separation. After running PC12 cell membranes on a 0-30% iodixanol gradient (133,000g for 1.5h), banded material was adjusted to 32.5% iodixanol; loaded beneath a second 0-30% gradient and centrifuged for 18h.

Nycodenz® has also been widely used in equilibrium density analysis of endocytosis. Combination gradients of Nycodenz® and polysucrose are particularly effective in resolving very late endosomes, endosomes/lysosomes hybrids and lysosomes. Analysis of endocytosis in equilibrium iodixanol gradients have been done on CHO cells, COS-7 cells, Embryo fibroblasts, HeLa cells, Neuroblastoma and Pheochromocystoma.

For a detailed protocol see Application sheet S44

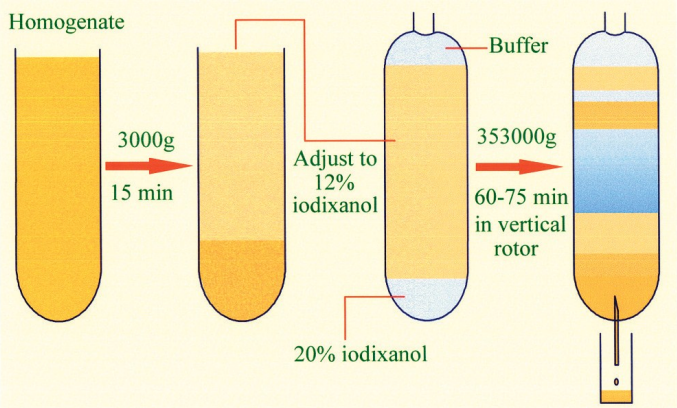
Analysis of the endocytic compartment using a self-generated gradient

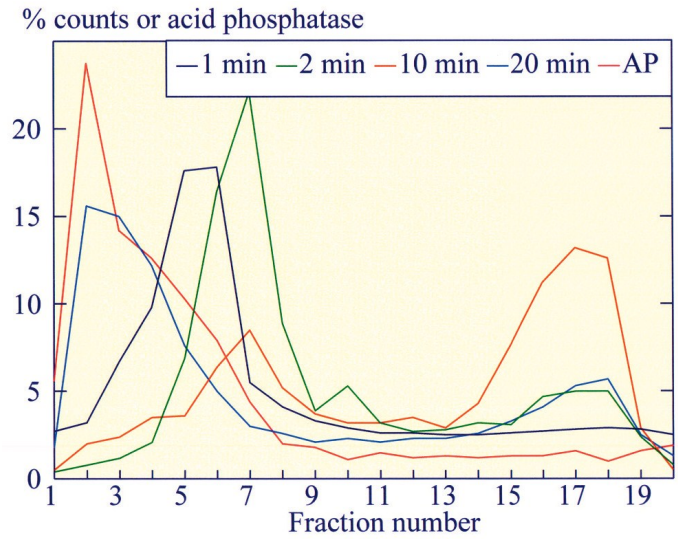
The flow chart that is the figure to the right, illustrates a different approach to the analysis of the endocytic process. By using OptiPrep™ in a self-generated mode, quite large volumes of a postnuclear (or post-heavy mitochondrial) supernatant can be simply mixed with the medium to a suitable iodixanol starting concentration and centrifuged for 1-3 h in a vertical or near-vertical rotor at approx. 350,000g.

By changing the centrifugation conditions the density profile of the gradient can be easily modulated for improved resolution of specific membrane vesicles.

The figure on the bottom right shows the analysis of endocytosis using the perfused rat liver method. The example given uses ^{99m}Tc-labelled neo-galactosyl-albumin as the ligand and following a 1 min pulse of the ligand, perfusion with unlabelled medium is continued for chase times of 1-20 min. The figure shows that these gradients can resolve dense (1-2 min chase) vesicles and light endosomes (10 min chase) and the lysosomes (20 min chase). The same system has been used to locate late endosome and lysosomal markers to vacuoles induced by *Helicobacter pylori* toxin.

In a modification to the procedure illustrated in the upper figure the postnuclear supernatant was adjusted to 30% iodixanol, layered under 20% and 10% iodixanol and centrifuged in a near-vertical rotor at approx. 380,000g for 3 h. An almost linear gradient is produced by a combination of self-generation and diffusion to separate early and recycling endosomes.





For a detailed protocol and references see S43 on the OptiPrep™ Applications CD or online at www.axis-shield-density-gradient-media.com

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In this leaflet we have presented some of the applications available for the Analysis of the endocytic compartment using Nycodenz® or OptiPrep™. More information can be found at: **www.axis-shield-density-gradient-media.com.** On the front page click on "Subcellular membranes and cell organelles".

Resolution of late endosome-lysosome events using polysucrose-Nycodenz® gradients

Polysucrose gradients have been widely used for the resolution of early and late endosomes, but Nycodenz® gradients provide far greater discrimination between late endosomes and lysosomes; moreover Nycodenz® gradients are able to resolve lysosomes and very dense endosomal compartments. This dichotomy has led to the use of hybrid polysucrose-Nycodenz® gradients for the simultaneous isolation of early and late endosomes and lysosomes. Gradients have also been developed for separation of lysosomes, very dense endosomes and other less dense endosomes. A popular approach in the study of endocytosis is the use of *in vitro* systems to follow the transfer of molecules between endosomes and lysosomes; this allows detailed study of the process in isolation from other events. Hybrid polysucrose-Nycodenz® gradients have been used to analyze the products and they have also permitted the isolation of endosome-lysosome hybrids subsequent to incubation of previously purified late endosomes and lysosomes.

For a detailed protocol and references see S55 on the OptiPrep/Nycodenz Application CD or online at: www.axis-shield-density-gradient-media.com

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