Intracellular exocytic vesicle trafficking and exocyst complex – a short methodological summary

1. Introduction

There are two areas of investigation where iodixanol gradients have been widely used in studies of exocytosis: (a) the control and organization of membrane trafficking within the cells that permits the movement of vesicles to, and ultimately their fusion with, the plasma membrane or a specific plasma membrane domain and (b) analysis of the process of cytokinesis and in particular the role of the evolutionary-conserved exocyst complex in controlling the final abscission process.

2. Membrane trafficking within the cytoplasm

2a. Cell homogenization

There is no particular consensus regarding the nature of the homogenization medium or the method of homogenization and a selection of examples is provided in Table 1.

Table 1 Homogenization procedure

Cell/tissue	Homogenization medium	Homogenization device	Ref #
3T3	0.25 M sucrose, 1.5 mM Mg Cl_2, 10 mM Tris-HCl, pH 7.5	Syringe needle (G25)	1
293T	0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5	Dounce homogenizer	2,3
Brain	0.32 M sucrose, 2 mM EDTA, 10 mM Hepes-KOH, pH 7.4	Dounce homogenizer	4
Carcinoma	0.25 M sucrose, 90 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH, pH 8.0	Ball-bearing	5
COS	0.25 M sucrose, 1 mM EDTA, 10 mM Hepes-KOH, pH 7.4	Ball-bearing	6
Endothelial	0.25 M sucrose, 90 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH, pH 8.0	Dounce homogenizer	7
HeLa	0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5	Syringe needle (G24)	8
	0.25 M sucrose, 0.5 mM EDTA, 10 mM Hepes-KOH, pH 7.5	Dounce homogenizer	9
Hepatoma	0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5	Syringe needle (G24)	8
MDCK	0.25 M sucrose, 90 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH, pH 8.0	Dounce homogenizer	10
		Ball-bearing	11
	0.25 M sucrose, 1 mM EDTA, 10 mM Hepes-KOH, pH 7.4	Syringe needle (G21)	12
	0.25 M sucrose, 78 mM KCl, 4 mM $MgCl_2$, 8 mM $CaCl_2$, 10 mM EGTA, 50 mM Hepes-KOH, pH 7	Dounce and Potter-Elvehjem homogenizer	13
		Glass bead agitation, Bio-Spec Mini-Beadbeater	14
Monocytic	0.25 M sucrose, 4 mM $MgCl_2$, 8 mM $CaCl_2$, 10 mM EGTA, 50 mM Hepes-KOH, pH 7	Dounce homogenizer	15
NRK	0.25 M sucrose, 90 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH, pH 8.0	Ball-bearing	16

- Any cocktail of protease inhibitors may be added to the homogenization medium

2b Pre-gradient processing

In most cases the fraction that is applied to the gradient, or incorporated into the gradient, is a post-nuclear supernatant (PNS). The single centrifugation may be carried out at any *g*-force from 500-1000 *g* for 5-10 min, although occasionally a post-heavy mitochondrial supernatant (3000 *g*) is used (see e.g. ref 6). Sometimes a more extensive differential centrifugation is carried out, for example an MDCK cell homogenate was centrifuged sequentially at 1000 *g* for 10 min, 5,000 *g* for 40 min and then 100,000 *g* for 2 h; the pellet from the last centrifugation was applied to the gradient [14]. Rather more rarely the differential centrifugation may be more extensive: e.g. 1000 *g* for 10 min, 5000 *g* for 10 min, 10,000 *g* for 20 min, 15,000 *g* for 20 min and then the final pellet at 100,000 *g* for 1 h was

resuspended and incorporated into a continuous gradient [13]. The advantage of using a PNS is the reduction in time between homogenization and gradient and losses of vesicles will be minimal. The disadvantage is the presence of all the other large organelles (mitochondria, lysosomes, peroxisomes) that will band in the denser regions of the gradient. Maybe a single 5000 *g* centrifugation, which will eliminate most of the mitochondria and some of the peroxisomes and lysosomes, might be a useful compromise.

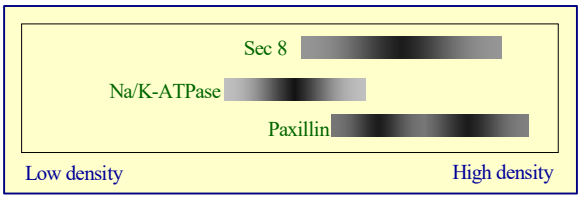

Figure 1: Approximate positions of protein markers from a MDCK cell PNS in the iodixanol gradient (adapted from ref 5). For more information see text.

2c. Self-generated gradients

- A detailed methodology of this technique can be found in Application Sheet S45

The 10%-20%-30% (w/v) iodixanol starting format for the preparation of gradients that are close to linear was first introduced by Yeaman et al [16] in 2001. These gradients are best prepared in a vertical or near-vertical rotor with a tube volume of no more than 13 ml; for example the Beckman VTi65.1 or NVT65 or the Sorvall 65V13. Smaller volume rotors (5-6 ml tubes) such as the Beckman VTi65.2 or NVT65.2 or the Sorvall 70V6 are equally acceptable. The method has often been by Yeaman and his colleagues in studies on the influence of the Sec6/8 exocyst complex in controlling membrane vesicle delivery to the plasma membrane of polarized cells [17,18]. In more recent experiments the gradient was modified: an MDCK cell PNS was adjusted to 30% iodixanol and layers of 25%, 20%, 15% and 10% (w/v) iodixanol layered on top, otherwise the centrifugation conditions were the same [5]. The gradient clearly resolved the Sec8 complex in the denser from the lighter Na^+/K^+-ATPase, while the paxillin marker was biphasic with only the lighter peak co-migrating with the Sec8 (see Figure 1). The standard 10%-20%-30% (w/v) iodixanol starting format was used in a study of the relationship between the Sec3 containing exocyst complex and desmosome assembly [11].

Kolesnikova et al [8] used the 10-20-30% iodixanol gradient to monitor the translocation of the VP40 matrix protein of Marburg virus in infected cells. At 7 h post-infection most of the VP40 was associated with the small vesicle fraction but as the infection progressed (up to 24 h) the gradient permitted the demonstration of a shift through the endosomal/ER zone to the plasma membrane.

A HeLa cell PNS was fractionated on the 10%-20%-30% (w/v) iodixanol gradient, with the PNS only in 30% layer and centrifuged at 330,000 *g* for 3 h: M-Sec and RalA formed a clear biphasic distribution, only the denser material co-fractionating with the Sec6/8 exocyst complex [9]. Using the same gradient and centrifugation format Chen et al [6] studied exocyst regulation of vesicle delivery to the centrosome prior to cytokinesis in COS cells: they observed a co-banding of RalA with TfR and Rab11 but not with early endosomes, Golgi or cytosol markers.

An additional layer of 15% iodixanol was inserted by Wang et al [12] who found that in PALS1 knockdown cells there was a significant shift in the banding of Sec8 and E-cadherin compared to wild-type cells.

- Similar self-generated gradients have also been used for the analysis of secretory proteins and the exocytic process for *Drosophila* [19] and in the study of the secretion of bone matrix proteins by osteoclasts [20].

2d. Gradients in swinging bucket rotors

Leblanc et al [1] working with 3T3 cells used a three-layer 10-30% (w/v) iodixanol gradient, again with the PNS in the densest layer in small volume (4 ml) swinging-bucket rotor at 260,000*g* for 3 h. A continuous gradient will form during the centrifugation mainly by diffusion, although some self-generation may also occur. The separation of dense small vesicles from lighter endosomes and plasma membrane was similar to that in ref 8.

Other gradients have conformed to the traditional format of top-loading of pre-formed gradients centrifuged at lower *g*-forces. A microsomal fraction placed on top of a continuous 10-40% (w/v) iodixanol gradient, centrifuged at 90,000 *g* for 18 h to study the delivery of TGF-α to the basolateral surface [13] and the targeting of exosomes to the same surface domain [14] of MDCK cells. The method in Ref 14 provides a particularly impressive purification of vesicles containing Naked-2-enhanced green fluorescent protein). A 5-30% (w/v) iodixanol gradient centrifuged under the same conditions was used in a study of miRNA effector proteins in exosomes derived from multivesicular bodies in monocytic cells [15].

A simple flotation discontinuous density gradient can separate soluble cytosolic proteins from a total vesicle fraction [21]

For some recent publications of studies of exocytic vesicles in polarised cells see refs 22-24.

3. References

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