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Review

Subcellular fractionation, electromigration analysis and mapping of organelles

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Abstract

Subcellular fractionation has provided the means required to analyze the composition and properties of purified cellular elements. In particular, subcellular fractionation has helped to define membrane boundaries and became necessary for the development of cell-free assays that reconstitute complicated cellular processes. Although cell fractionation techniques have improved over the last decades the purification of organelles to homogeneity is still a barely accessible goal in cell biology. In this article, we will first briefly review the basic principles of subcellular fractionation, and the establishment of different organelle fractions by density centrifugation, using tissue culture cells as a paradigm. Then we will discuss some of the intrinsic problems and will compare gradient purification of cellular extracts with electromigration analysis. Finally, we will describe alternative approaches, such as immunoisolation and flow cytometry to purify organelles from tissue culture cells.

Keywords: Reviews; Organelles

Contents

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1. Introduction

Subcellular fractionation was initially applied to separate organelles derived from rat liver and, in many cases, allows the separation of organelles based on their physical properties. Subcellular fractionation consists of two major steps: (1) disruption of the cellular organization (homogenization) and (2) fractionation of the homogenate to separate the different populations of organelles. This homogenate can then be resolved by differential centrifugation into several fractions containing mainly: (1) nuclei, heavy mitochondria, cytoskeletal networks and plasma membrane; (2) light mitochondria, lysosomes and peroxisomes; (3) Golgi, endosomes and microsomes (ER) and (4) cytosol. Each population of organelles is characterized by size, density, charge and other properties on which the separation relies. However, two major problems have impeded the development of standardized and ready-to-go procedures for subcellular fractionation. First, subcellular compartments share similar physical properties and co-fractionate at least to some extent in conventional gradients. Secondly, tissue culture cells are now more commonly used for fractionation, since cells can be manipulated in a manner impossible to achieve in animal-derived tissue. However, after homogenization tissue culture cells are more difficult to fractionate than most tissues, presumably because of differences in the cytoskeletal organization. It is essential to point out that complete purification is – with few exceptions – hardly possible. Therefore, many laboratories began to combine traditional fractionation procedures with alternative methods.

Density shift methods, which rely on altering the density of a specific compartment by the introduction of a perturbant, have proven useful for the isolation of fractions from the endocytic/lysosomal pathway. These perturbants can be loaded easily into these compartments from the extracellular space. This experimental approach was pioneered by the group of de Duve [1] to isolate lysosomes from rat liver on gradients after density modification with the detergent Triton WR-1339. This technique was further developed using membrane receptor ligands coupled to horseradish peroxidase (HRP). As another example may serve the density shift purification of diaminobenzidine-crosslinked endosomes containing HRP [2]. Likewise, endocytic/phagocytic organelles can be accessed by particles (e.g. latex beads, gold), plasma membranes can be labeled with a conjugate of wheat germ agglutinin and colloidal gold at 4°C before homogenization, and accordingly shifted to higher density gradients [3,4].

In contrast to those techniques immunoisolation relies on the unique localization of an antigen exposed on the outer surface of the compartment of interest. Isolation combines pre-fractionation on density or velocity gradients with the specific retrieval of membrane vesicles by using relevant antibodies bound to solid support (e.g. magnetic beads, cellulose fibers, acrylamide beads, *Staphylococcus aureus* cells, etc.) [5–7]. The immunoisolation conditions have to be optimized to obtain maximal specific recovery of the desired marker with minimal non-specific binding. This can be a serious problem when dealing with small amounts of material and with the mild washing conditions required to maintain membrane integrity.

Alternatively, flow electrophoresis separates subcellular organelles on the basis of charge [8]. This technique can also be combined with gradient centrifugation and was shown to be particularly useful for the separation of lysosomes and endosomes which are deflected towards the anode in the electric field [9].

In this review we will focus on classical subcellular fractionation, immunoisolation and electromigration analysis. We will document and discuss problems common to those methods and will finally describe alternative approaches for subcellular fractionation, such as fluorescent activated organelle sorting (FAOS) [10].

2. Classical subcellular fractionation

This chapter illustrates the separation and molecular analysis of different subcellular fractions from tissue culture cells. Taking mammary epithelial cells as an example, we briefly describe the homogenization and the separation of membrane vesicles on sucrose gradients. A typical fractionation experiment of tissue culture cells is outlined in Fig. 1. The resulting membrane fractions are analyzed for organelle-specific enzymatic activities and for the

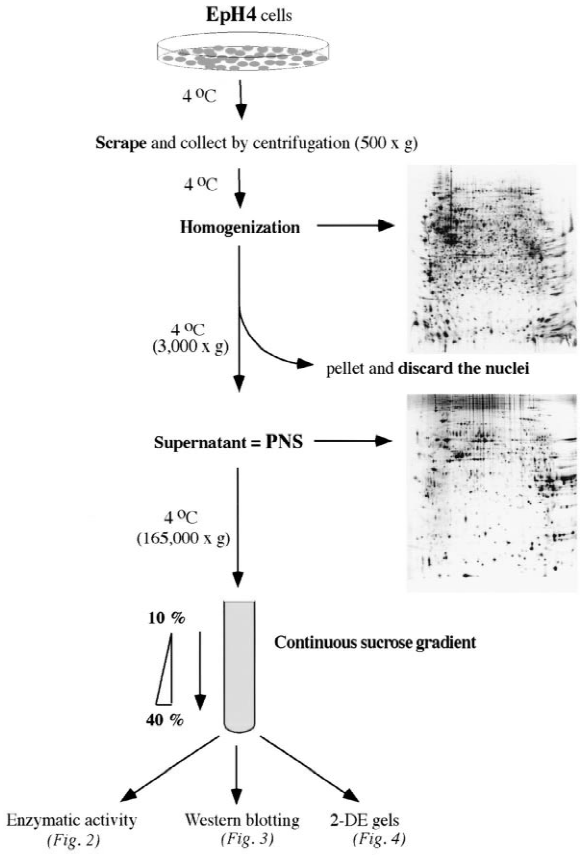

Fig. 1. Schematic outline of the preparation of organelle fractions from EpH4 cells. The murine mammary epithelial cell line EpH4 was grown on permeable filter supports and subsequently homogenized in 3 m*M* imidazole, pH 7.4, 250 m*M* sucrose, 1 m*M* EDTA, protease inhibitors and 10 μg/ml cycloheximide using a 22G needle and a 1-ml syringe. The nuclei were pelleted and the resulting PNS was loaded on top of a 10–40% sucrose density gradient to allow separation of subcellular organelles. Decrease in polypeptide pattern complexity from the homogenate to the PNS is indicated by the miniaturized 2-DE icons.

presence of established marker proteins (Fig. 2). To further characterize the gradient fractions we applied high-resolution two-dimensional gel electrophoresis (2-DE) (Fig. 3).

2.1. Homogenization

One major limitation in the successful fractionation of tissue culture cells is the production of an ''ideal'' homogenate, that is, the release of organelles and other cellular constituents as a free suspension of intact, individual components. Very often cytoplasmic aggregates are observed which contain cytoskeletal elements as well as various organelles. However, aggregates can reflect some pre-existing cellular organization, particularly due to the cytoskeleton, which may cause the cytoplasm to maintain some degree of organization after homogenization. Consequently organelles remain associated with the cytoskeletal elements surrounding the nucleus and/or become entrapped in large aggregates which readily sediment. A potential source for those are nuclei which break under harsh homogenization conditions and subsequently release DNA. This in turn will result in significant loss of components of the homogenate during the initial centrifugation step for removal of nuclei. Since the cytoplasmic and cytoskeletal organization of different tissue culture cells varies enormously, homogenization conditions must be optimized for each cell line [11–17].

The quality of the homogenization should be assessed by morphological means; e.g., by phase contrast microscopy, it is possible to assess the extent of cellular disruption, i.e., the appearance of unbroken nuclei and the absence of large aggregates. Taking these precautions into consideration one can assume that following homogenization, the nuclei are totally removed by a low speed centrifugation step, together with cell debris, unbroken cells and some larger subcellular components. The post nuclear supernatant (PNS) contains the cytosol and the other organelles in free suspension and can then be further separated by gradient centrifugation or other means (see Section 2–5).

2.2. Gradients

Centrifugation is the most effective method for organelle/membrane isolation. A number of other techniques which exploit various physical parameters (e.g. electrical charge for free flow electrophoresis) or biological properties (e.g. ligand affinity for immunoisolation) have been investigated as ways of examining the complexity of organelles and membranes. However, even when using these other methods, often better results will be obtained if the material is first purified by centrifugal methods. In the following section we will briefly comment on the most important requirements and principles of cen-

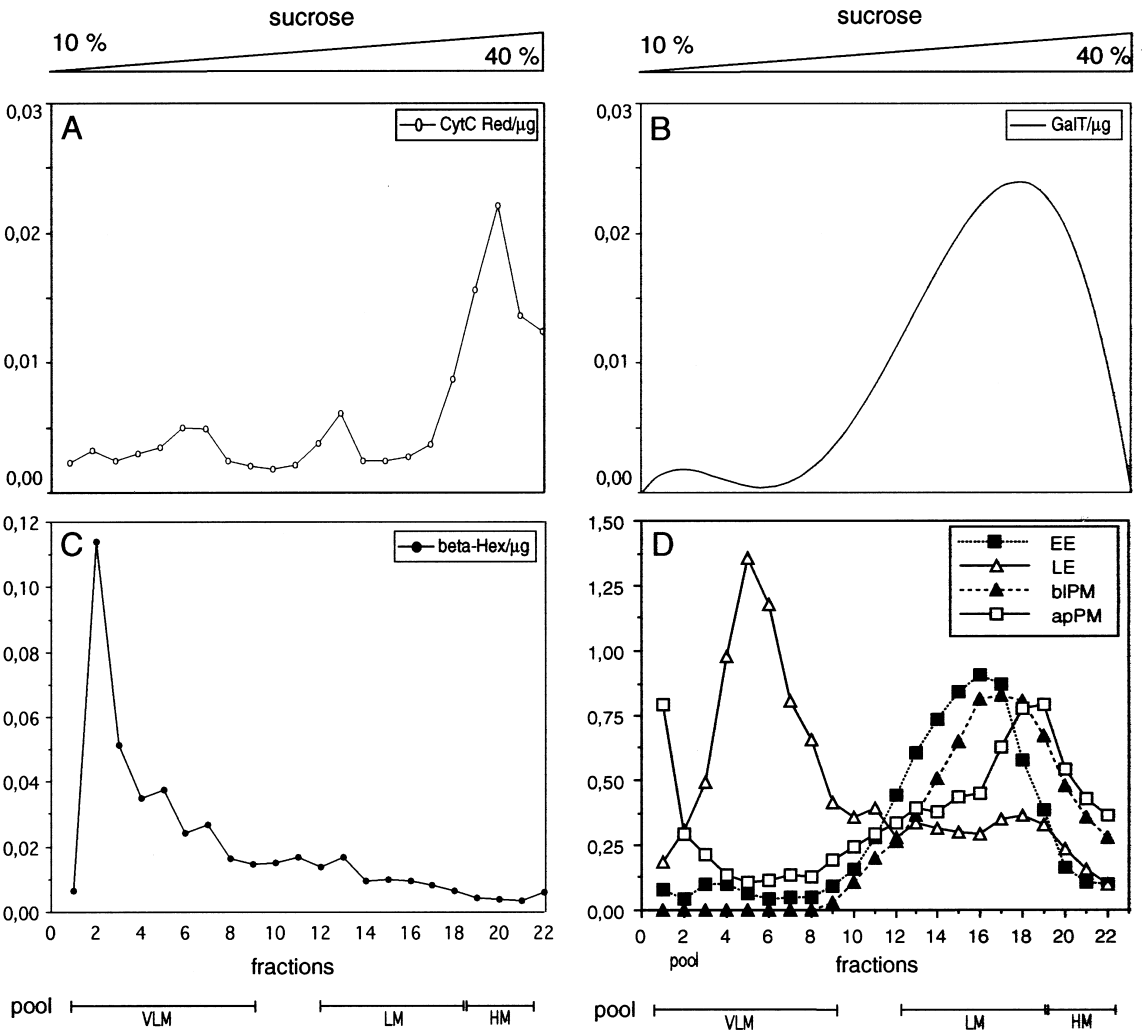

Fig. 2. Distribution of organelles from EpH4 cells in 10–40% sucrose gradients. The gradient fractions were analyzed for the presence of various organelle-specific enzymatic activities. Rotenone-resistant NADH-cytochrome C-reductase was measured as a marker for rough ER (A), β-1,4-galactosyltransferase as an enzyme localizing to the Golgi (B) and β-hexoseaminidase as lysosomal enzyme (C). For panel D, HRP was internalized as fluid phase marker for 5 min to label early endosomes (EE, filled squares) or internalized for 5 min and chased into late endosomes for 30 min. (LE, open triangles). Alternatively, the basolateral (blPM, filled triangles) or apical plasma membrane (apPM, open squares) was biotinylated and detected with avidin-HRP. In all cases the HRP-activity was used to determine the respective subcellular compartment. For a detailed description of all enzymatic assays and procedures see Fialka et al. [15]. In summary, very light membranes (VLM) represent lysosomes and LE, light membranes (LM) contain a mixture of EE, Golgi, TGN and plasma membrane and most likely light mitochondria (data not shown) and heavy membranes (HM) are mainly rough ER and heavy mitochondria (data not shown). Modified with permission from Fialka et al. [15].

trifugal methods. However, for a comprehensive review we would like to refer the reader to: Preparative Centrifugation, Practical Approach Series on CD-ROM, Book 51, Oxford University Press.

The position of membrane particles in density gradients is determined mainly by their ratio of lipid to protein contents; e.g., mitochondrial inner membranes are protein-rich and thus have a high density, whereas endosomal membranes are lipid-rich and are of low-density. Other parameters which determine

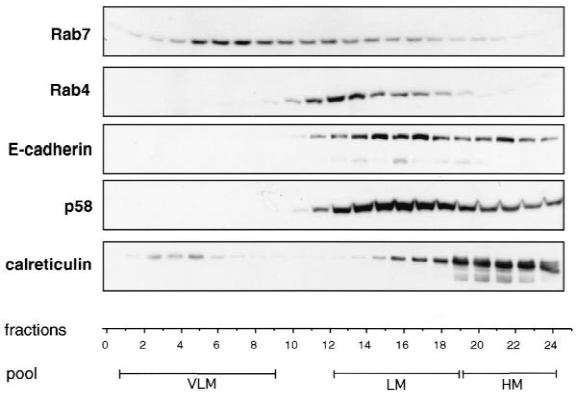

Fig. 3. Detection of organelle-specific proteins. Subcellular membrane fractions were prepared as outlined in Fig. 1. Proteins of equal volumes of gradient fractions were chloroform–methanol precipitated, separated on a 10% SDS–PAGE and blotted onto a nitrocellulose membrane. Immunodetection using antibodies specific for Rab7 (marker protein for LE), Rab4 (EE), E-cadherin (basolateral plasma membrane), p58 (ERGIC) or calreticulin (ER) shows the distribution of the respective compartments in the gradient. In agreement with the enzymatic determination, Rab7 is abundant in the very light membranes (VLM), Rab4, E-cadherin and p58 are present in the light membranes (LM) and calreticulin is highly enriched in the heavy membranes (HM). Modified with permission from Fialka et al. [15].

density include the contents of vesicles. For example, secretory low-density lipoproteins contained within Golgi vesicles render them more buoyant, whereas the protein contents of secretory granules increase their density (e.g. pituitary secretory vesicles). The presence of attached components (e.g. ribosomes on RER membranes and clathrin on coated vesicles) also affects the density of membranes. While differences in composition of subcellular components do determine relative densities, the degree of separation obtained also depends on the nature of the gradient medium. Although sucrose is most commonly used to form gradients, there are many other alternatives; e.g. Ficoll, Percoll, Nycodenz or Metrizamide [18,19]. The choice of the gradient might be cell type or tissue dependent. Discontinuous gradients as well as step gradients have been applied successfully for the separation of EE from LE [13,20–22]. Similar gradients were also applied for the purification of Golgi stacks [23,24] as well as Golgi-derived transport vesicles [14,25].

Separations can be achieved by two different centrifugation methods: velocity and equilibrium. In velocity separations particles move in the direction of the centrifugal force and separate according to size and density. Therefore, it is time dependent (because eventually everything will pellet). In equilibrium separations particles move to a position in the gradient which reflects their respective densities.

Centrifugation in step-gradients enriches different compartments at interfaces between different sucrose concentrations. In such gradients the sample is usually loaded on the bottom of the tube or on a sucrose cushion and membranous particles accumulate at the different interfaces. Enriched material can be collected in a small volume. However, small differences in density cannot be resolved.

For better resolution, equilibrium separations with continuous gradients are the method of choice. After centrifugation to equilibrium membranes distribute throughout the entire gradient according to their specific densities. A drawback of continuous gradients can be the low yield of organelles, resulting in rather diluted fractions. One can use normal H_2O or alternatively D_2O (heavy water) for the preparation of the sucrose solutions [20]. The sucrose concentration giving the same density is lower in D_2O than in H_2O. It was found that in certain cell types, such as BHK cells, for unknown reasons the contamination of plasma membrane in endosomal fractions is lower in D_2O-gradients than in H_2O-gradients [20].

2.3. Characterization of organelles: physical restrictions

In the given example, a PNS of cultured mammary epithelial cells [26,27] was loaded on top of a continuous H_2O-sucrose gradient (Fig. 1) and after centrifugation to equilibrium, fractions were collected and analyzed as described previously [15]. The typical distribution of specific enzymatic activities in continuous sucrose gradients is shown in Fig. 2. The results are plotted from top to bottom of the gradient (ranging from 1 to 22). It is important to emphasize that under mild homogenization conditions sealed vesicles and intact organelles are collected from such gradients and, therefore, lumenal proteins and enzymatic activities will be preserved in their respective cellular compartments [15,16].

The following markers are shown in Fig. 2A–C:

β-hexoseaminidase, a marker for lysosomes, β-1,4-galactosyltransferase (GalT), an enzyme residing in the *trans*-side of the Golgi, and rotenone-resistant cytochrome C-reductase, an enzymatic marker specific for the endoplasmic reticulum (ER). In such a representative experiment the lysosomal enzyme accumulated at the very top of the gradient, at a density of 1.038 g/cm^3 (10% sucrose). The enzymatic activity of GalT was detected at medium density levels starting in fraction 11 (about 23% sucrose, 1.094 g/cm^3) and reaching to the bottom of the gradients at a density of 1.166 g/cm^3 (38% sucrose). Finally, the enzymatic activity of cytochrome C-reductase was detected in the very dense gradient-fractions (1.146–1.176 g/cm^3, 34–40%). Most of the rough ER (RER) can be shifted into denser fractions by the addition of cycloheximide to all steps of the membrane preparations. Under these conditions, nascent polypeptide chains are trapped in the ribosomes and, thus, ribosomes remain bound to the RER [15]. Endocytic organelles can be identified in gradient fractions after internalization of HRP through the medium. Different chase times allow the selective labelling of EE and LE. Polarized epithelial cells are grown on semipermeable filter supports that allow experimental access to both plasma membrane domains. Accordingly, the apical or basolateral PM can be biotinylated on ice and detected with avidin-HRP.

Although such gradients efficiently separate very light from dense fractions, the problematic region comprises fractions of medium density (Fig. 2B). Golgi membranes, plasma membranes (both, the apical and basolateral plasma membrane (PM) in epithelial cells) as well as early endosomes (EE), all sediment at similar regions. However, late endosomes (LE), of similar densities as lysosomes [7], could easily be separated and distinguished from the bulk of other membranes (Fig. 2C). The overlapping distribution of Golgi, EE, PM, some parts of the ER, with its extension, the intermediate compartment (ERGIC), is confirmed by Western-blotting with antibodies against established organelle markers (Fig. 3). Antibodies to the LE-specific small GTPase Rab7 showed a distribution clearly distinct from EE detected by antibodies to Rab4. E-cadherin, a transmembrane protein of the basolateral plasma membrane, as well as p58, a marker protein of the ERGIC, distributed over a broad range, from the bottom to the middle area of the gradient. Calreticulin, a lumenal protein of the ER sedimented mainly to the bottom of the gradient but considerable amounts were also found throughout the entire gradient (Fig. 3).

This problem of overlapping distributions of medium density components becomes even more apparent when one analyses the overall protein composition of the fractionated samples. In this example we collected fractions of different densities from the gradient and generated three pools: very light membranes (VLM, fractions #4–9), light membranes (LM, fractions #12–18) and heavy membranes (HM, fractions #19–22). Samples were separated on high resolution 2-DE-gels as described previously [15,16], silver stained, scanned and digitized images compared by the help of the Melanie® software package on a Macintosh Power PC [28,29].

As expected, VLM (Fig. 4A) showed a rather distinct pattern when compared to LM (Fig. 4B) or HM (Fig. 4C). In the VLM fraction, 1679 silver-stained spots (with about 770 easily visible spots) were detected after scanning. Blue circles in VLM (Fig. 4A) highlight 80 proteins that were present exclusively in VLM when compared to LM. In the other hand, computer-assisted pattern analysis emphasized the largely overlapping protein distributions between HM (Fig. 4C) and LM (Fig. 4B). From 1798 detected in LM (Fig. 4B), 825 were unique to this fraction (Fig. 4B, green labels) whereas 973 were overlaping with HM. HM proteins revealed the most complex pattern with 2614 spots (Fig. 4C) within the same set of digital subtractions. Finally, a synthetic image was generated showing only those spots found in all three fractions (Fig. 4D). Although, the applied gradient separated well LE and lysosomes from the bulk of membranes, this did not exclude contaminations, as demonstrated by the 467 spots common to all three fractions (Fig. 4D).

Together, these results readily indicate that intact vesicles that migrate in the problematic middle density area of gradients, share similar physical properties and can not be separated from each other by conventional centrifugation. This is true for vesicles whether occurring naturally in cells, or formed by vesicularization of membrane fragments during the homogenization procedure. A detailed

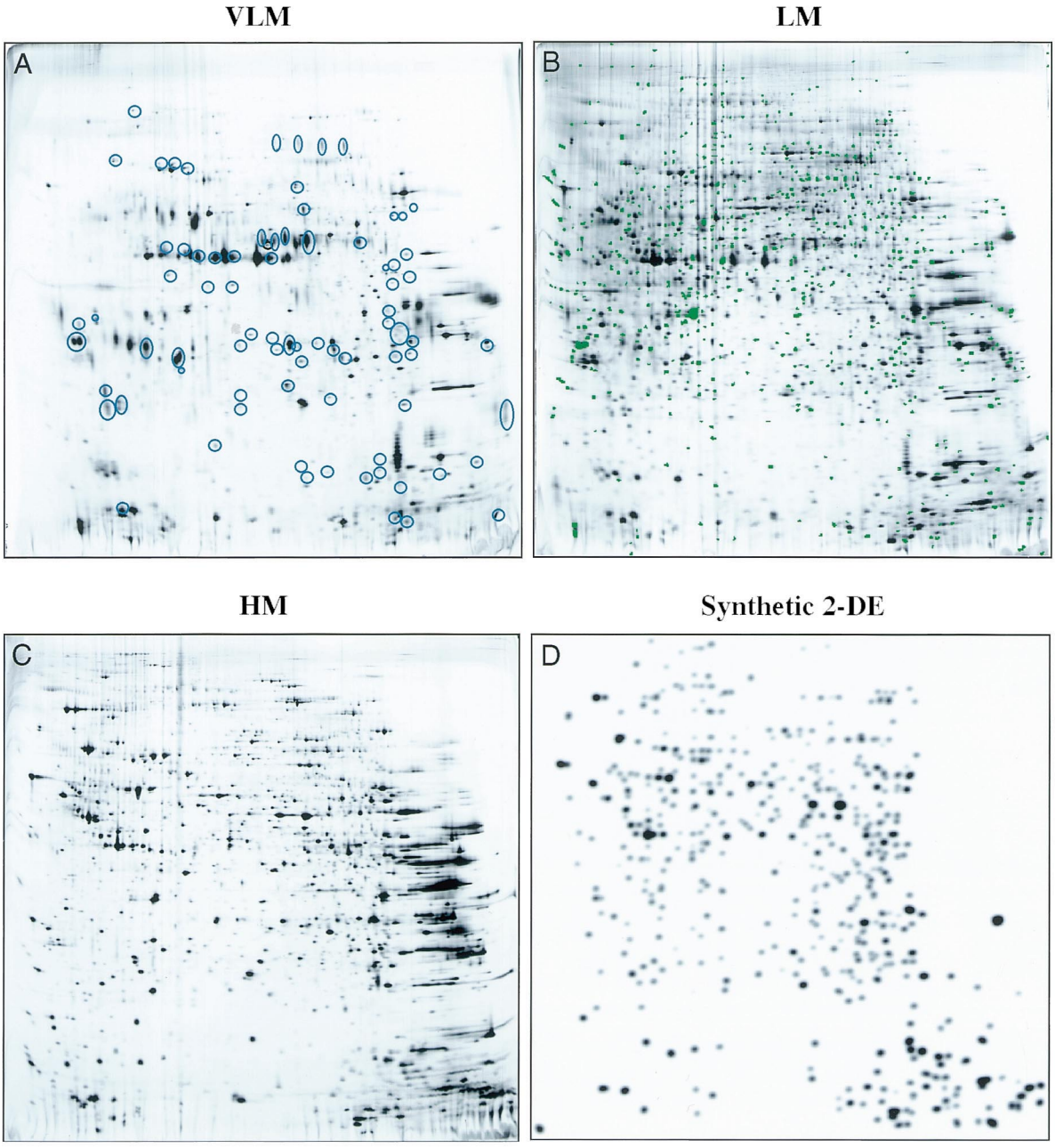

Fig. 4. Analytical silver-stained 2-DE gel of pooled organelle fractions. From left to right, acidic and basic proteins and from top to bottom, high (250 kDa) and low (10kDaX) M_r proteins. For sample loading, 60 μg of each pool of gradient fractions (VLM, LM, HM, see Figs. 2 and 3) were precipitated, solubilized and loaded during reswelling of nonlinear IPG strips as described previously [15,16]. Proteins were focused in the first dimension at 15°C until equilibrium (65 kVh). The second dimension was performed using 9 to 16% gradient SDS–PAGE followed by ammoniacal silver staining for protein detection [15,16]. Resulting images were digitized using a Personal Laser Densitometer and subsequently analyzed by the Melanie II software (BioRad). The overall membrane protein distribution of the pooled fractions was determined following three steps: (i) gel A (VLM) was compared to gel B (LM) in order to define spots only present in A (blue circles) or in B (green areas) (ii) gel A and B were matched against gel C (HM) and (iii) a synthetic gel (D) derived from the assembled mosaic of A, B, and C was created to represent all spots (features) which are common to the three images.

analysis of the experiments discussed here can be found in Fialka et al. [15]. As conclusion, only intracellular membranes with significant differences in density (e.g., lysosomes and LE, mitochondria, peroxisomes) and, to a lesser extent, size and shape (e.g., secretory granules, synaptic vesicles) can easily be separated from each other by conventional gradient centrifugation.

3. Immunoisolation

In the previous section we have discussed the principles and limitations of classical fractionation methods relying on sedimentation and velocity of organelles in gradients. However, these purifications are based on membrane densities rather than biological properties and have clear limitations. In this section we will expand the spectrum of applicable techniques towards purification of intracellular organelles by immunoisolation.

Immunoisolation techniques utilize the high specificity of antibodies to isolate subcellular organelles containing the cognate antigen. Usually, isolation protocols combine pre-fractionation by gradient centrifugation with immunoisolation. A prerequisite for immunoisolation is (1) to identify an appropriate antigen on the organelle of interest and (2) to have a high-affinity antibody that recognizes the antigen in its native state. Ideally, the antigen should be limited to the compartment that should be purified, but in cases where a broader distribution is expected, pre-fractionation could overcome this problem. The antigen should be of high enough abundance and available for antibody binding. Different solid supports are available for immunoisolation; e.g., monodisperse magnetic beads, *Staphylococcus aureus* expressing protein A, Eupergit particles, cellulose fibers [5,6].

The efficiency of immunoisolation for one particular antigen/antibody couple may vary with different immuno-adsorbents, depending on the abundance and accessibility of the antigen on the membrane of interest. Magnetic beads are now commonly used, because separation and retrieval with a magnet is rapid, efficient and considerable, avoiding fragmentation of vesicles or organelles. Another advantage of magnetic beads is that the isolated fractions can be introduced into and then retrieved from an in vitro reaction mixture at the end of a reaction, while bound to the solid support. This is an important consideration for all kinds of cell-free assay systems for measuring organelle function [5,6,11,20,30]. Other solid supports, such as cellulose fibers, can have problems with entrapment and fragmentation of bound fractions, which result in higher backgrounds and reduced efficiencies.

By analogy to all other techniques described in this article, the success of an immunoisolation is particularly dependent on the ability to produce a monodisperse input fraction from the tissue or cells of interest (see Section 2.1). Any amount of aggregates will increase the level of nonspecific components isolated. Therefore, many protocols prefer to couple the specific antibody first to the beads and then add the complex to the organelle fractions, as direct addition of the antibodies to the fraction may lead to aggregation of antigen-containing vesicles [11,20].

The purification of stacked Golgi fractions and exocytic transport intermediates may serve as classical examples for successful immunoisolations using magnetic beads [11,20,31–33]. The purified fractions have been extensively characterized, mapped and many new Golgi proteins have been identified. Immunoisolation has been successfully applied even in cases where no adequate antigen was present endogenously on the organelle of interest. In these experiments, the transmembrane glycoprotein G of vesicular stomatitis virus (VSV G) was first introduced into the plasma membrane and then internalized. Intact virions were attached to the cell surface and the VSV envelope fused to the plasma membrane at low pH. As a consequence, the G cytoplasmic protein is then exposed on the surface of endosomal elements after endocytosis and accessible to antibodies after homogenization [20,30]. Immunoisolated endosomes have functionally been characterized in cell-free assays and protein patterns have been mapped and characterized by high-resolution 2-DE-gel analysis and microsequencing [30].

In like manner, immunoisolation techniques have led to the purification of apical and basolateral transport vesicles that mediate the delivery of proteins from the TGN to the two plasma membrane domains of MDCK cells [14,25,34]. Using virally

infected cells, the purification of TGN-derived vesicles was possible by utilizing influenza hemagglutinin or VSV G protein as apical and basolateral antigens, respectively. Antibodies directed against the cytoplasmically exposed domains of the viral spike glycoproteins permitted the resolution of apical and basolateral vesicle fractions. These highly purified fractions were extensively analyzed on 2-DE-gels and served as an important source for the identification of molecules of the intracellular protein sorting and transport machinery [14,25,34–39].

These selected examples illustrate the usefulness of immunoisolation in combination with classical subcellular fractionation techniques. However, even in those highly purified fractions the use of 2-DE electrophoresis has relentlessly exposed considerable cross-contaminations, mainly with ER and plasma membrane proteins [25,30,39].

4. Electromigration analysis

Differences in electrophoretic mobility of various components of a mixed membrane fraction, due to the characteristic charge of membrane vesicles, offer an alternative way to prepare organelle fractions. In this section we discuss free flow electrophoresis (FFE) as a preparative technique, high resolution density gradient electrophoresis (DGE) and immune free flow electrophoresis (IFFE).

4.1. Free flow electrophoresis (FFE)

The development of free flow electrophoresis as a preparative technique [40,41] has meant that a variety of cells and subcellular organelles can now be separated on the basis of their unique charge densities. In FFE, a mixture of differently charged proteins, organelles or cells is injected into a liquid curtain of buffer that flows between two charged plates, a cathode and an anode. The electrophoretic mobility is largely independent of size and shape of membrane particles within the range of sedimentable particles found in cell or tissue homogenates, nor does it correlate exactly with the contents of negatively charged surface molecules [42]. From the work of Morre et al. it became apparent that the membranes with the greatest electrophoretic mobility (i.e. lysosomes, LE, *trans*-Golgi network vesicles, TGN) were those membranes with an inherent ability to acidify their interiors [42]. This generates a membrane potential, with negative charge outside and provides a basis to explain why certain membranes of very similar overall chemical composition may be separated by electrophoretic methods.

In the majority of cases FFE was successfully applied for enriching plasma membrane vesicles [43,44] and endosome populations from non-polarized cells [45–48]. The latter ones remain functionally intact after electromigration with respect to ATP-dependent endosome acidification. FFE has also been applied for organelle separation from tissues. The isolated perfused rat liver was used to internalize labeled ligands and endosome subpopulations were analyzed after enrichment by FFE [8]. In addition, FFE was successfully used as final step in the purification of clathrin-coated vesicles from bovine brain [49]. Tonoplast (vacuole membranes) and plasma membranes were isolated by free-flow electrophoresis from soybean (Glycine max) hypocotyls [50].

These examples show that mostly lysosomes and endocytic vesicles are deflected under the usual conditions of free flow electrophoresis, to such an extent that they appear well-separated from the bulk of organelles. This constitutes the main peak to be seen in a free flow electropherogram, with the possible exception of plasma membrane and tonoplast vesicles. However, membranes derived from Golgi, TGN, smooth ER, RER and light mitochondria are always major sources of cross-contamination depending on cell- or tissue-type of investigation as well as on the homogenization conditions applied.

Free-flow electrophoresis has a number of considerable advantages over other methods in the amount of material processed and speed of purification. However, in order to achieve separation of endosomes or lysosomes by FFE samples have to be treated with trypsin prior to electrophoresis. For microsomes and endosome enriched fractions from rat liver and various cell lines, treatment with 2–10 μg/ml trypsin for 5 min at 37°C is generally recommended to enhance separation [45–48]. The digestion is then stopped by returning the samples to 0°C and adding an excess of soybean trypsin inhibitor. The effects of trypsin on endosome and lyso-

some membranes that result in the increased electrophoretic mobility are still unclear. Although, authors have reported that there is no change in latency of internal markers, the buoyant density of the organelles or the ATP-dependent acidification activity [45–48], others have shown that treatment of early endosomal membranes with 0.5% trypsin essentially abolished fusion of these organelles in a well established in vitro assay [51]. In addition, the same authors have shown that the small GTPase Rab5, that regulates endosome–endosome recognition in vivo and in vitro is N-terminally cleaved with low trypsin concentrations to a 1kDa smaller polypeptide [51]. Therefore, it is desirable to have alternative methods to modulate the surface charges on specific organelles. Appropriate tools include lectins, specific antibodies, controlled use of proteases, or other enzymes, that allow subtle charge changes but interfere less or better not at all with the functional integrity.

In summary, it is unlikely that FFE will ever be a one-step technique for the purification of subcellular organelles, but in combination with other fractionation procedures it is a powerful addition to the approaches that can be applied and adapted for a particular organelle derived from a particular cell line or tissue.

In the next two sections we will discuss interesting developments and extended applications of FFE, including high resolution density gradient electrophoresis (DGE) and immune free flow electrophoresis (IFFE).

4.2. High resolution density gradient electrophoresis (DGE)

Density gradient electrophoresis has a long-standing record [52,53], but rather low resolution and long separation times were the rule. Basically, this method combines the principle of FFE with density gradients [9]. Recently, a modified method was developed for the isolation of endosomal/lysosomal membranes which is less time consuming and offered increased resolution [54]. After homogenization the organelle mixture is layered within a Ficoll or sucrose gradient. The gradient is separated by two electrodes, and upon electrophoresis, negatively charged membranes migrate upwards, while non-charged membranes lag behind in the gradient. As in FFE, endosomal and lysosomal membranes, being negatively charged, migrate towards the anode, and following fractionation these membranes can be recovered to a large extent separated from many other subcellular organelles [54]. Similar to classical gradient separation or FFE, this method has proven to be optimal for the purification of organelles that are part of the endosomal/lysosomal pathway. In addition DGE has allowed a dissection of distinct organelles involved in the generation of major histocompatibility (MHC) class II-peptide complexes [55,56]. DGE also separates efficiently EE from LE with the appearance of an intermediate compartment containing HRP after internalization from the medium [9,54]. Another interesting application is the recovery of phagosomes containing viable mycobacteria from unshifted fractions towards the cathode, separated from endosomes [57].

Though DGE effectively separates late endosomes/lysosomes from the bulk of other cellular constituents, careful analysis of the primary literature cited here revealed a problematic zone of poor resolution, analogous to the one described for classical gradient centrifugation [9,54,56]. As such, detection of EE by HRP activity, Golgi membranes by GalT activity Golgi, ER and plasma membrane by radioactive labeling revealed considerable overlap between all of them [56].

4.3. Immune free flow electrophoresis (IFFE)

Another extended version of FFE combines electrophoretic separation with the high specificity of antibody-coupling to an appropriate membrane-bound antigen. This concept was originally applied for cell separation (antigen-specific electrophoretic cell separation, ASECS) [58,59]. ASECS is based on the fact that immunoglobulins, at physiological pH values, are much less negatively charged than cellular membrane surfaces. The electrophoretic mobility of human lymphocytes was reduced by incubation with surface-antigen specific antibodies under non-capping conditions. This rendered sub-populations of human peripheral blood lymphocytes accessible to separation by free-flow electrophoresis and shifted cell populations could be separated with high yield, purity and vitality. Recently this method was adapted and modified for the separation of rat hepatic peroxi-

somes [60]. In this study crude peroxisomal fractions were prepared by differential centrifugation and organelles immunocomplexed prior to IFFE with an antibody directed against the cytoplasmic domain of the peroxisomal membrane protein PMP 70. Peroxisomes could be shifted away from mitochondria and lysosomes and appeared in electron microscopy as highly enriched, intact and as structurally well-preserved organelles. However, analysis of marker enzymes distributed across the electropherogram revealed a considerable contamination with microsomal esterase, an ER enzyme. [60].

In conclusion, although in principal appealing, there is only limited experience with IFFE and future experiments will have to prove if the method can be successfully adapted for other organelles too.

5. Flow cytometry and subcellular fractionation

Another alternative method for organelle purification, still under intense development, improvement and continuing change, is flow cytometry. In living cells intracellular organelles can be accessed and labeled from the outside and variables measured. However, a limitation of whole-cell measurements by FACS (fluorescence activated cell sorter) analysis is the lack of information regarding colocalization of marker proteins within subcellular compartments. This can be overcome by combining flow cytometric fluorescence analysis of individual intracellular organelles with conventional subcellular fractionation techniques.

The pioneering work of Robert Murphy has demonstrated the usefulness of flow cytometry for studying endocytosis on whole cells as well as at the level of a single organelle. Studies of binding, uptake, acidification and degradation of ligands including the identification and characterization of endocytosis mutants were performed with advanced flow cytometric and fluorimetric techniques [61–64]. Subsequently, several authors have adapted flow cytometry to sort and analyze intracellular organelles after labeling with fluorescent dyes and combined conventional subcellular fractionation techniques with high speed organelle sorting in a FACS. Labeling intracellular organelles, e.g. mitochondria, Golgi, ER, plasma membrane, phagosomes, endosomes, with fluorescent membrane dyes or fluorescently labeled ligands, allows purification due to biological properties rather than physical densities [10,13]. This new approach was named FAOS (fluorescent activated organelle sorting) [10].

For organelle sorting, sensitivity is obviously a major concern, since small structures, e.g. intracellular organelles, usually have only a small number of dye molecules associated with them. In general, commercially available flow cytometers have a threshold of detection in the range of several thousand dye molecules per particle [65]. Users with dedicated flow cytometers and/or special labeling protocols can increase sensitivity to single molecule detection [10,66]. Besides the physical properties of the dye (absorption coefficient, quantum efficiency) increased background signals can be a critical limitation.

A good example for organelle sorting are, once again, endosomes, since they can be accessed from outside the cell and loaded transiently with fluorescent membrane dyes or fluorescently labeled ligands under different conditions. Endosomes containing fluorescent signals can then be analyzed and purified based on their contents using flow cytometry [61–64,67,68]. Recently a protocol for the purification of endosomes from baby hamster kidney cells (BHK-21) that combines established subcellular fractionation techniques [20,69] with high speed organelle sorting in a flow cytometer was established [13].

Today a diverse array of cell-penetrating fluorescent stains that selectively associate with intracellular organelles or the cytoskeleton, in living cells, is available. Several of these probes are compatible with flow cytometric fluorescent instrumentation and have been successfully used for intracellular applications (for review see Ref. [10]). In addition, Mycobacteria expressing green fluorescent protein (GFP) of jellyfish *Aequorea victoria* were successfully used to study mycobacterial interactions with macrophages. These bacteria can be phagocytosed and, in combination with conventional subcellular fractionation, provided a powerful marker for the preparative sorting of phagocytic organelles [70]; a representative example is shown in Fig. 5.

Flow cytometry allowed the purification of a heterogeneous endosomal population of apical origin from rat renal cortex [71] and small-particle flow

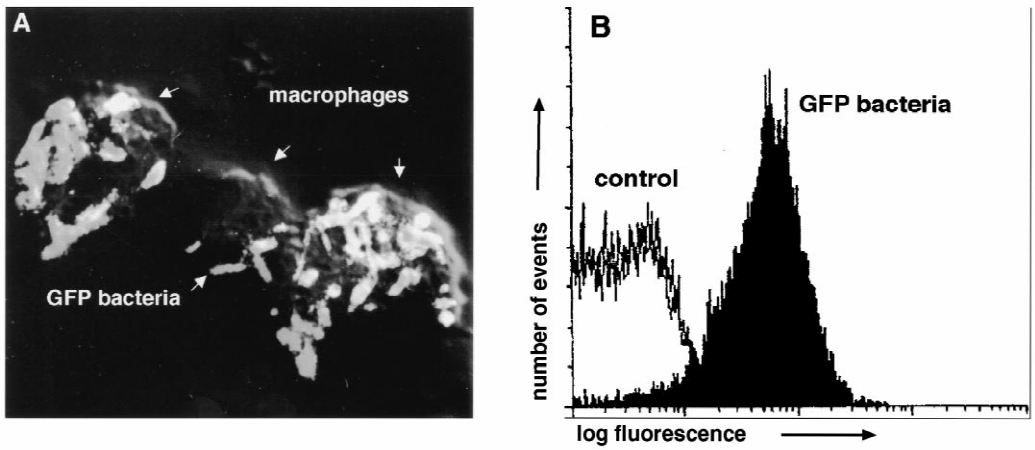

Fig. 5. Fluorescent activated organelle sorting (FAOS) of GFP-mycobacteria. J774 macrophages were infected with GFP-tagged *M. bovis* BCG and analyzed by confocal laser scanning microscopy (A). A representative confocal optical section of *M. bovis* BCG GFP-fluorescence was merged with the DIC image of the macrophage monolayer. Numerous GFP-labelled bacteria are present within the macrophages. (B) FAOS of GFP-tagged *M. bovis* BCG after homogenization of J774 macrophages. The graph representing analysis of input bacteria harbouring only the vector is also shown (line marked 'control'). Modified with permission from Dhandayuthapani et al. [70].

cytometry with fluorescein dextrans was used to enrich for vesicles from inter-microvillar clefts [72]. Furthermore, the labeling of Golgi cisternae with fluorescent lectins could be easily extended towards preparative sorting of *cis*- and *trans*-Golgi elements [73].

Flow cytometry offers an unique multiparametric tool to sort intracellular organelles after labeling with fluorescent dyes. However, experience with potential cross-contaminations is still limited and future experiments will show the feasability of it.

6. Conclusions

Subcellular fractionation and purification of organelles had always been a challenge for cell biologists. Most fractionation protocols take advantage of physical properties of intracellular membranes; e.g., their density or charge. Here we have discussed the most commonly applied experimental approaches for organelle enrichment concluding that it is almost impossible to purify a compartment to homogeneity. We can also conclude that certain organelles, such as endosomes can be more easily enriched than others. Interestingly, endocytic elements seem to be the ideal target for most of the techniques discussed here.

The combination of several approaches provides additional tools for increasing the resolution of separation. Preparative FFE and its satellite techniques provide a useful complementation to conventional centrifugation methods with important applications for the isolation of membrane compartments with charged surfaces; e.g., endocytotic compartments, clathrin coated vesicles, plasma membrane vesicles of differing charge, tonoplast membranes from plants and peroxisomes. Immunoisolation in combination with conventional gradient centrifugation has a successful record and flow cytometry could provide a sensitive and versatile tool for the preparative enrichment of rare organelle fractions.

The use of high resolution 2-DE gel electrophoresis uncovered the problem that is common to all of those techniques: considerable cross-contamination with other cellular membranes. However, working with 2-DE gels becomes a major advantage if many people are using them. Obviously, today we can take full advantage of the existing pool of information by matching our gels against protein patterns obtained by other groups and in public databases. Proteins that have been mapped already in one of the existing 2-DE-databases could simply be identified by the

help of computer-assisted gel analysis [74] or by co-migration experiments with a standardized sample mix from the database of choice [12]. Even though this approach is limited to experiments performed with the same standardized gel systems, immense information about contaminating organelles can be achieved [74]. Reference maps of different subcellular organelles will be available on database servers in the near future and the generation of specific ''organelle-finger-prints'' will be an important step towards our understanding and definition of subcellular membrane boundaries. Clearly, there are many promising techniques available and only the actual experiment will provide the only appropriate trial for selecting one or a combination of them.

7. Abbreviations

2-DE	two dimensional gel electrophoresis
ASECS	antigen-specific electrophoretic cell separation
DGE	density gradient electrophoresis
DIC	differential interference contrast
EE	early endosomes
ER	endoplasmic reticulum
ERGIC	ER-Golgi-intermediate-compartment
FACS	fluorescent activated cell sorter
FAOS	fluorescent activated organelle sorting
FFE	free flow electrophoresis
GalT	β-1,4-galactosyltransferase
GFP	green fluorescent protein
HM	heavy membranes
HRP	horseradish peroxidase
IFFE	immune free flow electrophoresis
LE	late endosomes
LM	light membranes
MHC	major histocompatibility complex
PNS	post nuclear supernatant
RER	rough endoplasmic reticulum
TGN	trans-Golgi network
VLM	very light membranes
VSV	vesicular stomatitis virus

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