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Review on Recent Advances in the Analysis of Isolated Organelles

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Abstract

The analysis of isolated organelles is one of the pillars of modern bioanalytical chemistry. This review describes recent developments on the isolation and characterization of isolated organelles both from living organisms and cell cultures. Salient reports on methods to release organelles focused on reproducibility and yield, membrane isolation, and integrated devices for organelle release. New developments on organelle fractionation after their isolation were on the topics of centrifugation, immunocapture, free flow electrophoresis, flow field-flow fractionation, fluorescence activated organelle sorting, laser capture microdissection, and dielectrophoresis. New concepts on characterization of isolated organelles included atomic force microscopy, optical tweezers combined with Raman spectroscopy, organelle sensors, flow cytometry, capillary electrophoresis, and microfluidic devices.

Keywords

Organelle isolation; organelle purification; subcellular; centrifugation; electrophoresis; dielectrophoresis; fluorescence; atomic force microscopy; microfluidics

1. Introduction

Isolation of subcellular compartments for analysis of their contents or function is widely used in bioanalytical chemistry and is the basis of many biomedical assays. Typically, cell homogenates prepared by mechanical homogenization contain a mixture of various organelle types that are then fractionated by procedures such as centrifugation. These organelle fractionation procedures usually yield fractions that are enriched in the organelle of interest. While these procedures have been well established since the 1950's, the growing demands in bioanalysis require better enrichment techniques, lower levels of contaminating organelles, isolation of biologically functional organelles, high-sensitivity analytical techniques compatible with low sample volumes, and in some instances, ability to prepare sub-fractions of a given organelle type.

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Following the first review on modern techniques for subcellular analysis that appeared in 1999 [1], our group published a review in 2005 on this theme [2]. To avoid duplication, this report covers developments on the isolation and characterization of isolated organelles that appeared during the 2006–2011 period. A Web of Science search using the terms “Organelle Analysis” or “Subcellular Analysis” during this time period yields more than 7900 hits. A large number of these hits refer to the use of existing and established technologies and are not covered here [3]. Instead the focus of this review is on novel, improved, and innovative methods that focus on the release of organelles from their cellular milieu, both fractionation and separation of isolated organelles, and characterization of such organelles. We put more emphasis on emerging, less-traditional techniques that are already in use and those that show potential for future applications of organelle isolation, fractionation, and analysis.

One of the most important applications of subcellular fractionation is in proteomics. We do not review the developments in this field because excellent reviews focused on both plant [4–6] and mammalian proteomics [7, 8] have appeared recently. We will not cover reports on subcellular imaging because there are multiple recent reviews on the topics of colocalization in images [9], throughput of imaging techniques [10, 11], secondary ion-mass spectrometry [12], 3D imaging [13], technique comparisons [14] and recent developments on direct organelle analysis by mass spectrometry [15].

2. Methods for organelle release

Organelle release is the first step in the preparation of subcellular fractions. Procedures used to release organelles from their cellular milieu include: mechanical homogenization, nitrogen cavitation, chemical disruption, and electrical disruption. Salient developments on this topic include methods that provide better control and reproducibility of organelle release [16], methods specific to unique organelle types [17–20], and methods compatible with the observation of single organelles after their release [21].

Gross et al. applied the use of a PCT Shredder followed by a barocycler to release mitochondria from rat kidney and skeletal muscle tissue without exposing the tissue to the sheer forces caused by traditional mechanical homogenizers [16]. The PCT shredder works by pushing tissue through lysis discs instead of compressing the tissue as done in traditional grinding. Only a single pass through the PCT-shredder was needed for mitochondria release. While the PCT shredder has been well established for the release of proteins, RNA, and DNA, this method shows the first application of the PCT shredder for organelles. The barocycler works by changing the pressure applied to the sample in cycles that alternate between atmospheric and high pressure. For the release of mitochondria, only ~2 minutes is needed with maximum pressures of 10,000 psi. The PCT shredder and traditional homogenization produced mitochondria with the same proteomes, membrane potentials, and sequestered calcium levels. When the PCT shredder was used in combination with the barocycler, the yield of isolated mitochondria matched that of traditional homogenization. As assessed with transmission electron microscopy, the released mitochondria had the same morphology. Successful preparations of mitochondria from two very different tissues, mouse kidney and skeletal muscle, showed that the procedure is applicable to multiple tissue types. Since only a single pass through the lysis disk of the PCT shredder was needed for isolation, the chances to over-homogenize the mitochondria were also decreased. While this procedure only matches the results of traditional tissue homogenization, further developments in the use of the PCT shredder in combination with the barocycler could improve the quality of organelle preparations.

Schlesinger et al. reported a unique method for releasing nematocysts from sea anemones [17]. Nematocysts found in cnidarians (e.g., jellyfish, coral, anemones) are organelles that

store and deliver venoms. These venoms are attractive targets for drug design. Due to the variability within cnidarians and the small amount of venom within a nematocyst, large amounts of nematocysts from a single cnidarians need to be collected to study the venom. In this report, the authors demonstrated the release of nematocysts from the excrement of the snail *S. neapolitana* that feeds on sea anemones. Their excrement contains intact nematocysts and are collected, pooled, filtered, and separated with density centrifugation [22]. This method yielded large amounts of nematocysts while avoiding tedious tissue dissection of sea anemones. Even though the method described is very specific for nematocysts in cnidarias, its large increase in organelle yield and decrease in time spent dissecting tissue is noteworthy.

Isolation of the plasma membrane is particularly challenging because it spans the entire cell surface. Techniques such as cavitation break the plasma membrane into shards and do not allow for isolation of intact membranes. An alternate method by Bezrukov et al. isolated plasma membranes using cellular adhesion [18]. Glass plates were incubated with poly-lysine that strongly promotes cell adhesion. Mouse embryonic fibroblasts were cultured directly on the plates and disrupted with ice-cold water washes. There were neither additional chemical nor mechanical processes needed to release the plasma membrane from the cell. This procedure left the plasma membrane attached to the glass, which was then analyzed by microscopy and treated with a 2:1 chloroform:methanol mixture water to extract lipids, including cholesterol. This isolation technique allows for a high yield of plasma membrane while keeping its structure intact. This also allows for easier biochemical analysis of the membrane due to the high yield with the additional bonus of morphological features being explored due to the intact plasma membrane on the glass surface.

Polarized cells have specialized membranes at different surfaces. For instance, apical membranes face the lumen in endothelial cells. Selective release of apical membranes is practically impossible using conventional techniques. For example, the chemical release with polyethylene glycol often results in enriched fractions containing membranes from other parts of the cell. Fong-ngern et al. released apical membranes by layering Whatman filter paper or cover slips onto canine kidney epithelial cells and then peeling them off [19]. When the paper or slip was peeled from the cell culture, it removed the apical membrane with it. Western blot analysis revealed that paper was better than coverslips in the removal and purity of the apical membranes. However, both methods were suitable to prepare membranes for direct imaging or determination of the chemical makeup in different parts of the membrane [18, 19]. Similar methods may be suitable to isolate and enrich apical membranes from other cell lines as well.

Symbiosomes refer to associations of cells from two different organisms. Release and analysis of membranes defining the interface between the two organisms is needed to understand the basis of symbiosis. Traditional homogenization techniques are not effective at isolating the organelles of symbiosome cells due to their high resistance to disruption. Previously, Trautman *et al.* developed a method to prepare symbiosome membranes at low yield [23]. Kazandjian et al. improved on this method releasing large amounts of intact symbiosome membrane from *Z. robustus* while maintaining the purity levels previously achieved [20]. Symbiosomes were exposed to mechanical disruption using very high shear forces. Centrifugation methods were then used to enrich the membrane fractions into a highly pure fraction as indicated by Western blot analysis. This method may be useful to investigate other symbiotic interactions.

Investigating subcellular heterogeneity requires carrying out measurements at the individual organelle level. One advance in this area was reported by Kometani et al. who developed nano-tools to release single chloroplasts from individual *E. densa* leaf [21]. The main

features of the nano-tools were a cell wall cutting tool and a filtering unit to collect organelles. Scanning and transmission electron microscopy revealed nano-tools were indeed effective at isolating single chloroplasts. To our knowledge, this is the first organelle release device on the nano-scale. Future nano-tool designs may be adapted to isolate other plant organelles and characterize their heterogeneity. While the throughput of the method is currently limited due to the time it took to collect organelles for analysis, its potential for determining biological heterogeneity among chloroplasts and other plant organelles is high.

3. Organelle Fractionation

Organelle fractionation is commonly based on centrifugation protocols such as differential centrifugation and density gradient centrifugation [24–29]. However, it may also be accomplished by magnetic capture [30–37], free flow electrophoresis [38, 39], flow field-flow fractionation [40], fluorescent organelle sorting [41, 42], laser capture microdissection [43], and dielectrophoresis [44]. Because fractionation protocols have been reviewed recently [1, 7, 45], here we include only reports that were not included in these reviews. A summary of these reports is included in Table 1.

3.1 Centrifugation

Centrifugation remains a traditional and often reliable method for enriching organelle fractions. However, the method can be time consuming and does not always result in a pure fraction as in the case of mitochondria-associated membranes. Reports on subcellular fractionation based on centrifugation that are included in this review comprise description of organelle-specific procedures [24–27], utilization of a single protocol to isolate two different organelle types [28], and systematic optimization of procedures for preparation of isolated mitochondria [29].

The endoplasmic reticulum and mitochondria commonly associate within each other to participate in processes such as lipid synthesis [46]. Investigation of such processes requires isolation of mitochondria with bound vesicles originating from the endoplasmic reticulum called mitochondria-associated membranes (MAM). Most methods used to prepare MAMs are not suitable because they produce fractions containing both MAMs and mitochondria [47]. Wieckowski et al. adapted a previously developed method from rat liver mitochondria to fractionate MAMs from cell cultures [24]. They demonstrated that, with properly designed sequence of centrifugation steps and large volumes of cell cultures, fractions containing enriched MAMs could be prepared in only 2–3 hours. Western blot analysis of the enriched MAM fraction showed MAM and endoplasmic reticulum markers and the absence of contaminating mitochondria, cytosol, and nuclear markers, confirming the success of this preparation procedure.

With the study of autophagy becoming increasingly important and to determine its role in subcellular function, it is important to have reliable techniques for autophagy related organelle fractionation. Autophagosomes are organelles that degrade subcellular components. Isolated autophagosomes and other autophagy-related structures are difficult to separate in high purity [48]. Seglen et al. optimized a centrifugation protocol for the isolation of autophagosomes after treatment of rat hepatocyte cell culture with vinblastine, which halts autophagosome disappearance and reduces formation of contaminants such as amphiosomes [25]. This procedure is well suited to increase the yield and purity of fractionated autophagosomes.

While most studies have focused on animal cells, Takatsuka et al. reported the first fractionation of autolysosomes from tobacco seedling cell cultures [26]. Autolysosomes are formed from the fusion of autophagosomes and lysosomes [49]. The autolysosomal fraction

was identified by the presence of marker enzymes for acid phosphatase and vacuolar H^+-ATPase as well as transmission electron microscopy. Trace contaminations of microbodies and mitochondria may be present in the enriched fractions, if at all. The fractionation of autolysosomes is an important advancement for future studies on factors affecting autophagosome-lysosome interactions.11

Secretory lysosomes are specialized lysosomes of some cell types that, upon external stimulation, participate in simultaneous cellular secretion and degradation. Schmidt et al. used centrifugation to fractionate secretory lysosomes from hematopoeitic cells [27]. Previous complications due to the presence of silica-based density gradient material [50] were eliminated when using a density gradient made of Iodixanol. Electron microscopy revealed that the majority of organelles maintained their wide-range morphologies, while Western blot analysis demonstrated decreased levels of contaminating Golgi, mitochondria, and peroxisome markers. This appears to be the first report specific for secretory lysosome enrichment from both cell culture and tissues.

Most subcellular studies on algae have been based on isolation of organelles from cell cultures. Lang et al. described the first procedure to obtain both chloroplasts and two different densities of mitochondria fractions from algal tissues with high yields [28]. Western blot analysis was used to confirm the purity of both fractions. The authors suggest that isolation of both chloroplast and mitochondria directly from tissue is of tremendous advantage to understand the biochemistry and metabolic pathways of algae.

With increasing numbers of studies on brain tissue such as organelle makeup and organelle-organelle interactions, it is critically to have reliable means of fractionating mitochondria originating from brain. However, most of the preparations based on Percoll density gradient centrifugations still contain large amounts of both synaptosomes and myelin. A key development to decrease the levels of these impurities was the use of discontinuous Percoll gradients [51]. Based on this study, Sims and Anderson described several centrifugation protocols for the fractionation of rat brain mitochondria that are adaptable to meet the needs of many studies using brain mitochondria [29]. The authors note that the use of digitonin increases the mitochondria yield by separating the mitochondria from the biological matrix and reduces contaminant levels. Its use compromises the outer mitochondrial membrane, however, and leaves the mitochondrial non-function for some studies. The authors provide several fractionation techniques that are optimized for many variables: This reference is an excellent guide to develop and optimize mitochondria preparations from neurons using centrifugation because it considers variations in the starting amount of brain tissue, time allowed for fractionation, desired level of mitochondrial function, and the target yield of the preparation.

3.2 Affinity Purification

Although affinity purification of organelles has been practiced for many years [52, 53], a limiting factor for their widespread use has been that affinity purifications rely on the availability of antibodies against proteins found on the surface of organelles of interest. The recent increase in the number of suitable antibodies and the ability to assess the functional status of the immunopurified organelles has begun changing this landscape. Most of the recent reports use a magnetic field to retain organelles that bind to the antibodies attached to magnetic beads. After washes and removal of unwanted contaminants, the magnetic field is removed to recover a fraction containing the organelle of interest. In the period reviewed here, salient reports on affinity purification of organelles included those used to isolate plasma membranes [30], synaptic vesicles [54], chloroplasts [32], mitochondria [33], peroxisomes [34], and lysosomes [35].

Lawson et al. enriched plasma membranes from rat liver and two different hepatocellular carcinoma cell lines using immuno-affinity [30]. The inspiration for this report was a previous report by Chang et al., who enriched neutrophil plasma membranes using a similar technique [55]. A crude preparation of plasma membranes was incubated with magnetic beads coated with monoclonal antibodies specific for membranes proteins. After enrichment the immunocaptured material was solubilized with a detergent. The membrane proteins present in the enriched fraction were characterized by Western blotting, mass spectrometry, and gel electrophoresis. The method produced a more pure fraction and with a higher yield when compared to other protocols designed for isolation of plasma membranes [56–58].

The immuno-purification procedure for fractionation of synaptosomes from adult rat brain was first published by Mariano et al. [54] and utilized shortly after in a follow up study by Burre et al. who characterized enriched synaptosome preparations with SDS-PAGE [31]. They incubated a crude synaptosomal fraction enriched with sucrose density gradient centrifugation with magnetic beads coated with anti-synapsin I antibodies. Western blot analysis of the enriched fraction confirmed the enrichment of the synapsin I isoform. There was no detectable contamination from other organelles such as endoplasmic reticulum and peroxisomes.

Immuno-purification of organelles may also be accomplished using antibodies that target proteins that are non-native to the organelles. Truernit et al. described the first affinity purification of chloroplasts expressing yellow fluorescent protein-OEP-14 (YFP) on the surface of *A. thaliana* and *N. tabacum* leaves [32]. For immunopurification they used magnetic beads coated with anti-YFP antibodies. The authors expressed YFP in only certain types of cells thereby selectively isolating chloroplasts from only those cell types. The overall yield was high, there was no visible contamination from other organelles, and close to a half of the organelles had their double membranes intact. These are impressive results when compared with previously reported isolations of chloroplasts. Most importantly, this study shows that in the absence of a native protein, a genetically engineered protein designed to target the surface of a specific organelle type could be expressed and then be targeted via immunopurification.

Hornig-Do et al. immuno-purified mitochondria for the first time from human embryonic kidney epithelial, human cervix epithelial, and human osteosarcoma cell culture lines using superparamagnetic beads coated with anti-TOM22 antibodies [33]. This procedure did not require preliminary differential centrifugation steps, produced an enrichment similar to those obtained with standard centrifugation procedures [59, 60], and was completed in 1–2 hours. Flow cytometry reported a 2–4 fold enrichment in mitochondria. The purity of the enriched mitochondria fraction was assessed with Western blotting. When compared to a mitochondria fraction prepared with standard centrifugation techniques, the immuno enriched fraction had a similar contamination from the nucleus, endoplasmic reticulum, Golgi, and endosome to that of density gradient centrifugation and an improved enrichment compared to differential centrifugation as assessed with Western blotting. The immuno-based fractionation was much faster compared to the centrifugation based fractionations. The yield from the immuno-based fractionation was 2–4 fold increased compared to either centrifugation method.

The use of labeling peroxisomes with antibodies to facilitate their magnetic enrichment was first introduced by Luers et al. [61]. In this early study, peroxisome subpopulations labeled with anti-PMP70 antibodies bound to magnetic beads were separated from non-labeled organelles in a free-flow magnetic separation device. The enriched fraction was confirmed to be peroxisomal with scanning electron microscopy and immuno-histochemistry of peroxisome markers. This report did not determine if the peroxisomes were metabolically

active. Using the same antibodies Wang et al. immuno-purified peroxisomes from cultured L6 rat myoblasts [34]. Cell lysates were treated with magnetic beads coated with the anti-peroxisome antibodies. After labeling, peroxisomes were magnetically captured while the non-magnetic organelles were simply separated by aspiration of the liquid. They confirmed that the enriched peroxisomes were metabolically functional as indicated by β-oxidation metabolism of palmitoyl-CoA. Peroxisome fractions were deemed to be highly enriched as contamination from mitochondrial was not detectable and the lysosomal contaminants were 60–70 fold reduced compared to the unretained fraction. While Wang's assay is an easy and time-effective fractionation method, the different subpopulations of peroxisomes cannot be fractionated from each other. An interesting future application of this work would be to investigate the metabolic properties of peroxisomes fractionated via the method by Luers et al. to determine if peroxisome-specific metabolism varies between the peroxisome subpopulations.

Nylandsted et al. enriched lysosomes using immunopurification with anti-V-ATPase antibodies for the first time [35]. This was possible because of the recent discovery of V-ATPase's expression on the surface of lysosomes [62]. Antibodies were treated with a crude fraction of human breast carcinoma cell culture line. The organelles retained within a magnetic column were acidic in nature. The subcellular localization of enriched proteins was largely from lysosomes and also some endosomes as indicated from proteomic analysis of the recovered samples. Only one mitochondria-related protein was observed and there was no detection of any Golgi, plasma membrane, and the endoplasmic reticulum integral membrane proteins, suggesting that this procedure is adequate to purify lysosomes.

The magnetic enrichment of lysosomes with endocytosed magnetic nanoparticles have been previously enriched on mesh, magnetic columns [63]. This early study did not determine if magnetically retained organelles had the acidic pH characteristic of late endosomes and lysosomes. To further investigate the pH status in magnetically isolated endocytic organelles released from cultured rat myoblasts after endocytosis of dextran-coated iron oxide, Satori et al, captured organelles with a magnetic cargo as these flowed in a magnetic flow-through device [36]. Following introduction of the sample, buffer was passed through the tube to remove any retained, non-magnetic material. Finally, the magnets were removed and additional buffer was passed through the tube to elute the retained fraction. Peroxisomal and mitochondrial contaminants were not detected in the retained fraction. The analysis of individual organelles by laser induced fluorescence detection revealed that the retained organelles indeed had an acidic pH. Future adaptations of this method may be used to investigate functional properties of acidic organelle subpopulations, which may be separated by coupling of the setup for magnetic retention with devices designed for free flow electrophoresis or isoelectric focusing studies.

3.3 Free flow electrophoresis and Flow Field-Flow Fractionation

Free flow electrophoresis (FFE) is a continuous separation technique that delivers organelles with different electrophoretic mobilities to different collection points. An electric field, applied perpendicularly to the direction of a laminar flow profile, causes displacement of the organelles along the electric field. This displacement is related to the organelles' surface properties that are associated with their electrophoretic mobilities. Thus, FFE can be used to separate and identify different organelles subtypes possessing different electrophoretic mobilities. As reviewed by Islinger et al, FFE has been used previously as an analytical tool to separate organelles and organelle subpopulations [64]. Two reports in this review that appeared in the time period covered here illustrate the potential of FFE to enrich organelles and separate organelles into subpopulations : (1) FFE to enrich peroxisomes [38] and (2) FFE to separate subclasses of mitochondria [39].

Previously, peroxisomes has been labeled with anti-PMP70 antibodies prior to the FFE separation [65]. This method, termed immuno free flow electrophoresis, was first introduced in 1997 to separate peroxisomes labeled with the antibodies from the rest of a post-nuclear rat liver homogenate prepared by centrifugation [66]. By specifically labeling density-gradient centrifugation-enriched peroxisomes with antibodies, their electrophoretic mobilities were changed and no longer overlapped with the mobilities of unlabeled contaminating organelles, making it possible to collect peroxisomes into a fraction with lower levels of contaminants. A caveat of such method was that prior to FFE, the cellular material was prefractionated into two separate differential centrifugation fractions which required separate FFE analysis that in turn had low yields. The low yields impeded a comprehensive characterization of the peroxisomes after FFE. More recently, Islinger et al. separated peroxisome subpopulations from rat liver tissue by FFE, without using labeling with anti-peroxisomal antibodies prior to the FFE separation [38]. Surprisingly, the recovered peroxisomes had minimal contamination from other organelles (3.4% mitochondria, 0.4% lysosomes, 4.7% microsomes). FFE also showed the presence of various subpopulations with sufficient yield for further analyses. Subpopulations were characterized based on Western blotting for the presence organelle markers, transmission electron microscopy for morphological changes, and mass spectrometry to determine proteins present. While the peroxisomes did appear to have similar morphologies, Western blotting and mass spectrometry indicated proteins were expressed differently in both fractions.

The second salient report was by Zischka et al. who analyzed yeast mitochondria using FFE [39]. They observed that subpopulations appeared when mitochondria experienced alterations in respiratory activity, hyperosmotic stress, or controlled proteolysis. Although the subpopulations were not fully separated, mitochondria with diminished respiratory activity clearly had different electrophoretic mobilities from those of normally respiring mitochondria. Because mitochondria were recovered in sufficient yield for further analysis, these fractions were analyzed by transmission electron microscopy and Western blotting. Further optimization of the FFE fractionation of mitochondrial subpopulations could make possible additional studies characterizing the biomolecular composition of mitochondria subpopulations.

Field-flow fractionation (FFF) is another promising technique for the separation and enrichment of organelles because it performs similarly to centrifugation-based techniques but does bulk fractionations of organelles without centrifugation mediums (e.g. Percoll). Similar to FFE, a laminar flow elutes analytes into an open tube. A cross flow pushes analytes perpendicular to the laminar flow against the side of the wall. The analyte also experiences a diffusion force, pushing it away from the wall. Different analytes will find their equilibrium between the diffusion and cross flow forces and are eluted at different locations of the side of the open tube [67]. Kang et al. reported the first FFF study to enrich mitochondria from rat liver with sufficient yield for additional analyses [40]. They used frit-inlet asymmetrical FFF to isolate four different fractions of mitochondria of different diameters and protein profile. Eluted fractions contained mitochondria with different morphological properties such as shape and area as determined with fluorescence microscopy. The first two fractions appeared to have similar mitochondria which were small. Mitochondria size increased in fraction 3 and additionally in fraction 4. Mitochondria homogenate from each fraction was analyzed further by two-dimensional gel electrophoresis and liquid chromatography coupled to mass spectrometry. The protein profiles of the first two and last two fractions were qualitatively very similar as assessed with SDS-PAGE. Unfortunately, all the fractions also had large amounts of protein from contaminating organelles.20

3.4 Fluorescence activated organelle sorting

Fluorescence-activated organelle sorting (FAOS) uses a flow cytometer to detect and sort organelles with specific fluorescence and scattering characteristics [68–70]. The technique requires organelle specific fluorescent probes including chemical reagents, fluorescently-labeled antibodies and fluorescent proteins. The latter is only applicable to cell cultures and animal models in which fluorescently proteins have been expressed. Here we present two salient examples using FAOS to fractionate secretory granules [41] and vesicles [42].

Gauthier et al. enriched dense-core secretory granules from mouse anterior pituitary cell culture using FAOS [41]. In their study, the instrument's flow pressure was lowered to minimize shear and tear forces on the organelles. A GFP-fusion protein was expressed and targeted with FAOS. Western blot analysis confirmed that the fluorescently sorted fraction had low levels of contaminations of other organelles such as Golgi, nuclei, and mitochondria. When compared to density gradient centrifugation methods, this protocol was completed in a shorter amount of time (less than one hour) and needed less starting materials. Furthermore, this is the first application of FAOS to secretory granules.

The G2A Naked2-associated vesicles are a type of low abundance exocytic vesicles that are transient due to fusion with other subcellular membranes. Due to their transient nature and low abundance, their fractionation has been challenging. Cao et al. used FAOS to isolate G2A Naked2-associated vesicles from cultured canine kidney epithelial cells for the first time [42]. By expressing GFP-labeled Naked2 protein and labeling with a lipophilic tracer, FAOS enriched organelles that had this dual label. Initially, only 64–74% of the peaks were dual labeled. Following FAOS, 99% of the peaks were dual-labeled. Furthermore, electron microscopy confirmed that the isolated material were indeed vesicles of uniform size.

3.5 Optical tweezers and laser capture microdissection

Optical tweezers (OT) apply an infrared laser beam at a target object to manipulate its position. Differences in the refractive index between the laser and the target object trap the target in the laser beam and allows for its manipulation. Laser capture microdissection (LCM) has been traditionally used to capture single cells from tissue [71–75]. In LCM, a laser is focused onto a target covered by a thermoplastic film that melts the film trapping the cell of interest. Pflugradt et al. recently extended the use of these techniques to individual organelles and compared performance of FAOS, LCM, and OT to fractionate mitochondria from a mixture of murine and porcine liver mitochondria [43]. The assessment of a single mitochondrial fractionation from FAOS, LCM, and OT was based on the detection of the polymerase chain reaction product specific to mitochondrial DNA from either porcine or murine liver detected by real time-quantitative polymerase chain reaction. Detection of one and only one type of mitochondrial DNA was indicative of successful single mitochondrion fractionation. While all three methods were able to fractionate single mitochondria, OT had the most success, i.e. highest yield. OT was also the slowest, compared to LCM and FAOS. Easier control of fractionation was facilitated by optical control, used by both OT and LCM. Depending on the priorities of the type of experiment being performed, this paper provides an excellent discussion on which of the emerging techniques (OT, FAOS, or LCM) is the best choice for single mitochondria fractionation.

3.6 Dielectrophoresis

Dielectrophoresis (DEP) uses an inhomogeneous electrical field to create a dielectrophoretic force, which depends on the polarizability of the analyte. DEP has been used at length to separate cells [76] and to separate mitochondria from nuclei, however, separations of organelles of similar size, e.g. mitochondria and peroxisomes, were not successful. Furthermore, DEP has been done in microchips that do not provide sufficient material for

organelle analyses [77]. Moschallski et al. described the first separation of mitochondria from other organelles of similar size using a dielectrophoresis device that provided sufficient material for subcellular analysis [44]. Using mitochondria released from cultured human lymphomablastoid cells, their approach offered a 2.5-fold enrichment of mitochondria relative to the cell homogenate, a 6-fold decrease in endoplasmic reticulum-related proteins and no detectable levels of lysosomes. Furthermore, their protocol produced micrograms of enriched organelles, sufficient for multiple analysis e.g. SDS-PAGE and Western blotting.24 Because changes in the shape of the AC field, its intensity, and its frequency, this technique has high tunability and great potential for separating different organelle types. On the other hand, there are several difficulties that must be overcome. They are: device clogging, joule heating due to high salt content, and selection of buffers that are both suitable for DEP and organelle stability.

4. Characterization & detection

Classical methods for the characterization of organelles included qualitative methods, such as those based on Western blots and enzymatic marker assays, and morphological methods such as those based on scanning electron microscopy. These methods will not be reviewed here. In this review we focus on techniques that were more recently introduced. These include atomic force microscopy [78–80], optical tweezers [81, 82], organelle sensors [83], flow cytometry [84, 85], capillary electrophoresis [36, 86–98], and microfluidic approaches [99–101]. The reports included in this review are summarized in Table 2.

4.1 Atomic force microscopy

Atomic force microscopy (AFM) uses a nanometer-size tip to probe and analyze surfaces, thus producing 3D images with sub-molecular resolution. AFM has been successfully applied to study properties of DNA, proteins, bacteria, viruses, cells and organelles [102].

Lee et al. used AFM for the first time to characterize morphological and nano-mechanical changes in isolated rat heart mitochondria after myocardial infarction [78]. By topographic and force-stance curve measurements, the authors found that ischemic stimuli led to mitochondrial swelling and decrease in adhesion forces. This brings new insights on morphological changes that occur to the outer mitochondrial membrane during apoptosis.

Awizio et al. developed a non-destructive AFM method to probe shape, size and stiffness of synaptic vesicles from rat brain [79]. They compared the properties of vesicles with and without synapsin I. Vesicles with synapsin I were usually larger, had a broader size distribution, and had an increased tendency to cluster. These results suggest that electrostatics is the predominant stabilizing force in these vesicles.

Guo et al. characterized age-related changes in topography and mechanical properties of retinal pigment epithelium (RPE) melanosomes [80]. The RPE melanosomes have a unique elliptical shape (1 – 2 μm in length, 0.8 μm wide) and are responsible for synthesis and storage of melanin. RPE melanosomes from elder donors showed significantly higher adhesion to the AFM scanning tip compared to those from young donors. This was attributed to the presence of lipofuscin on their surface. These findings demonstrate the potential of AFM to characterize surface changes in isolated organelles.

4.2 Optical tweezers

Optical tweezers refers to the use of highly focused laser beams to create force fields capable of capturing and manipulating nanometer- and micrometer-size objects. The Raman spectra of objects that are manipulated with IR laser-based optical tweezers can be obtained without needing a second laser. Tang et al. published the first application of optical tweezers

and Raman spectroscopy to examine physiological changes in individual mitochondria from rat liver, kidney, and heart [81]. A single mitochondrion was optically trapped and its Raman spectrum was collected in real time. The authors observed spectral changes resulting from calcium-induced mitochondria swelling. This technology may be suitable to study changes in mitochondrial composition and physiology in response to drug treatment and toxin exposure.

Reiner et al. used optical tweezers to extract a single mitochondrion from a human peripheral blood myeloblastic cell and then deliver it for PCR analysis [82]. One cell labeled with the mitochondria stain Mitotracker Green was lysed with a pulsed UV laser. An IR-laser was used to select and trap a mitochondrion that was delivered to a femto-pipette for PCR. The authors determined that the single mitochondrion had both mutated and wild-type mitochondrial DNA.

4.3 Sensors

Quarato et al. monitored membrane potentials in individual mitochondria [83]. They deposited isolated mitochondria from mice liver onto a poly-lysine coated glass and incubated the mitochondria with the membrane potential probe tetramethylrhodamine ester (TMRE). The fluorescence signal of each mitochondrion was detected using laser scanning confocal microscopy. By measuring multiple mitochondria, they were able to describe basal heterogeneity of the mitochondria and the effects of substrates and inhibitors on the membrane potential of mitochondria.

4.4 Flow cytometry

Flow cytometry is used to detect fluorescent and scattering properties of particles as they are hydrodynamically focused through a laser beam. The technique is high throughput as it can analyze up to thousands particles per second [103]. It has been commonly used to analyze microbes [104], cells [105], and organelles [106]. There were two salient reports during the review period.

Yang et al. used flow cytometry to describe the heterogeneity in the membrane potential of maize leaf mitochondria [84]. After staining with Rhodamine 123 flow cytometry revealed two distinct mitochondrial sub-populations. Further analyses showed that these populations differed in their mass and DNA content. This report suggests that different mitochondrial sub- populations may have different roles during the maize leaf development.

Hu et al. used flow cytometry to characterize the membrane potential of mitochondria in two types of Honglian cytoplasmic male sterility (HL-CMS) rice seedlings [85]. They identified mitochondrial subpopulations with high or low membrane potential as revealed by the fluorescence intensity of rhodamine 123. The abundance of the high membrane potential population increased with the higher ROS content in the plants. This report brought new insights into the role of mitochondria subpopulations in crop breeding.

4.5 Capillary electrophoresis techniques

Electrophoretic separations provide the electrophoretic mobilities of organelles, which are determined by their size, surface charge density, and overall morphology as well as the ionic strength of the separation medium [107]. In capillary electrophoresis (CE), the separation takes place in a narrow bore fused silica capillary using high electric fields. CE separations are rapid, have high separation efficiency, and consume small volumes of samples and buffers. Because of its sensitivity, laser induced fluorescence (LIF) has been the detector of choice for CE analysis of fluorescently labeled organelles, making it possible to detect

individual organelles. The combination of CE and LIF, termed CE-LIF, describes both fluorescent and surface charge density properties of individual organelles.

Poe et al. compared flow cytometry and CE-LIF for the analysis of mitochondria from rat muscle [86]. The signal-to-noise ratio for mitochondria labeled with nonyl acridine orange was similar for both techniques. Flow cytometry was superior in sample throughput. CE-LIF was superior in sample requirements: while CE-LIF required 500 picoliters of sample, flow cytometry used 24 microliters. Furthermore, CE-LIF described the electrophoretic mobility of mitochondria. CE-LIF is the method of choice for the analyses of small volume samples and for characterizing surface properties of organelles.

Andreyev et al. described a dual light scattering, LIF detector for CE analysis of individual mitochondria from rat muscle [87]. By combining fluorescence and scattering plots, the authors detected changes in the mitochondrial morphology caused by their cryogenic storage. This was not possible by observing just fluorescence intensity.

Reliable determinations of organelle electrophoretic mobilities require capillaries with negligible interactions between their walls and the organelles. Covalent coatings with hydrophilic polymers, such as poly(acroylaminopropanol), are the most effective way to minimize these unwanted interactions, but their preparation is more cumbersome. In order to simplify the coating procedures, Whiting et al. dynamically coated capillaries with poly-vinyl alcohol (PVA) for the separation of mitochondria from both mouse and rat liver [88]. They showed that the PVA-coated capillaries can be successfully used for the analysis of both mitochondria without significant carryover.

The CE-LIF analysis of organelle samples with a high density number of organelles over time results in organelles being detected simultaneously (peak overlap), which biases the interpretation of fluorescence intensities and electrophoretic mobility data [89]. Davis and Arriaga used statistical overlap theory to predict conditions under which peak overlap of individual organelles is negligible [90]. A simple calculation procedure requires only information about the number of peaks observed in a defined region of an electropherogram and the standard deviation of peak intensity in order to predict if the peak overlap affect the results.

Isoelectric focusing separates analytes based on differences in their isoelectric point (p*I*). Wolken et al. described for the first time the measurement of isoelectric points of individual mitochondria fractionated from rat skeletal muscle myoblasts using capillary isoelectric focusing with LIF detection [91]. A mixture of mitochondria labeled with nonyl acridine orange and low molecular weight p*I* markers were focused in the pH gradient and then mobilized. The p*I* of individual mitochondria was determined using the p*I* markers as standards. The resolution of the method was 0.03 pH units. This method could be equally applicable to other organelle types.

Dual channel fluorescence detection extended the possibilities of CE-LIF analysis of individual organelles. Three different approaches reported here include the analysis of mitochondrial DNA [92, 93], cytoskeletal-mitochondrial binding [94], and pH of organelles [36, 95].

Navratil et al. reported on a method for the quantitation of the absolute mitochondrial DNA content in individual mitochondria fractionated from rat skeletal muscle myoblasts [92]. The authors identified mitochondria because of their red fluorescence resulting from the DsRed2 protein. pDsRed2-Mito was genetically engineered to localize in the mitochondrial matrix. Mitochondrial DNA was detected and quantified using the intercalating dye PicoGreen. They reported that the some mitochondria have one single copy of DNA, while most of

them have multiple copies. Further development of this approach incorporated a collection of mitochondria after LIF detection, which made possible to carry out quantitative PCR for quantification of mutated and wild-type mitochondria DNA [93].

Cytoskeleton supports the mitochondria network of cells. When mitochondria are isolated they may remain tethered through cytoskeleton fragments, which complicated individual organelle analysis. Kostal et al. characterized binding of cytoskeleton remnants to the surface of isolated individual mitochondria fractionated from rat skeletal muscle myoblasts using dual detection CE-LIF [94]. Mitochondria expressing DsRed2 in their matrix were labeled with Alexa488-phalloidin, a fluorescently tagged dye that selectively binds to F-actin, a cytoskeletal protein. The authors found that 79% of isolated mitochondria did not contain detectable levels of cytoskeleton on their surface, while the remaining 21% contained on average about 2 zeptomoles of F-actin.

Chen et al. described for the first time the determination of pH in individual acidic organelles isolated from human peripheral blood lymphoblasts using dual detection CE-LIF [95]. After endocytosis of the ratiometric dye fluorescein tetramethlrhodamine dextran (FRD), separate detection of fluorescein (pH sensitive) and tetramethylrhodamine (pH-insensitive) made possible to calculate a fluorescence ratio for each detected organelle. The ratio for each organelle was compared against a pH calibration curve that used FRD. The authors observed that individual acidic organelles from drug resistant cells have lower pH than those from drug sensitive cells. Subsequent work using the same strategy established that magnetically purified endocytic organelles maintain their expected acidic pH [36].

The CE separation capillary may also be used as a microinjection device and a chemical microreactor. The capillary tip was mounted in a micromanipulator, which facilitates precise position of the injection end of the capillary for sampling single cells or from tissue cross-sections. Following injection, the material introduced into the capillary may be further processed (e.g. exposed to cell lysis or labeling reagents) prior to separation and detection of the organelles.

Johnson et al. analyzed fluorescently labeled mitochondria isolated from a single rat skeletal muscle myoblast [96]. The cell was flanked by two plugs of digitonin and trypsin, which dissolve the plasma membrane and weaken the cytoskeletal network, respectively. Following chemical treatment, the CE separation began by the application of an electric field. The method is suitable to count organelles and to describe the fluorescence and electrophoretic properties of the individual organelles contained within single cells.

Another application of the CE-LIF analysis of organelles from single cells by Chen et al. was focused on endocytosis of fluorescently labeled dextran [97]. Single cell analyses were done of human peripheral blood lymphoblasts after endocytosis of Alexa Fluor 488 Dextran, which is tracer of the endocytic process. Simultaneous detection of the fluorescent drug doxorubicin indicated that this drug also accumulates in endocytic organelles in a highly heterogeneous fashion.

Ahmadzadeh et al. analyzed mitochondria sampled directly from the rat muscle fiber cross-sections [98]. A picoliter-volume sample was removed from the tissue cross-section using the injection end of the CE-LIF capillary. Fluorescence labeling of the mitochondria sample was accomplished by flanking the sampled volume with nonyl acridine orange and allowing for sufficient time (5 minutes) for diffusion of this labeling reagent into the sampled volume. The CE-LIF analysis began when a high electric field was applied. The spatial resolution of the method was defined by the size of the inner diameter of the capillary (50 μm). This method may be used for the analysis of mitochondria and other organelles in multiple tissue types.

4.6 Microfluidics

Microfluidic technologies have become powerful tools in many fields, such as cell biology, clinical diagnosis and environmental monitoring. Compared to capillary systems, they offer faster separations, higher throughput, and the potential of integration of the whole analysis workflow (i.e. sample preparation, enrichment, separation and detection) on a single platform. Here we cover reports on the use of techniques for individual organelle analysis [99–101].

Duffy et al. demonstrated an electrophoretic separation of isolated mitochondria from rat skeletal muscle myoblasts in a glass microfluidic chip [99]. Mitochondria from bovine liver were labeled with nonyl acridine orange, separated in the PVA coated channel and individually detected with the LIF detection system of the microfluidic chip. A five-fold decrease in separation time was achieved compared to CE analysis.

While these reports analyzed the properties of intact mitochondrion based on the overall fluorescence of each particle, Allen et al. demonstrated for the first time the analysis of the content of single mitochondria from B cells [100]. After labeling with Oregon green diacetate succinimidyl ester, a membrane permeable, amine reactive dye, mitochondria were loaded into a microchip device and lysed with a single, nanosecond laser pulse. After lysis, the content of each mitochondrion was analyzed by CE-LIF. The authors were able to analyze amine content of a single mitochondrion within 5 milliseconds with a duty cycle of about 1 minute. This approach may be suitable to classify organelles based on their unique biomolecular signature.

While FFE is adequate for preparation of organelle fractions (see Section 3.3) micro-free flow electrophoresis (μ-FFE) has great potential for analytics and preparation of organelle fractions from small sample volumes [108]. Kostal et al. demonstrated for the first time separation of fluorescently labeled mitochondria from rat skeletal muscle myoblasts using microchip free flow electrophoresis equipped with on-line detection [101]. Compared to CE-LIF analysis of mitochondria, μ-FFE decreased the separation time from about 20 minutes to about 30 s and described electrophoretic mobilities of mitochondria at low electric fields. This study suggests that the electrophoretic analysis of other organelle systems such acidic organelles and cytoskeleton-mitochondrial aggregates previously analyzed by CE-LIF could be accelerated by using μ-FFE. [94, 109]

5. Concluding remarks

While the use of isolated organelles to elucidate biological function is controversial, it is still one of the pillars of modern bioanalytical chemistry [110]. The analysis of isolated organelles must carefully consider the suitability of the methods used for cell disruption, organelle release, and organelle analysis, if the goal is to obtain reliable information on key biological processes. This review is a compilation of recent reports on such methods (see Tables 1 and 2).

Reports on methodologies to release organelles from cells were few. Except for specialized techniques, such as those used to isolate membranes, most techniques do not offer the macroscopic control that may be needed to minimize stress to subcellular environments upon their isolation [110].

Subcellular fractionation methods include exciting developments in fields such as immuno-affinity purifications, FAOS, electrophoretic and dielectrophoretic separations. Implementation of affinity purifications is straightforward when affinity reagents (e.g. antibodies) are available. Unless the organelles can remain bound to the affinity reagent,

release of organelles is an added complication. This is an area of research that needs further expansion. FAOS relies on fluorescent labels specific to antibodies and the multiple examples in the literature point to its feasibility. Although organelle recovery is more straightforward for FAOS than for affinity purifications, the method still requires concentration of the collected organelles usually done by centrifugation), which may compromise organelle function. Electrophoretic separations have also been successful at fractionations of organelles. However, the purity of the isolated fractions is not as high as for FAOS or affinity purifications. On the other hand, electrophoretic techniques have great potential to separate sub-populations of a given organelle type. Dielectrophoresis is an emerging approach to fractionate organelles, with a proof-of- principle report on mitochondria fractionation. It is anticipated that future reports will demonstrate the feasibility of applying it to other subcellular compartments.

The characterization of isolated organelles has demonstrated these are heterogeneous, regardless of the property being observed or the technique being used. The techniques covered here (AFM, optical tweezers, flow cytometry, capillary electrophoresis and microfluidics) will likely continue being used as work horses to describe biological heterogeneity. Some of them will require further development to improve throughput and make them more rugged. On the other hand, all of them will likely be faced the need for robust platforms to analyze complex data. The authors of this review anticipate that bioinformatics is going to address some of these needs.

Indeed, it is likely that other techniques currently developed for particles or artificial organelles (liposomes) will be adapted for organelle analysis. Among these, it is anticipated that laser-capture microdissection [111] and CE with electrochemical detection [112] will provide new vistas on the analysis of isolated organelles.

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Highlights

- Advancements in organelle release
- New approaches to fractionate organelles
- Updates on new techniques to characterize of isolated organelles

Table 1

Release and fractionation of isolated organelles

Organelle	Source	Release	Frac	Key point	Ref
MAM	Rat cell culture, tissue	MH	Cent	First fractionation from cultured cells	[24]
Autophagosomes	Rat liver	MH	Cent	Increased enrichment of autophagosomes	[25]
Autolysosomes	Tobacco plant cells	CL	Cent	First fractionation of organelle in plants	[26]
Secretory lysosomes	Human blood mononuclear cells	MH	Cent	First characterization of this organelle	[27]
Mitochondria & chloroplasts	Moss	MH	Cent	Simultaneous fractionation of both organelles	[28]
Mitochondria	Rat brain	MH	Cent	Optimization of fractionation based on tissue amount, time, and application	[29]
Plasma Membrane	Rat liver	SO, MH	IC	Higher yield and better purity	[30]
Synaptosomes	Rat brain	MH	IC	High yield and better purity	[54]
Chloroplasts	Arabidopsis thaliana and tobacco plants	CL	IC	Antibody that targets cell-type specific surface protein	[32]
Mitochondria	Human cancer cell culture	MH	IC	Improved fractionation in less time	[33]
Peroxisomes	Rat skeletal muscle cell culture	MH	IC	Isolation of functional peroxisomes	[34]
Lysosomes	Human breast sarcoma cell culture	MH	IC	First immuno-isolation of lysosomes	[35]
Endocytic Organelles	Rat skeletal muscle cell culture	MH	Mag	Isolation of organelles with acidic pH	[36]
Peroxisomes	Rat Liver	MH	FFE	Analysis of subpopulations	[38]
Mitochondria	S. cerevisiae	NA	FFE	Analysis of subpopulations	[39]
Mitochondria	Rat Liver	MH	FFF	Similar to density gradient centrifugation without density material	[40]
Secretory Granules	Mouse pituitary cell culture	MH	FAOS	Short analysis time, smaller sample requirements	[41]
Exocytic Vesicles	MDCK Tet-off cell culture	GB	FAOS	First enrichment of basolateral exocytic vesicles	[42]
Mitochondria	Murine and Porcine liver	MH	LCM, OT	Comparison of techniques	[43]
Mitochondria	Lymphoblastoid cell culture	MH	Dielec	Optimization of fractionation method	[44]

This table is relevant to Sections 2 and 3 and covers reports on methods to release and fractionate organelles. Abbreviations for organelle release methods: CL = chemical lysis; FP = French press; GB = glass bead agitation; MH = mechanical homogenization; NA = not available; SO = sonication. Abbreviations for organelle fractionation methods (Frac): Cent = centrifugation; Dielec = dielectrophoresis; FAOS = fluorescence activated organelle sorting; FFE = free-flow electrophoresis; FFF = field flow fractionation; Frac = fractionation;IC = immunocapture; LCM = laser-capture microdissection; OT = optical tweezers; Mag = magnetic capture without affinity purification, Ref = reference.

Table 2

Characterization of isolated organelles

Organelle	Source	Isolation/ fractionation	Char	Key point	Ref
mitochondria	rat heart	Cent	AFM	Swelling during ischemic stress	[78]
Synaptic Vesicles	rat brain	Chrom	AFM	Surface properties	[79]
melanosomes	human eye	Cent	AFM	Changes in melanosome structure with aging	[80]
mitochondria	maize leaf	Cent	FCM	Subpopulations with different membrane Potential	[84]
mitochondria	rice	Cent	FCM	Membrane potential differences	[85]
mitochondria	rat muscle	Cent	CE-LIF	Technique comparison	[86]
mitochondria	rat muscle	Cent	CE-LS-LIF	Hyphenated detector	[87]
mitochondria	rat liver, mouse	Cent	CE-LIF	Dynamic PVA coating	[88]
mitochondria	rat muscle	Cent	CE-LIF	Peak overlap	[89]
mitochondria	rat muscle	Cent	CE-LIF	Peak overlap	[90]
mitochondria	cultured L6 cells	Cent	CE-LIF	*pI* of individual organelles	[91]
mitochondria	cultured L6 cells	CL	CE-LIF	Organelles from single cells	[96]
acidic organelles	Cultured CCRF cells	CL	CE-LIF	Organelles from single cells	[97]
mitochondria	rat skeletal muscle	DTS	CE-LIF	Organelles sampled from muscle cross-sections	[98]
mitochondria	cultured L6 cells	Cent	μ-CE	Fast measurement of mobility distributions	[99]
mitochondria	cultured B cells	Cent	μ-CE	Single mitochondrion content analysis	[100]
mitochondria	cultured L6 cells	Cent	μ-FFE	Fast determination of electrophoretic mobility	[101]
mitochondria	cultured L6 cells	Cent	CE-LIF	Heterogeneity of mtDNA content	[92]
mitochondria	cultured L6 cells	Cent	flow-LIF	Capture and PCR analysis of single mitochondria	[93]
mitochondria	cultured L6 cells	Cent	CE-LIF	Cytoskeleton binding to mitochondria	[94]
acidic organelles	cultured CCRF-CEM cells	Cent	CE-LIF	Distributions of pH and doxorubicin contents in acidic organelles	[95]
lysosomes	cultured L6 cells	Mag	flow-LIF	pH of magnetically purified endocytic organelles	[36]
mitochondria	liver, kidney, heart	Cent	OT	Shape and size of individually trapped mitochondrion	[81]
mitochondria	cultured HL-60 cell	LL	OT	PCR analysis of single mitochondrion	[82]
mitochondria	mouse liver	Cent	sensor	Membrane potential of deposited mitochondria	[83]

This table covers organelle characterization methods relevant to Section 4. Abbreviations: AFM = atomic force microscopy; Char =characterization; Ref = reference; Cent = centrifugation; Chrom = controlled-pore glass chromatography; CE-LIF = capillary electrophoresis with laser-induced fluorescence detection; CE-LS-LIF = capillary electrophoresis with light scattering/laser-induced fluorescence

detection; CL = chemical lysis; DTS = direct tissue sampling; FCM = flow cytometry; flow-LIF = flow with laser-induced fluorescence detection; LL = laser lysis; Mag = magnetic capture; μ-CE = microfluidics CE; μ-FFE = micro free-flow electrophoresis; OT = optical tweezers; PCR = polymerase chain reaction; PVA = polyvinyl alcohol.