Three dimensional electron microscopy of cellular organelles by serial block face SEM and ET

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INTRODUCTION

The knowledge of complex 3D-structures of cells and cell organelles in their natural context is important for understanding the structure-function relationship. Recently evolved 3D techniques at the electron microscopy (EM) level have enabled a new insight into the morphology of tissues, cells and cell organelles that has not been conceivable before. During the last couple of decades the development of computers and the automatization of transmission electron microscopes (TEM) have enabled electron tomography (ET) to be available to numerous EM laboratories, and that has made ET the most widely applied method for obtaining 3D information at the EM level. In addition to ET, new methods utilizing scanning electron microscopy (SEM) have been developed given researchers more freedom to choose the technique that best suits them. These methods, serial block face SEM (SBF-SEM) [1] and focused ion-beam SEM (FIB-SEM) [2], have lower resolving power compared to ET but the capability to analyze bigger volumes.

In ET, several images are collected from the same object at different viewing angles using TEM. Subsequently aligned projection images are combined to form the tomogram using back projection or Fourier methods [3]. The contrast in TEM micrographs is formed according to specimen's mass-thickness and since biological material possess very little differences in mass densities, the structures are usually stained by electron-dense materials, e.g. osmium, uranium and lead. For structural studies, the intact preservation of the specimen is crucial, and the best possible structural preservation methods of thick biological specimens rely on high pressure freezing (HPF) followed by freeze substitution (FS). The low contrast of some cell organelles in specimens prepared by HPF/FS can be challenging but for most organelles this method is very suitable. Since ET utilizes TEM, the thickness of the section is limited, mainly according to the accelerating voltage of the microscope; however, in case bigger volumes are required, the tomograms from several consecutive sections can be combined [4].

The preparation of ET specimens is the same as conventional TEM specimens with the exception of cutting of semi-thick consecutive sections, which is more challenging. The image acquisition, alignment and reconstruction prior to viewing of the tomographic slices generally takes a couple of days. ET can be expanded to be utilized with frozen hydrated specimens (cryo-ET or cryo-ET of vitrified sections) where the only specimen fixation/preservation step is freezing [5]. In the future this might be the ultimate in 3D-EM methods in studies of cells and cellular organelles.

Both SBF-SEM and FIB-SEM are new types of serial-section imaging where the surface of the block is imaged instead of the section. For these techniques the biological specimens have to be fixed and embedded in plastic. The new block face is produced inside the SEM chamber by cutting with a microtome or by milling with an ion beam in SBF-SEM or FIB-SEM, respectively. In these methods the uncoated block face is repeatedly imaged using low accelerating voltage and backscattered electrons are recorded by a sensitive detector, which is specifically designed to perform at low voltages. After contrast reversal, the images look similar to traditional TEM micrographs (for comparison see Figures 1a and 2c). Both methods benefit from a higher amount of contrast-forming atoms in the specimen; however since SBF-SEM is implemented at low accelerating voltages, high contrast is a requisite, and therefore HPF/FS for specimen preparation is not applicable, yet.

Good contrast in FIB-SEM can already be achieved using standard TEM staining protocols, while in SBF-SEM enhanced staining is usually required. The enhanced contrast in the specimen is achieved by several heavy-metal staining steps using ferrocyanide reduced osmium tetroxide, thiocarbohydrazide-osmium liganding, en-block uranyl acetate and lead aspartate staining [6]. The extra staining lengthens the specimen preparation for one day compared to standard TEM processing; however, after adjusting the imaging parameters the acquisition of datasets is fully automated and can be done without human resources. Since the images are acquired from the block face only minor jittering is observed between the images, especially in SBF-SEM, making the alignment process more effortless or even redundant.

These new techniques have opened novel opportunities to achieve 3D data from biological materials. As all of the methods have their pros and cons, it is important to discuss how different methods are chosen to best answer specific biological questions. In this article we will focus on the morphological study of cell organelles and demonstrate what can be achieved with the two techniques, SBF-SEM and ET, which we are applying in our laboratory using plastic-embedded biological specimens. In the studies investigating the morphology of the endoplasmic reticulum (ER) and its interactions with other cellular organelles we have encountered several good examples of the applications of these techniques. The suitability of ET and SBF-SEM for morphological studies of the ER and its interactions with autophagosomes as well as for immunolabelling, will be presented and discussed.

EXPERIMENTAL PROCEDURES

SERIAL BLOCK FACE SCANNING ELECTRON MICROSCOPY (SBF–SEM)

Huh-7 (human hepatoma) or NRK-52E (normal rat kidney) cells were grown on glass coverslips. When indicated, the Huh-7 cells were transfected to transiently express an ER marker for cytochemical staining of the ER lumen [7] and autophagy was induced in NRK-52E cells

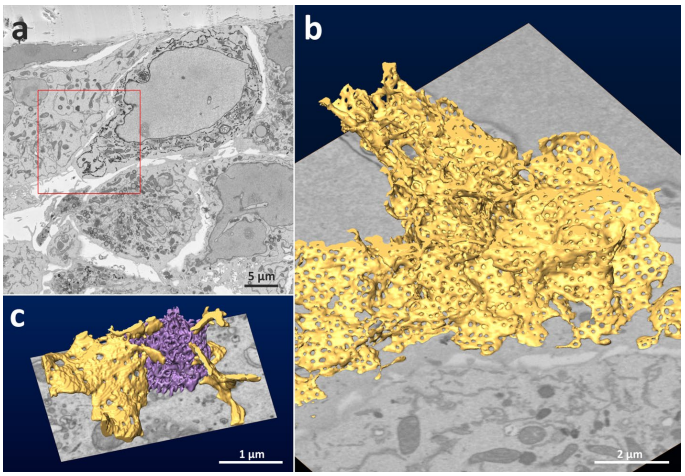


Figure 1
Huh-7 cells expressing ER marker were cyto-chemically stained and imaged using SBF-SEM at 3,000x, voxel size 14.3x14.3x30 nm. (a) A slice from the original image stack showing overall view from non-stained and cytochemically stained cells. (b) The fenestrated ER sheets (yellow) modelled from the area shown in (a) (14.9x14.8x2.5 µm). (c) Model depicting ER sheets (yellow) and tight network of smooth ER tubules (lilac). Images collected at 30,000x, voxel size 4.7x4.7x25 nm.

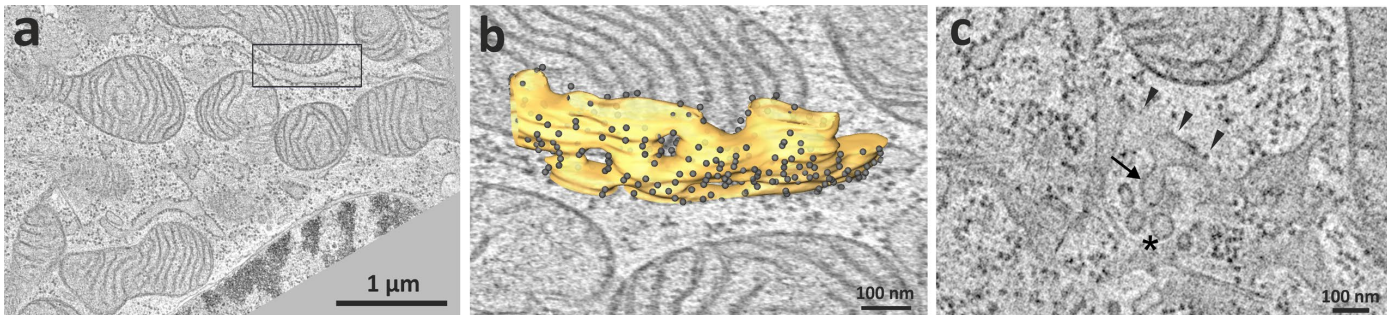


Figure 2
Huh-7 cells were prepared by HPF/FS and a 250-nm-thick section was subjected to ET. (a) A tomogram slice showing profiles of nucleus, ER, mitochondria and ribosomes. (b) A model of ER with membrane bound ribosomes from the area shown in (a). (c) An ER exit site where the ribosome-containing side of the ER sheet is marked with arrow heads, a vesicle with an asterisk and a budding tubular profile with an arrow.

by starvation by incubating cells in serum and amino acid-free medium for 1 hour. The specimens were prepared according to a modified protocol from Deerinck et al. [6]. After trimming the specimen was glued to a specimen pin, and the sides of the block were covered with silver paint and the whole specimen was platinum coated to improve conductivity. The images were acquired with an FEI FEG-SEM Quanta 250, equipped with Gatan 3View at an accelerating voltage of 2.5 kV, dwell time 20 µs (except 15 µs in Figure 3a), spot size 3.0 and pressure 0.25 Torr. The datasets were segmented using a self-developed program written under Matlab environment, and visualized using Amira (FEI).

ELECTRON TOMOGRAPHY

For HPF/FS, Huh-7 cells were grown on sapphire discs and processed as described earlier [8]. NRK-52E cells grown on glass cover slips were chemically fixed and embedded in Epon [9]. 250-nm-thick sections were subjected to ET and the images were acquired at 200 kV accelerating voltage in an FEI Tecnai F20, ±62° tilt at 1° intervals using a dual axis approach [4]. The tilt series were acquired with a Gatan Ultrascan 4000 CCD camera, except in Figure 3b where a Gatan Multiscan 794 CCD camera was used, at 9600x magnification providing a 2-binned pixel size of 2.3 nm, or at magnification of 14,500x with a pixel size of 1.3 nm. The alignment and reconstruction were done using IMOD software package [10] and the segmentation and modeling with Amira (FEI).

IMMUNO–EM SPECIMENS FOR SBF–SEM AND ET

For pre-embedding immuno-EM, Huh-7 or NRK-52E cells were grown on glass coverslips and transfected to transiently express ER marker coupled to GFP when indicated. The cells were fixed and permeabilized using saponin prior to antibody treatments [11]. Primary antibodies recognizing GFP, GM130 and Rtn4B were from Roche, BD Biosciences and Kinasource, respectively, and 1.4-nm-nanogold conjugated secondary antibodies were from Nanoprobes. Silver enhancement was done according to manufacturer's instructions (Nanoprobes) and specimens were plastic embedded as described above. For SBF-SEM, the uranyl acetate en-block staining was omitted because it precipitates with enhancement chemicals. In ET, tilt series were acquired using a magnification of 7800x, providing a pixel size of 2.8 nm.

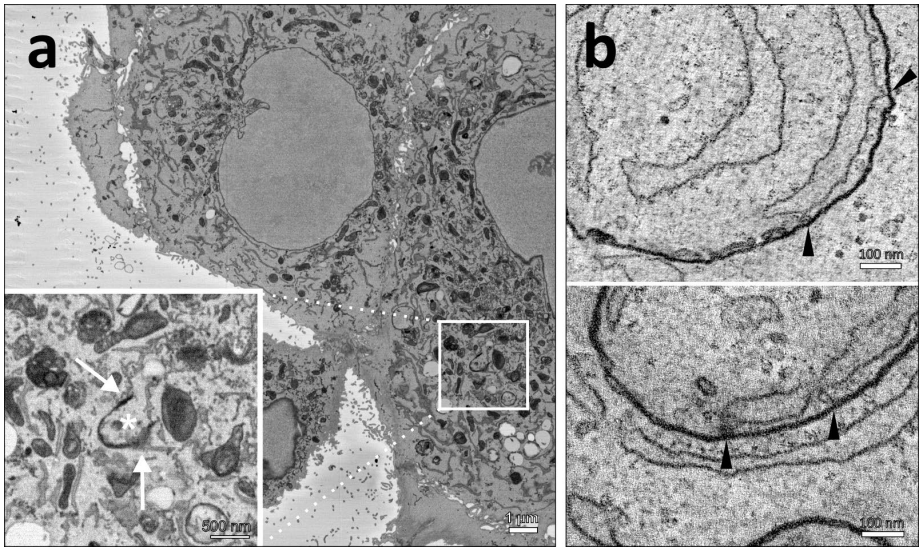


Figure 3
(a) NRK-52E cells were imaged using SBF-SEM at 3,500x, with a voxel size of 11x11x40 nm, showing an overall view of the cell. A forming autophagosome (*) and surrounding ER (arrows) are highlighted in the inset. (b) ET revealed the phagophore and ER connection sites (arrow heads). The tilt series was acquired at 14,500x.

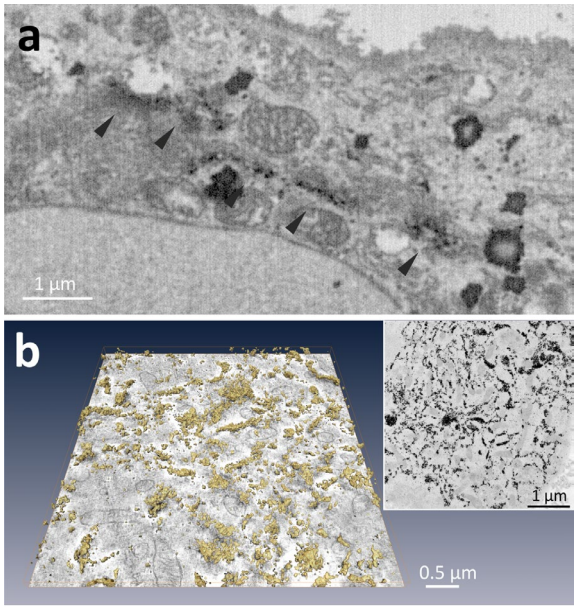
RESULTS

SBF–SEM AND ET REVEAL DIFFERENT ASPECTS OF THE ENDOPLASMIC RETICULUM

To fully appreciate the complex structure of the ER network, the 3D-EM techniques have to be applied [7, 8]. First, in thin section TEM projection images, due to the dimensions of the ER substructures, cross-sections of sheets and tubules have similar profiles to tubules and vesicles, respectively. Second, the connectivity of the 3D-network of ER sheets and tubules spread all over the cell cytoplasm is broken and only a collection of small separate profiles is seen in each section. We prepared chemically fixed or HPF/FS specimens of Huh-7 cells with or without cytochemical staining of the ER, and analyzed them with both SBF-SEM and ET. Cytochemical staining of the ER was used to verify that the structures belonged to the ER and to make the segmentation of the ER for 3D-modelling easier.

The ER seemed to be an extremely suitable target for SBF-SEM because it enables imaging of several cells at a resolution sufficient to define different structural components of the ER network, i.e. tubules, sheets and fenestrations (Figure 1). The majority of the ER appeared as large sheet structures containing numerous fenestrations of about 75 nm in diameter [8]. Even though the ribosomes could not be observed in the SBF-SEM datasets, the smooth ER could be identified from its distinct morphology of tight network of thin tubules (Figure 1c).

The same morphological features observed by SBF-SEM were evident by ET too. In addition to the overall morphology of the ER, ET revealed membrane-bound ribosomes enabling quantification of them in different structure categories of the ER (Figure 2 a and b). These results indicate that ribosomes exist at higher density on non-curved membranes of ER sheets compared to curved membranes of sheet edges and tubules [8]. The designation of ribosomes to a certain ER structure is much more reliable from tomograms compared to thin-section TEM images where the difference between sheet and tubular profiles is not obvious. ET analysis of the ER and ribosomes revealed that in ER exit sites, which are special sites on the ER network where vesicles bud off to be transported further in the secretory pathway, one side of the ER sheet is decorated with ribosomes whereas the opposite side, the vesicle budding site, was lacking ribosomes (Figure 2c).



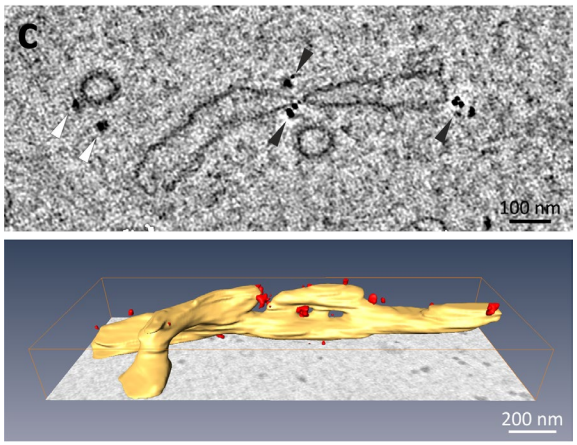


Figure 4
(a) Pre-embedding immuno-EM labelling of Huh-7 cells combined with SBF-SEM reveals enhanced gold precipitation decorating one side of the Golgi ribbon (arrow heads). Pre-embedding immuno-EM combined with ET demonstrates the localization of ER proteins in NRK-52E (b) and Huh-7 (c) cells. With a higher quantity of label the detection of the membranes next to the label is nearly unfeasible; however the staining can be used to model the ER network (b). (c) To visualize the ER membranes the amount of label (arrow heads in tomographic slice) has to be low for reliable modelling of the organelle (in yellow) together with the label (in red). White arrow heads in tomographic slice point to glycogen particles which have only slightly higher grey level values than the gold label.

SBF–SEM AND ET ANALYSIS OF PROXIMITY OF MEMBRANE BOUND ORGANELLES IN LARGE VOLUMES AND SPECIFIC MEMBRANE CONTACT SITES

Autophagocytosis is a catabolic mechanism by which the cells can recycle cellular components through the lysosomal degradation pathway. Even though the biogenesis of autophagosomes has been a target of extensive studies, the origin of the phagophore membrane has remained unclear [12]. Biogenesis of autophagosomes was studied in starved NRK-52E cells. SBF-SEM datasets revealed that first, numerous autophagosomes were found throughout the cytoplasm and second, forming autophagosomes always resided in the vicinity of the ER (Figure 3a and inset). Since SBF-SEM can be used to produce models from several cells and quantification of surface areas and volumes is feasible, this is a powerful method to produce quantitative data on the distribution of organelles within cell and/or their proximity to other organelles, for example the amount of ER in close vicinity of the autophagosomes in respect to different phases of the autophagosome biogenesis.

ET, on the other hand, is a valuable technique to study tiny fusions between membranes that cannot be detected by SBF-SEM and are easily missed utilizing traditional thin-section TEM. ET revealed direct contacts between the forming phagophore membrane and the ER locating either inside or outside of the forming autophagosome (Figure 3b). The distance between the membranes was irregular, and the sites of direct membrane contacts were tiny and infrequent. Observed contact sites suggest that the ER is involved in the biogenesis of autophagosomes and may function as a lipid source for phagophore membrane [8]. Several other equivalent membrane contacts between different cellular organelles have been reported, for example between mitochondria with ER and lipid droplets, lipid droplets and ER, and ER and plasma membrane. Such membrane contacts cannot be reliable resolved with any current light microscopy technique, and based on our experience on using SBF-SEM it seems that ET will remain the best suited technique to study membrane contacts between organelles.

PRE–EMBEDDING IMMUNO–EM COMBINED WITH SBF–SEM AND ET

In many biological studies the localization of the target proteins has an important role; however, immuno-EM techniques are highly challenging and occasionally not even practicable due to the low affinity/specificity of given antibodies. Combining the pre-embedding immuno-EM with 3D-EM methods introduces some new challenges resulting from the use of electron-dense metal atoms as visualization probes. In pre-embedding immuno-EM, nanogold particles are used to ensure efficient penetration, and are then enhanced with silver to form larger, detectable electron dense precipitates. High contrast in SBF-SEM specimen increases the signal to noise ratio; however, heavily contrasted cell structures hamper the detection of the label. Therefore, in SBF-SEM the contrast levels have to be optimized in order to distinguish the label from the structures with the lowest intensities, e.g. glycogen particles. A structural Golgi matrix protein GM130 was immunolabelled, and cells were subjected to double osmication and lead aspartate staining prior to embedding and SBF-SEM analysis (Figure 4a). Mild fixation and permeabilization of the cells impair the fine structural preservation of the specimen; however, the silver precipitate decorating the *cis*-side of the Golgi stacks is readily detected (Figure 4a, arrowheads).

Pre-embedding immuno-EM can be combined with ET as demonstrated. The amount of label is always an important factor in immuno-EM; however, when combined with ET, it becomes critical. The gold inside of the tomogram volume introduces reconstruction artifacts which can cover up all the fine structures nearby (Figure 4b, white halo surrounding the silver precipitates). The localization of two ER proteins was examined in Huh-7 and NRK-52E cells. In one example we show that when the signal coming from immunolabeling is strong, a 3D model from the corresponding organelle can be made by filtering the enhanced gold precipitate (Figure 4b). On the other hand, when localization of the protein in respect to the finest morphological features is the aim, the amount and size of the label have to be optimized to enable the visualization of the associated membranes, as shown in Figure 4c. In conclusion, by optimizing the labelling conditions and specimen preparation, pre-embedding immuno-EM can be combined with both SBF-SEM and ET.

GREATER CONTRAST DIFFERENCES OF SBF–SEM DATASETS FACILITATES MODELLING

The amount of information obtained by these 3D methods is overwhelming and requires modelling and analysis to reveal the specific information. The segmentation and modelling can be time consuming and laborious as automated software for segmentation can seldom be applied. The variation in grey levels between different organelles and the bounding membranes allows or at least greatly facilitates automated segmentation and is worth optimizing.

The intensity variation within ET and SBF-SEM images (Figures 5a and 5b) was analyzed by segmenting 10 to 80 small random areas belonging to each of the analyzed structures and calculating the average intensities (Figures 5c and 5d). The variation of grey levels in different organelles in SBF-SEM dataset was smaller compared to those in tomograms. In a SBF-SEM dataset the relative grey levels of all organelles were lower than that of cytosol (the dashed line in Figure 5c), which allows segmentation of all organelles from the cytosol (background). Moreover, the variation of intensities of the organelles allows segmentation from each other almost solely according to the grey levels. The relative contrast levels in different cell organelles can be affected by using different combinations of staining steps (data not shown), or cytochemical staining of certain organelles (e.g. ER in Figure 5a, the upper cell, orange bar in panel c) to facilitate the segmentation and modelling even further (Figure 1).

In ET, the relative grey level values do not differ remarkably between different organelles hampering the use of automated software (Figures 5b and 5d). The intensity variation of

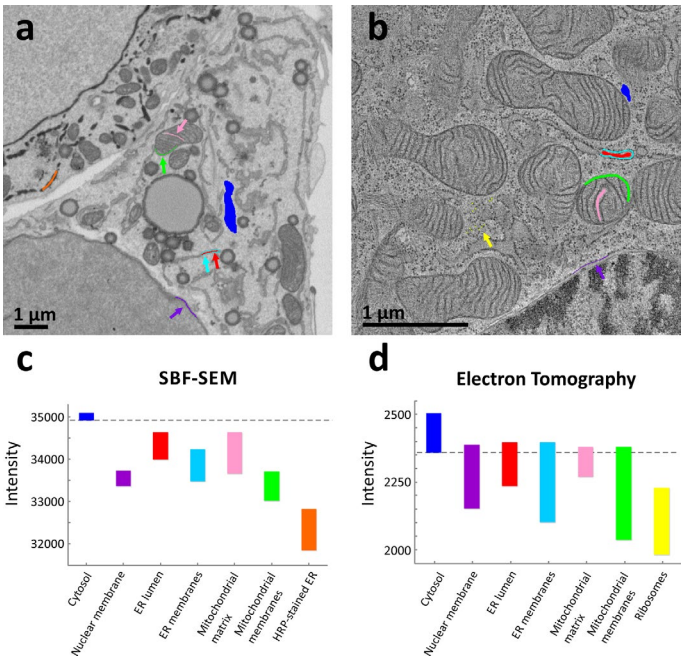


Figure 5
Different cell organelles in SFB-SEM datasets can be readily distinguished according to grey levels (a and c), but in electron tomograms the grey level variation is not sufficient (b and d). Large variations between grey levels in SBF-SEM dataset from Huh-7 prepared according to modified protocol from Deerinck et al. [6] demonstrate the readiness for dataset segmentation (a and c). Almost no variation in relative grey level in different structures is observed in a tomogram slice from Huh-7 cell prepared by HPF/FS (b and d).

cytosol in the ET is larger than that in SBF-SEM and its lower limit overlaps the higher intensity limit of most cellular organelles, which hinders segmentation. In addition, the cytosol is full of soluble ribosomes and other protein complexes, which do not allow the separation of organelles from each other. We should note that application of the cytochemical staining combined with ET does not bring enough contrast to automatic segmentation of the ER.

CONCLUSIONS

Novel methods using SEM have increased the selection of 3D methods at the EM level and thereby offer alternatives to choose the best method for each research project. These techniques do not just use different types of electron microscopies but they also differ from each other in respect of achievable volume and resolution. Typical volumes imaged by SBF-SEM and ET are thousands and tens of cubic micrometers, respectively. SBF-SEM has clearly demonstrated its power in imaging large tissue specimens [1, 13, 14], and we have shown that it has high potential for cell biological specimens too. SBF-SEM can be used to analyze large extended organelle networks (e.g. ER and mitochondria), to quantitatively map the occurrence and distribution of different cell structures (e.g. autophagosomes and lipid droplets) in large numbers of cells, and to reveal the organelles in close proximity to each other.

The resolving power of SBF-SEM is not adequate to resolve tiny structures such as membrane connections, filaments and ribosomes which are more suitable targets to be studied by ET. ET is not applicable at tissue levels and it is very laborious when applied to studies of whole mammalian cells. The other drawbacks of ET are the missing wedge and use the sections that suffer from compression due to sectioning. Since in serial imaging SEM methods the block face is imaged instead of the section, these methods are free from compression artifacts. In biological studies, immunolabelling is an important tool, and both ET and SBF-SEM can be combined with pre-embedding immuno-EM; even though much optimization is required to find a functional combination in specimen preparation.

Further new developments in the field of serial imaging SEM will result in improved resolution. The equipment of the facility at times governs the methods to be utilized; however, whether in studies of plastic-embedded specimens serial imaging SEM methods can displace ET completely remains to be seen. Currently, since both tiny fine structures and their placement into correct context can be equally important in biological studies, ET and serial imaging SEM methods are complementary rather than rivals.

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BIOGRAPHIES

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Dr Eija Jokitalo (centre) has a PhD in biochemistry from the University of Helsinki. She was a post-doctoral fellow at the Imperial Cancer Research Fund in UK, and has been the head of the EM unit since 2001.

ABSTRACT

Novel serial imaging methods using scanning electron microscopy may be used for 3D morphological studies of plastic embedded biological specimens. Serial block face SEM has already proven an undisputable position in 3D-EM studies of tissues, but it has a lot of potential in cell biology applications too. Electron tomography can be used to complement those studies where higher resolution is needed. In this article we present examples of utilizing these two 3D-EM techniques to study cellular organelles.

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