Multiscale imaging of neurons grown in culture: From light microscopy to cryo-electron tomography

Vladan Lucić a, Albrecht H. Kossel b, Ting Yang a, Tobias Bonhoeffer b, Wolfgang Baumeister a,*, Anna Sartori a

a Max-Planck Institute for Biochemistry, Department of Structural Biology, Am Klopferspitz 18, D-82152 Martinsried, Germany
b Max-Planck Institute for Neurobiology, Martinsried, Germany

Received 19 October 2006; received in revised form 2 August 2007; accepted 13 August 2007
Available online 29 August 2007

Abstract

Cryo-electron tomography (cryo-ET) allows the visualization of supramolecular architecture in cells preserved in a close-to-physiological state. In order to supplement the structural information obtained by cryo-ET with the functional state of the molecules involved based on fluorescent labeling we developed a method of correlating light microscopy and cryo-ET. This method is suitable for investigating complicated cellular landscapes such as mature neurons grown in culture. It has the advantage that a correlation is obtained without exposing a feature of interest to additional electron irradiation, and that it does not rely on visual recognition of features. Different modes of correlation are presented here: a feature identified on a light microscopy image is used to guide the cryo-ET investigation, and cryo-tomograms are correlated to light microscopy images. Cryo-tomograms of a neuronal synapse and of an isolated presynaptic terminal are shown as examples of the correlative method. The correlation method presented here can be expected to provide new insights into the structure–function relationship of supramolecular organization in neurons.

Keywords: Cryo-electron tomography; Correlative microscopy; Neuronal cultures; Endocytosis; Synapse

1. Introduction

Cryo-electron tomography (cryo-ET) combines the potential of three-dimensional (3D) imaging at molecular resolution with a close-to-life preservation of biological samples. Rapid freezing followed by the investigation of the frozen-hydrated samples avoids the artifacts caused by chemical fixation and dehydration procedures. Furthermore, the biological material is observed directly, without heavy metal staining, avoiding artifacts caused by the unpredictable accumulation of staining material (Baumeister, 2002; Al-Amoudi et al., 2004b). A problem in cryo-ET is the high sensitivity of ice-embedded specimens to electron irradiation. The automated acquisition of electron tomograms helps to keep the total exposure during the recording of a cryo-tomogram within acceptable limits (Koster et al., 1997). However, minimization of the electron dose used during the search for a feature of interest in cryo-ET remains an important issue that needs to be addressed.

Light microscopy (LM) has been correlated to conventional electron microscopy (EM) in order to obtain higher resolution information about structures detected in LM (Nakata et al., 1998; Ahmari et al., 2000). Combinations of immuno-labeling with electron-dense markers and fluorescent labeling have been used for detection of molecules at higher resolution than LM can provide (Mironov et al., 2001; Gaietta et al., 2002). These methods are effective on a cellular scale, however, they either rely on an extensive search by EM, thereby
increasing the cumulative electron dose, or on the presence of heavy metals, which renders them unsuitable for studies on a molecular scale.

Cryo-ET of whole cells has the advantage that supramolecular architecture can be studied in unperturbed cellular environments (Medalia et al., 2002; Lucic et al., 2005a; Murphy et al., 2006). The cryo-preparation of adherent cells, such as neuronal cells, is more challenging than the cryo-preparation of cells in suspension. Recently, two different approaches were taken to investigate neurons in a frozen-hydrated state: cryo-sections of organotypic neuronal cultures were studied by cryo-EM (Zuber et al., 2005), and cryo-ET was used for the investigation of neurons in culture at an early developmental stage (Garvalov et al., 2006). Studying mature neuronal cultures by cryo-ET adds another layer of complexity arising from the presence of cell bodies together with many long and highly interconnected neuronal processes. This makes it difficult to locate features of interest and a prolonged search can contribute substantially to the total electron dose. We circumvented this problem by using light microscopy to assist a cryo-ET search and to provide cues for identification of larger cellular structures, thus establishing a correlative light and cryo-ET approach. The purely software-based approach, presented in this paper, differs from a correlative procedure developed by Sartori et al. (2007) in that it requires minimal extra electron dose to be spent on a feature of interest and is therefore consistent with the requirement of minimizing the electron dose. Our procedure does not rely on a visual recognition of features, and so it can be reliably applied to mature neuronal cultures that often show dense networks of interconnecting neuronal processes. Cryo-tomograms of a neuronal synapse and of an isolated presynaptic terminal are shown as examples of the correlative method. We also show that this correlative approach can be used to supplement structural information at the molecular level obtained by cryo-ET with functional information derived from fluorescent labeling.

2. Materials and methods

2.1. Neuronal cultures

Gold finder EM-grids (type NH2A by Plano, Wetzlar, Germany) coated with Quantifoil (TM) R2/2 were sterilized in ethanol for 10 min, then washed in H2O, and transferred to culture dishes. Both grids and dishes were coated with 1 mg/ml poly-L-lysine (in water) for 1 day, washed in D-MEM medium (Gibco 61965), and placed in D-MEM with B27 supplement prior to plating. Whenever a liquid had to be removed completely from a dish during washing and medium exchange steps, grids were transferred into another dish containing the appropriate liquid in order to prevent grids from drying. Sideways submersion of grids into the media greatly reduced the risk of ablation of the sensitive grid coating.

Primary hippocampal neuronal cultures were dissected from E18 Sprague–Dawley rats in accordance with the procedures accepted by Max-Planck Institute for Neurobiology, and dissociated as described previously (Goslin and Banker, 1991). Briefly, hippocampal neurons were dissected from embryos and were dissociated after incubation for 30 min in an EDTA/trypsin solution (0.25% trypsin, 1 mM EDTA). After washing with D-MEM and 10% FBS, neurons were plated on the poly-L-lysine coated dishes and EM grids (kept in the dishes). Cultures were kept at 37°C in 5% CO2 and investigated at 10–15 days in vitro (DIV). In some cases (tomogram shown in Fig. 3) the cultures were treated with 50 ng/ml BDNF (Chemicon) for 24 h before freezing.

2.2. Fluorescent labeling and live light microscopy

Cultured neurons grown on EM grids were first labeled for 90 s with 15 μM FM1-43 equivalent (synaptogreen C4, Sigma) in HBSS solution (Gibco 14025050) with the addition of 50 mM KCl, 2 mM CaCl2 (3.3 mM Ca2+ total), and 10 μM CNQX (Tocris). This step was followed by 60 s incubation in 15 μM FM1-43 equivalent in HBSS, at 30–35°C to allow time for the completion of endocytosis. Depolarization-induced FM1-43 unloading was done for 60 s in HBSS solution with the addition of 50 mM KCl, 2 mM CaCl2, and 10 μM CNQX.

The grids were imaged in HBSS in glass-bottom culture dishes (MatTek P55G-1.5-7-c-grid) mounted in a chamber at 35°C with a Zeiss Axiovert 200M inverted light microscope equipped with motorized stage and using a Zeiss air 10× Plan-NeoFluar Ph1 objective with 0.3 N.A., a Zeiss air 20× Achromplan Ph2 objective with 0.4 N.A., and a fluorescence filter set (Zeiss) for FM1-43 (470/40 nm bandpass excitation, 510 nm long-pass beamsplitter, 540/50 nm bandpass emission). In order to compensate for the fact that grids are usually slightly bent and to ensure that the whole grid is imaged in focus, several images comprising a z-stack were taken at each position. Typically, 10–15 images with 5 μm z-increment at 20× were taken. Images covering a whole grid and z-stacks were recorded using Mosaix and z-stack modules of Zeiss Axio Vision 4.1 software. In-focus information was manually extracted from individual z-slices and individual images were combined to show a wider area using TNImage (free software distributed under the GNU public license, http://brneurosci.org/timage.html) and GIMP (free software developed by The GIMP Team and distributed under the GNU public license, http://www.gimp.org/) image manipulation software.

2.3. Cryo-electron microscopy and tomographic reconstruction

BSA tracer 15 nm gold (Aurion) was repeatedly spun with a tabletop centrifuge at 13,000 rpm (15–30 min total), the supernatant was discarded and the soft pellet...
resuspended in HBSS. Equal parts of 10 nm and 15 nm BSA-gold prepared in this way (3 µl total, prewarmed to 30–35 °C) were applied to grids to serve as fiducial markers for tomographic alignment. Excess liquid was blotted and the grids were rapidly frozen in liquid ethane (Dubochet et al., 1988). In some cases (tomogram shown in Fig. 3), grids were kept in a humid atmosphere during the blotting procedure using a custom-made humidifying device (Cyrklaff et al., 1990). Grids were stored in liquid nitrogen before they were mounted on a cryo-holder (Gatan).

Tilt series were recorded at 300 kV on a Philips CM 300 microscope equipped with FEG, a computer-controlled stage, a post-column energy filter (Gatan GIF 2002) operated in the zero-loss mode, and a fiber-coupled 2k × 2k Gatan CCD camera. Pixel size at the specimen level was 0.82/1.15 nm. The defocus was −9/−12 µm, corresponding to the first zero of the contrast transfer function at 4.2/9.4 nm. The angular increment was 3° and the tilt range was −60° to 60°/−60° to 42° for tomograms shown in Figs. 3 and 4/5. Typically, the total electron dose for the whole series was 15–20 e/Å2. Tilt series were aligned using fiducial markers. Reconstruction of tomograms was done using the weighted back-projection algorithm. During the reconstruction, images were binned twice to reduce the noise (final voxel size 3.3/4.6 nm) and low-pass filtered at the post-binning Nyquist frequency. The alignment and reconstruction of tomograms were done using the ‘EM’ software (Hegerl, 1996).

2.4. Denoising and segmentation

The tomograms shown in Figs. 3e and 4a were slightly denoised (in 3D) using the improved anisotropic nonlinear diffusion algorithm (Frangakis and Hegerl, 2001; Fernandez and Li, 2003). A software implementation of this algorithm was provided by J.J. Fernandez (University of Almeria, Spain). Denoising of the tomograms shown in Figs. 3e/4a/5b/5c was done using the following parameters: edge-enhancing diffusion threshold \( K = 0.05/0.13/0.11/0.16 \), coherence-enhancing diffusion threshold \( C = 6.2 \times 10^{-5}/1.0 \times 10^{-5}/2.1 \times 10^{-5}/7.6 \times 10^{-5} \), CED/EED balance 0.75, CED along second eigenvector 0.75/0.5/0.5/0.5, initial \( \sigma = 0.25 \), averaged structure tensor sigma 4.0, time interval 0.04/0.01/0.01/0.01, 6/20/10/10 iterations, while for the initial tomogram the average voxel value was −0.001/−0.004/−0.006/0.000 and the standard deviation was 0.126/0.118/0.187/0.207. All features observed in the denoised tomograms, such as the actin filaments and the connections between the vesicle and the neuronal processes were also present in the non-denoised tomogram.

The segmentation shown in Fig. 3f was done as follows. Membranes were segmented in the Amira visualization software (TGS), starting from the manually assigned points around a membrane and using the intelligent scissors algorithm. Extracellular vesicle-bound molecular complexes were segmented using the automated procedure based on thresholding and connectivity (Lucic et al., 2005b). Surface rendering of the segmented tomogram was done in Amira.

2.5. Coordinate transformation

A coordinate transformation from a point \((x_1, y_1)\) in one coordinate system to \((x_2, y_2)\) in another is described by the following equation:

\[
\begin{pmatrix} x_2 \\ y_2 \end{pmatrix} = s \begin{pmatrix} \cos(\alpha) & \sin(\alpha) \\ -\sin(\alpha) & \cos(\alpha) \end{pmatrix} \begin{pmatrix} 1 & 0 \\ 0 & p \end{pmatrix} \begin{pmatrix} x_1 \\ y_1 \end{pmatrix} + \begin{pmatrix} d_x \\ d_y \end{pmatrix}
\]

The parameters defining the transformation are: magnification \(s\), rotation \(\alpha\), mirror reflection \(p\) (1 for reflection and 0 for no reflection), and translation \((d_x, d_y)\). The error of establishing a coordinate transformation is calculated as:

\[
\text{error} = \sqrt{\frac{\sum_{i=1}^{n} ((x_i - x_i')^2 + (y_i - y_i')^2)}{n-1}}
\]

where \(n\) is the number of reference points, where \((x_i, y_i)\) are real coordinates of \(i\)th marker and \((x_i', y_i')\) are the coordinates of the \(i\)th markers calculated using a given transformation.

3. Results

3.1. Cryo-preparation of neuronal cultures

We first verified that mature neuronal cultures are suitable for the cryo-preparation. We followed the standard procedure for preparing dissociated rat hippocampal cultures, with the exception that neurons were plated on dishes containing EM-grids, so that some neurons adhered to the carbon film attached to the grids. During the initial grid preparation steps, such as sterilization, washing, and coating with polylysine, grids were handled very carefully in order to avoid damaging the carbon film. In our preparations, grids remain in cell culture medium for about two weeks, much longer than the few minutes to few days required for other cell types or for neurons at early developmental stage (Garvalov et al., 2006). Small handling-incurred defects on the carbon film can easily cause a major portion of the film to detach from a grid during the time spent in culture. The density of neuronal cells on a grid is kept relatively low, because the cell bodies are too thick to be investigated by cryo-EM and they obscure neighboring processes. We verified that neurons grown on grids developed well and showed typical morphology at a mature stage (at DIV 10–15), as seen by the well-defined shape and strong processes of a pyramidal neuron in the lower left square in Fig. 1a. These neurons also show normal, punctate staining by fluorescent membrane-bound dye FM1-43, both at the cell body and processes, indicative of functional endocytotic processes (Fig. 1b). Furthermore, the high potassium depolarization-induced destaining of the same dye (Fig. 1c) points to synaptic vesicle exocytosis. We note
that the destaining is quite thorough, even though the destaining induced by high potassium concentrations is not expected to be complete (Rizzoli and Betz, 2004). Together, these observations indicate the presence of functional presynaptic terminals capable of stimulation-induced synaptic vesicle release and recycling.

The neurons grown on grids were preserved in vitreous ice using the standard technique: the excess liquid was first blotted with filter paper and the grids were rapidly frozen by plunging in liquid ethane (Dubochet et al., 1988). Because a thick, electron-opaque layer of ice is usually formed around cell bodies, we focused our attention on regions where neuronal processes were less dense and which were devoid of cell bodies. Neuronal processes embedded in 200–500 nm thick ice showed features characteristic for cryo-preserved material, such as smooth and well defined membranes and a wealth of intracellular and membrane-bound molecular complexes (Dubochet and Sartori Blanc, 2001; Al-Amoudi et al., 2004a), while processes embedded in thinner ice often have a rugged and faceted appearance, more similar to those prepared for EM using chemical fixation and dehydration.

We also observed that the type of carbon film that covers EM grids has some influence on the growth of neuronal processes. Axons do not appear to require much adhesion; they grow well even on holey carbon films that cover around 10% of the grid area and provide some support at every 1–2 μm, as is the case with the lacey-carbon film. Dendrites, especially larger dendrites, adhere better when a carbon support covers 50% of a grid or more, such as the Quantifoil R2/2 film (see 2 μm circular holes in Figs. 1a and 4b, c), or in dense environments containing many neuronal processes.

3.2. Correlative light microscopy and cryo-electron tomography: the procedure

Our procedure for correlating a spot on a light microscope image with a cryo-EM tomogram is completely software-based. We developed a software procedure that allows us to establish a coordinate transformation between two coordinate systems in two-dimensions (2D) and to use this transformation to map a point in one system to a corresponding point in another system. This coordinate transformation includes translation, rotation, (isotropic) magnification, and mirror reflection (all in 2D), and was therefore sufficient to directly relate positions in two coordinate systems, such as pixels on LM images with goniometer positions at which EM images are taken.

In our case, a typical correlation consisted of two steps: rough and precise correlation (Fig. 2). In the rough correlation, we identified the field (square) on the EM grid where the feature of interest was located. We used finder grids (grids with symbols) to facilitate this process. When the grid symbols in the vicinity of the feature of interest were covered by thick ice so that they were hard to see in EM, we recorded EM coordinates of symbols anywhere else on the grid and used the coordinate transformation software to identify the field where the region of interest is located (Fig. 2a). In few cases, no symbol could be identified on the grid due to thick ice (few μm’s or more). This ice thickness is transparent to light, so we solved this problem by using a cryo-holder adapted for LM (recently designed and built in our department (Sartori et al., 2007)) and by comparing the ice-pattern on the grid observed using the cryo-LM holder with the pattern observed in cryo-EM.

For the precise correlation, we identified well-defined reference points in the vicinity of the region of interest, such as the corners between grid bars that defined the field identified in the first step. In order to find EM coordinates of corners one needs to illuminate only the narrow region along the grid bars, which is not suitable for tomography anyway. The EM coordinates of these reference points and their coordinates on a corresponding LM image allowed us to establish a coordinate transformation between the EM coordinate system and the LM image coordinates. We then used this transformation and the coordinate transformation software to either find the position on the LM image that corresponded to the EM coor-

![Fig. 1. Neurons cultured on EM grids. (a) Phase-contrast image showing a pyramidal neuron in the lower left square showing typical morphology, scale bar 100 μm. (b) Fluorescence image showing FM1-43 labeling of the neuron shown in the lower left square of (a). Note the punctate staining of the cell body and some of the processes, scale bar 20 μm. (c) Fluorescence image of neurons loaded with FM1-43 (above) show strong distinct puncta, unlike the image showing the same area after the FM1-43 dye was unloaded (below), scale bar 20 μm.](image-url)
In order to make sure that any feature imaged in cryo-EM can be correlated with LM, the whole grid was imaged, usually both in phase-contrast and fluorescence, typically before freezing, using a 10× (0.3 N.A.) or a 20× (0.4 N.A.) air objective. To allow for the fact that grids were usually slightly bent, and to ensure that each part of the grid was imaged in focus, a z-stack instead of a single image was taken at each position. It is not advantageous to use higher magnification objectives, because the number of images taken becomes prohibitively high (more images are needed to cover the whole grid, and smaller z-stack increment is required to image all features in focus), so only a part of the grid can be imaged.

This two-step procedure allowed the use of reference points that are close to the feature of interest (in the second step), producing a more precise correlation because it reduced the errors due to imprecise determination of the symbol coordinates, mechanical deformations of the grid, and the possible misalignment between the low magnification EM mode (used to identify the symbols on the grid) and the high magnification mode (used for imaging the feature of interest). We also applied corrections for the imprecise positioning of the goniometer that occur during EM investigations, and for the image shift between TEM (transmission electron microscopy) mode that is used for identification of marker points and EFTEM (energy filtered TEM) mode used for the recording of tomograms. The correction of these errors reduced the correlation error.

Therefore, this correlation procedure provided reliability and minimization of electron dose. That is, this procedure did not rely on the recognition of a feature of interest or its surroundings in EM and visual identification of the same area in LM images in order to guide EM search to the feature of interest. Consequently it can be applied to neuronal cultures having a dense network of interconnecting neuronal processes, as is often the case with mature cultures. Furthermore, the procedure does not cause any additional electron irradiation of a feature of interest, and may reduce the electron dose spent during EM search.
3.3. Test examples

Here we show two examples of correlative light and cryo-EM that illustrate the procedure described above. In addition to the correlation procedure described above, we also recorded EM images covering a wider area around a correlated position that allowed us to determine the correlation error, that is the distance between the EM position calculated using the correlation procedure and the exact position of the correlated feature.

In the first example, we used the correlation procedure to find the EM coordinates of a spot that showed a strong fluorescence signal (arrow in Fig. 3b). Using the easily identifiable features of the neighboring processes (Fig. 3c) and the pattern on the carbon film, we determined the exact position in EM that corresponds to the fluorescence spot and recorded a tomogram at that position (Fig. 3d, e). Therefore, without analyzing its content, we established that the tomogram shown in Fig. 3d, e corresponds to the fluorescence spot labeled by arrow in Fig. 3b.

---

**Fig. 3.** Test example of LM and cryo-ET correlation where LM was used to guide cryo-ET. (a) Montage of 10× FM1-43 images covering the entire grid, scale bar 500 μm. The marked area is enlarged in each panel in (b). (b) Phase-contrast (above) and the FM1-43 (below) images of the region where a tomogram was taken, scale bar 100 μm. The marked area is enlarged in (c). The arrow points to the spot with high FM1-43 fluorescence where the tomogram (shown in panel d) was recorded. (c) Collage of EM images of neuronal processes surrounding the area where the tomogram was taken, scale bar 10 μm. The marked area is enlarged in (d). (d) Tomographic slice showing two neuronal processes (labeled ‘NP’), an extracellular vesicle connected to one of the processes and to a protrusion (labeled ‘PR’) from the other one, and an endocytotic invagination on the protrusion, scale bar 200 nm. (e) Tomographic slices showing an extracellular vesicle forming multiple connections with two neighboring processes (yellow and orange arrows), vesicular membrane bound molecular complexes on the exterior (green arrows) and in the interior (red arrows), and an endocytotic pit (black arrows). Scale bar 100 nm. (f) Surface rendering showing the extracellular vesicle (blue), neuronal processes (gray), and some of the vesicle-bound molecular complexes (color are the same as in e). The vesicle is shown with one side open in order to expose the complexes in its interior.
relation error was 3 μm, while the error associated with establishing the correlation was 0.6 μm.

The tomogram shown in Fig. 3d shows two neuronal processes. An Ω-shape invagination showing fine structures radiating away from the invagination (shown in Fig. 3e, black arrows), as well as two other membrane invaginations (not shown) at a protrusion from one of the neuronal processes suggest endocytotic activity (Vaughn, 1989; Brodin et al., 2000; Higgins and McMahon, 2002). In addition, an extracellular vesicle with a diameter of 80 nm lies between the two processes. The presence of many vesicle-bound molecular complexes both in the vesicle interior and exterior (shown in red and green in Fig. 3c, f), and several molecular connections (bridges) formed between the vesicle and both processes (yellow and orange in Fig. 3c, f) argue against the possibility that the vesicle is a preparation artifact. All together, the data in this tomogram point to a high endo-/exocytotic activity. This conclusion is further supported by the fluorescence. Namely, the tomogram was exactly correlated to a fluorescence spot (see above) that was obtained using FM dye (Cochilla et al., 1999), which under our experimental conditions labels endocytotic processes (not only synaptic vesicle endocytosis). Therefore, this example shows that functional data from LM can contribute to the understanding of the structures detected by cryo-ET.

Our second example illustrates the reverse approach: we first recorded three tomograms at the same bundle of neuronal processes (Fig. 4a) and then correlated the tomogram positions to phase-contrast LM images (arrows in Fig. 4c).

Upon close inspection of the tomograms and the position of the bundle of neuronal processes in EM (Fig. 4b) and LM (Fig. 4c) with respect to the pattern (circular holes 2 μm in diameter) on the carbon film we determined that the distances between actual and correlated tomogram positions (correlation error) were below 2 μm (the error of establishing a correlation was 2.2 μm). The EM images of the whole bundle (Fig. 4b) were not used for the correlation, they only allowed us to calculate the correlation error. As another check of the correlation we recorded a phase-contrast cryo-LM image of the same region (Fig. 4d) and correlated the tomograms to that image. Despite the blurring and the loss of resolution in the cryo-LM (Sartori et al., 2007) we can see that the correlation points to the same bundle of neuronal processes.

Each of the three tomograms recorded shows a bundle of interwined neuronal processes. In particular, a filopodium protruding from a large neuronal process is visible in a tomographic slice shown in Fig. 4a. Filamentous structures, one consistent with an actin bundle in the filopodia and the other with cortical actin at the base of the filopodia (Medalia et al., 2007) were recognized in this image.

In general, the correlation error consists of the error of establishing a correlation and of the error arising from imprecise movements of the goniometer. In the examples presented above, it seems unlikely that the error of establishing a correlation is significantly influenced by possible deformation of a grid or due to a slanted position of a grid during the investigations, because we did not observe a grid...
deformation or a difference in focal value that would be high enough to cause an error higher than 1 μm. This leaves the error of determining corner positions in EM and the goniometer error the major contributors to the correlation error.

3.4. Application examples

The low-dose requirement of cryo-ET, combined with generally thick ice in the plunge-frozen neuronal cultures, renders the search for features of interest, such as synapses, more involved than in the conventional EM. The proximity of cell bodies, or larger dendrites to many synapses (resulting in thick ice surrounding those synapses) makes the search for synapses even more difficult. Here we show applications of our correlative procedure that helped us overcome these difficulties. We recorded two tomograms at positions that were correlated to FM fluorescence puncta (Fig. 5a) (the error of establishing correlation was below 1 μm). One of the tomograms shows a synapse (Fig. 5c) while the second shows an isolated presynaptic terminal (Fig. 5b). Both presynaptic terminals contain large number of synaptic vesicles (more than 80), so it appears that in these cases the FM signal was due to synaptic vesicle endocytosis.

The synapse (Fig. 5c) was recognized by classical morphological criteria: the presence of synaptic vesicles on the presynaptic side, a dense thickening on the postsynaptic side termed postsynaptic density (PSD) and a synaptic cleft in between. Judging from the thickness of the PSD, the synapse is asymmetric (having stronger density on the postsynaptic side) and is therefore most likely an excitatory

Fig. 5. Application of LM and cryo-ET correlation. (a) Montage of two 20x cryo-LM images showing FM1-43 puncta (arrows) that were correlated to the tomograms shown in (b) (upper left arrow) and (c) (lower right arrow). Scale bar 20 μm. (b) Tomographic slices showing the correlated isolated presynaptic terminal. Arrows indicate smooth endoplasmic reticulum (blue), dense core vesicle (red) and synaptic vesicle associated complexes (orange). Scale bar 200 nm. (c) Tomographic slices showing the correlated synapse. Arrows indicate actin network (green), filaments and shorter molecular connections between them in the PSD (orange), and microtubules (blue). Pre- and postsynaptic terminals are labeled by “Pre” and “Post”, respectively. Scale bar 100 nm.
synapse. The presynaptic terminal is formed on an axonal varicosity, which also contains microtubules and larger vesicles in its interior. We note that we are looking here at 4.6 nm thick tomographic slice. Since microtubules do not lie flat they disappear from the slice shown (to continue in other slices). Also, not all synaptic vesicles are cut in the middle by the tomographic slice, so they appear very heterogeneous in size. In addition to these ultrastructural features, this tomogram shows elements of molecular architecture. The PSD can be seen to contain both filaments running along the membrane and shorter molecular connections that are approximately perpendicular to the filaments. Also, actin network can be recognized in the cytoplasmic area of the postsynaptic terminal.

The other tomogram shows an abundance of synaptic vesicles in an axonal varicosity, but this axon does not form a synaptic junction with any of the neighboring processes, so the tomogram shows an isolated presynaptic terminal. Such terminals were characterized before, both during development and at mature stage (Ziv and Garner, 2004; Krueger et al., 2003). This isolated presynaptic terminal contains similar structures as the presynaptic terminal of Fig. 5c. A dense core vesicle and tubules and sacs of smooth endoplasmic reticulum are shown in Fig. 5b. On the molecular level, membrane-bound molecular complexes are found both on the extra- and intravesicular sides of synaptic vesicles (Fig. 5b).

4. Discussion

Correlative approaches combining light microscopy with various forms of electron microscopy are important for the integration of structure and function in cell biology (Koster and Klumperman, 2003). Cryo-ET of cells not only has the power to investigate supramolecular architecture in unperturbed cellular environments, but it also provides structural information at the molecular level. Considering that LM-based methods such as fluorescence resonance energy transfer allow monitoring of different functional states of molecules in living cells, it is clear that the correlative light and cryo-ET approach has a potential to identify structural correlates of many functional states.

Here we build on related work that established a correlative light and cryo-EM technique (Sartori et al., 2007). Unlike the approach taken by Sartori et al. that is based on the use of a cryo-LM holder and a procedure that scans the whole grid in cryo-EM (and therefore requires that the grid-scan procedure is integrated with a microscopy software), our correlation procedure is completely based on the use of the (stand-alone) 2D coordinate transformation software. In this approach, a correlation is established in two sequential steps to allow relatively high precision with a typical error of 1–3 μm. The approach described in this communication minimizes preirradiation of a feature of interest (most of the relatively low electron dose required to establish a correlation is spent outside the feature of interest) and is therefore consistent with the requirement of keeping the total electron dose low. In addition, this procedure does not rely on visual recognition of features, so it can be reliably applied to mature neuronal cultures that often show a dense network of interconnecting neuronal processes. However, it is more complicated to use, more time-consuming and perhaps less intuitive. We showed examples of using both “live” and cryo-LM images. Clearly, applications to motile cells or fast-moving processes require cryo-LM imaging.

The examples of the application of correlative LM and cryo-ET to neurons grown in culture presented here show different modes of the application of this method. In one mode the correlation was used to guide cryo-ET investigation to a feature that was identified on an LM image, while in the other cryo-tomograms were first recorded and subsequently correlated to neuronal processes on a LM image. Some other correlation strategies may be more involved. For example, cryo-LM investigation after cryo-tomography may be limited to phase-contrast because of the apparent bleaching of FM1-43 dye by electron irradiation. Also, video-LM immediately followed by cryo-preparation has the possibility to link a known time point in a process observed with LM to EM (Mironov et al., 2000) or cryo-ET with temporal resolution of few seconds, but it would require higher reproducibility of cryo-preparation or the investigation of larger number of grids to succeed. In addition, if the cryo-LM holder was used to image a feature of interest after recording a tomogram of that feature, only the region on the grid where the tomogram was recorded would need to be imaged, instead of the whole grid. However, this method appears not to be well suited for use with fluorescence, because we observed that an extensive electron illumination appears to cause bleaching of FM1-43 fluorophore. We assume that is it related to the fact that extensive electron irradiation causes formation of free radicals, and that chemical interactions between some of these radicals with fluorophore causes bleaching (Lichtman and Conchello, 2005).

We showed that mature neuronal cultures are suitable for cryo-ET investigation. Neurons grown in culture on EM grids adhere to grids, develop well, and show normal morphology at the mature stage (10–14 DIV). They can be embedded in 200–500 nm thick vitreous ice by rapid freezing (Dubochet et al., 1988), and they typically show characteristic features of good structural preservation (Dubochet and Sartori Blanc, 2001; Al-Amoudi et al., 2004a). Using the correlative approach described here, we recorded tomograms of a neuronal synapse and of an isolated presynaptic terminal (Fig. 5). In addition to the ultrastructural features consistent with the earlier EM-investigations, these tomograms show a full three-dimensional view of synaptic macromolecular complexes in their unperturbed cellular environment. This opens up the possibility to investigate the molecular architecture of the postsynaptic density, a large complex playing a central role in synaptic transmission and plasticity, a task that requires further work. The presence of many membrane-bound, as
well as cytoplasmic complexes in neuronal processes together with the long and highly branched structure of the processes naturally support compartmentalization and active transport. In such an environment, the precise cellular localization and spatial arrangement of macromolecular complexes is essential for their function. Currently, with the resolution that is estimated to be around 5 nm (Baumeister, 2002), cellular cryo-ET is well suited to investigations of neuronal supramolecular architecture. With the prospect of reaching a resolution of 2 nm it will be possible to obtain pseudo-atomic maps of some of the neuronal signaling complexes.

The ability to supplement structural information at the molecular level obtained by cryo-ET, with functional information from fluorescent labeling is at the core of the correlative LM and cryo-ET. The use of FM dyes, commonly used to monitor endo- and exocytotic processes in neurons, led us in one of the examples to record a tomogram of a process with high endocytotic activity and allowed us to observe molecular details of these processes. Interestingly, the tomogram showed a medium sized (80 nm in diameter) vesicle carrying many membrane-bound complexes in the extracellular space, between two processes (Fig. 3). The presence of several molecular connections to both processes surrounding the vesicle may indicate that the vesicle was involved in signaling between these processes. We rarely see such extracellular vesicles: up to this point we have three examples out of more than forty tomograms. However, less then ten tomograms were correlated to a FM fluorescence spot. Furthermore, we normally record a tomogram only if we recognize a promising feature in an image taken before we start recording a tomographic series (the tomogram showing the extracellular vesicle being an exception, we recorded it because it was correlated to a fluorescence spot and despite the lack of interesting features in the image recorded before the series), so there is a possibility that we missed many extracellular vesicles of this kind because they are hard to recognize in pre-tomogram images. Therefore, we have too few examples to conclude if the correlative approach could lead us to record more tomograms of such extracellular vesicles. Nevertheless, finding a more specific marker, of in combination with cryo-ET, offers numerous possibilities. Following the delivery of proteins involved in the establishment and maturation of both pre- and postsynaptic sides, observing the changes in cytoskeleton that might accompany morphological changes of dendritic spines caused by the overexpression of AMPA receptor to postsynaptic density are just some of the possible applications. The correlation of light and cryo-electron microscopy provides an experimental platform for performing studies of this kind.

Acknowledgments

We thank Gabriela J. Greif and Andrew Leis for critical reading of the manuscript and Frank Voss for the preparation of neuronal cultures. This work was supported by the Max-Planck Society, Human Frontiers Science Program Research Grant, DFG SPP 1128 Grant and the Max-Planck-Forschungspreis.

References


