60th Anniversary Issue: Biological

Insights into the molecular organization of the neuron by cryo-electron tomography

Rubén Fernández-Busnadiego, Nikolas Schrod, Zdravko Kochovski, Shoh Asano, Dimitri Vanhecke, Wolfgang Baumeister* and Vladan Lučić

Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany

*To whom correspondence should be addressed. E-mail: baumeist@biochem.mpg.de

Abstract

Despite great progress in the identification and characterization of the key molecular players in neuronal function, remarkably little is known about their supramolecular organization. Cryo-electron tomography (cryo-ET), providing three-dimensional views of the molecular components of the cell in their native, fully hydrated environment, is uniquely positioned to elucidate the native architecture of the molecular machinery of the neuron. In our laboratory, we employ cryo-ET to study neuronal morphology in a variety of experimental systems and develop methods to extract quantitative and functional information from tomographic data. This approach has allowed us to shed light onto the intricate organization of the molecules of the synaptic cleft and the presynaptic cytomatrix, providing evidence for their functional roles. Also, cryo-ET of cultured neurons is beginning to open new perspectives on neuronal ultrastructure and the architecture of synaptic complexes in situ. Here, we will review these findings and discuss future directions towards the elucidation of the molecular landscape of the neuron.

Keywords

cryo-electron tomography, neuron, synapse, presynaptic cytomatrix, automated segmentation, correlative microscopy

Received 6 January 2011, accepted 15 March 2011

Introduction

Neurons and synapses

The neurons of our brain form a complex network that coordinates our actions, perceptions and thoughts. The pioneering work of Ramón y Cajal showed that neurons were discrete cells consisting of a cell body and specialized extensions (i.e. axons and dendrites) and that they communicated with each other at junctions today known as synapses. Most synapses in the mammalian central nervous system function by converting electrical signals traveling along axons into the release of chemical substances (neurotransmitters) from presynaptic terminals. Neurotransmitters bind receptors on the postsynaptic terminal of the partner cell, thus establishing synaptic communication. This functional asymmetry translates into synaptic ultrastructure. The presynaptic terminal contains neurotransmitter-filled synaptic vesicles and the machinery required for their exocytosis, as well as other organelles such as mitochondria and endoplasmic reticulum (ER). Exocytosis at presynaptic terminals occurs at the active zone (AZ), a specialized region of the presynaptic membrane exactly opposite to the postsynaptic side. The arrangement of synaptic vesicles at the presynaptic terminal is generally described in terms of functional pools. The so-called readily releasable pool (RRP) consists of vesicles immediately capable of releasing neurotransmitters upon Ca2+ influx. Vesicles released under moderate stimulation form the recycling pool. The reserve pool consists of vesicles that can be
mobilized only during intense stimulation. The postsynaptic side, normally located on dendrites, harbors a dense array of receptors, ion channels and signaling and scaffolding proteins known as the postsynaptic density (PSD). The synaptic cleft separates pre- and postsynaptic sides and contains cell adhesion molecules that are responsible for tight coupling and further signaling between the terminals.

Electron microscopy has played a pivotal role in our understanding of synaptic function. Early studies demonstrated the discrete nature of neurons, providing conclusive evidence for Ramón y Cajal’s neuron doctrine. The first details on synaptic ultrastructure were revealed by the visualization of synaptic vesicles [1], which were immediately hypothesized to mediate the observed quantal nature of neurotransmitter release [2]. Later studies showed (docked) vesicles making direct contact with the AZ [3] and correlated neurotransmitter release with vesicle exocytosis [4] (Fig. 1b). The observation of the thickening of the postsynaptic membrane [5] gave rise to the concept of PSD.

Cryo-electron tomography

In spite of the wealth of information obtained from those and other EM studies, the sample preparation protocols used were based on dehydration and heavy metal staining, not allowing structural analysis at the molecular scale. The aim of cryo-electron tomography (cryo-ET) is to visualize and identify three-dimensional (3D) biological structures at molecular resolution in a fully hydrated environment [6,7]. The native structure of biological samples is preserved through rapid cooling (vitrification) by either plunge-freezing, previously removing excess liquid by blotting with a filter paper (sample thickness <20 µm) [8] or high-pressure freezing (sample thickness <200 µm) [9]. Samples exceeding 0.5 µm in thickness require further thinning steps, such as cryo-ultramicrotomy [10] or focused ion beam milling [11] before imaging in the electron microscope (EM). Tomographic series acquisition consists of recording electron micrographs at different sample orientations, while adjusting for positioning and focusing errors. Most often, the sample is rotated around an axis perpendicular to the
electron beam. A 3D reconstruction (tomogram) is obtained by computationally merging the tomographic series.

Vitrified biological samples are particularly sensitive to electron irradiation, restricting the total electron dose applied to the specimen and consequently limiting the signal-to-noise ratio (SNR). This is aggravated by the low difference in density of the biological material and the embedding vitrified water (amorphous ice). In addition, several technical parameters (e.g. tilting geometry or detector performance) limit the resolution in cryo-tomograms and make it anisotropic. Various computational methods, such as denoising algorithms, are used to alleviate some of these limitations, increase the usable resolution and aid data analysis and interpretation.

Experimental systems
Three experimental systems widely used for neuroscience studies have so far been instrumental in the ultrastructural study of mammalian central nervous system synapses and neuronal processes by cryo-ET: isolated nerve terminals (synaptosomes), primary neuronal cultures and hippocampal slice cultures [12–15].

Synaptosomes consist of isolated synapses, detached from cells, that are obtained by fractionation of brain homogenates. They allow functional studies on neurotransmitter release and pharmacological treatments, as they retain physiological activity for several hours [16]. Synaptosomes are also advantageous for cryo-ET because they can be vitrified by plunge-freezing.

The high interconnectivity of primary neurons in culture and the relatively low cell density are among the features that make this system suitable for investigations of synaptic transmission, development or transport processes. Primary neurons, as well as some neuronal cell lines [17], can be cultured directly on EM grids and vitrified by plunge-freezing, making these systems suitable for cryo-ET.

Lastly, hippocampal slice cultures offer a direct view into nervous tissue, but due to their thickness they require high-pressure freezing and cryosectioning [18]. The difficulty of obtaining cryosections of good quality, the artifacts associated with the sectioning process and the need of using cryoprotectants to achieve complete vitrification of brain tissue hinder the routine use of this system in cryo-ET.

The molecular architecture of the synapse
Native organization of the presynaptic terminal
The presynaptic cytomatrix
In the presynaptic terminal, vesicles are embedded in a dense filamentous network known as the presynaptic cytomatrix. Both in frozen-hydrated synaptosomes (Fig. 2a) and hippocampal slice cultures (Fig. 2b), most synaptic vesicles were linked to each other by short (<40 nm) filaments (connectors, upper insets in Fig. 2a and b) and most vesicles in the proximity of the AZ were linked to it by similar strands (tethers, lower insets in Fig. 2a and b) [12]. Longer filaments were rarely observed, indicating that connectors and tethers are the most prominent features of the presynaptic cytomatrix in vitrified specimens (visualized in three dimensions in Fig. 2c).

Synaptic vesicle clustering
Short filaments interlinking vesicles were previously observed in dehydrated samples [19–22] (Fig. 1c and d). Some of these studies also reported longer strands identified as actin, resulting in a model in which vesicles were linked to each other and to actin filaments through short connectors, thus restricting vesicle movement in resting synapses. It was proposed that the connectors consisted of synapsin and that synaptic activity caused phosphorylation of synapsin and its dissociation from actin filaments, thus freeing vesicles from the restraining matrix and allowing fusion. Later work showed that synapsin is not the only molecule implicated in connector formation, as some of these structures persisted in triple synapsin knockout mice [22].

The low abundance of actin filaments in both frozen-hydrated synaptosomes and hippocampal slices argues against a major role of actin filaments in synaptic vesicle clustering. Furthermore, the extensive vesicle connectivity observed in untreated synaptosomes and hippocampal slices was
drastically reduced upon prolonged membrane depolarization by KCl and phosphatase inhibition by okadaic acid. Thus, our observations call for a revision of the classical model of vesicle clustering, indicating that this process is mainly mediated by the short inter-vesicular connectors. Whereas in resting synapses, most vesicles are connected to at least one of their neighbors, a large number of connectors are removed upon synaptic stimulation (Fig. 2d).

Even though the molecular identity of the connectors remains to be determined, our data suggest that connectors are formed by more than one molecular species and that these are differentially distributed within the presynaptic terminal and behave differentially upon phosphorylation. While synapsin is likely to be involved in vesicle organization away from the AZ [20,22], synaptobrevin might be implicated in connector formation in the proximal region to the AZ (0–45 nm), as suggested by the strong reduction of connectivity in this area upon tetanus toxin treatment.

Fig. 2. Presynaptic morphology visualized in tomograms of frozen-hydrated synapses. Synaptic vesicle (SV), mitochondrion (mit), synaptic cleft (SC) and PSD. (a) Synaptosome. Upper inset: connector linking two vesicles (black arrowhead). Lower inset: tether linking a vesicle to the AZ (white arrowhead; same vesicles as in the upper inset at another z-slice). (b) Organotypic slice. Synaptic vesicles are compressed along the cutting direction (white arrow). Upper inset: connector linking two vesicles (black arrowhead). Lower inset: tethers linking a vesicle to the AZ (white arrowheads). Scale bars: 100 nm in main panels and 50 nm in insets. (c) 3D rendering of a synaptosome and corresponding tomographic orthoslices. Synaptic vesicles (yellow), connectors (red), tethers (blue), mitochondria (light blue), microtubule (dark green), plasma membrane (purple), cleft complexes (green), PSD (orange). Proposed models for connector (d) and tether (e) function in synaptic vesicle mobilization and fusion. The number and topology of connectors and tethers shown in (d) and (e) are based on average values. Shaded vesicles in the bottom panel of (d) represent the decrease in vesicle number upon prolonged stimulation.

Synaptic vesicle association with the AZ, exo- and endocytosis

At the ultrastructural level, the mechanisms of vesicle association and fusion at the AZ remain controversial because of the disparity of the results obtained with different EM sample preparation techniques. Chemically fixed and heavy metal-stained samples showed a regular arrangement of dense projections at the AZ around which vesicles would organize and fuse [23] (Fig. 1a). However, quick freezing revealed that those dense projections likely represented collapsed filaments and that
filaments of different length link vesicles to the AZ [19,22,24]. In heavy metal-stained samples, vesicles making direct membrane contact with AZ are commonly referred to as ‘docked’, and a subset of them is thought to form the RRP.

In frozen-hydrated synaptosomes and hippocampal slices, vesicles making direct membrane contact with the AZ were extremely scarce, whereas a large majority of vesicles in the vicinity of the AZ were linked to it by up to 40 nm long tethers. Most of these tethers were in fact shorter than 10 nm, suggesting that they might have been obscured by heavy metal staining in previous studies. Therefore, our results indicate that direct membrane contact between vesicle and AZ occurs only transiently during vesicle fusion and that tethering is the main mechanism of vesicle association with the AZ. Furthermore, even though densities were observed on the AZ of vitrified synapses, they did not form a regular arrangement and there was no correlation between their positions and those of tethered vesicles.

Two distinct populations of synaptic vesicles were differentiated based on the effects of hypertonic sucrose (causes exocytosis of the RRP) and tetanus toxin [blocks soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex assembly] on the length and number of their tethers. Long tethers were not affected, indicating that they do not participate directly in vesicle fusion. Under both treatments, the number of vesicles with multiple short tethers was significantly reduced, suggesting that these vesicles constitute in fact the RRP and that the formation of short tethers requires SNARE complex assembly. The model of vesicle tethering and fusion emerging from these observations is summarized in Fig. 2e.

In the first step, long tethers, which do not require full SNARE complex zippering for their assembly, mediate the initial association of the vesicle to the AZ. Once the vesicle is held in the proximity of the AZ, shorter SNARE complex-dependent tethers may form. The formation of a sufficient number of these short tethers (more than two under our experimental conditions) constitutes the priming step, by which vesicles become readily releasable. Synaptic stimulation causes a Ca²⁺ influx that triggers direct membrane contact of primed vesicles and the AZ, immediately followed by fusion.

Similar models have been proposed for tethering and fusion of vesicles at the ER [25], Golgi apparatus [26] and chromaffin cell periphery [27]. In all these cases, the initial vesicle tethering is not mediated by a fully assembled SNARE complex. Furthermore, our estimate on the number of SNARE complex-dependent short tethers necessary for vesicle fusion is in perfect agreement with recent data [28]. In addition, the model arising from our cryo-ET observations clarifies the discrepancy between vesicle docking and availability for release: whereas in dehydrated samples, all vesicles in the vicinity of the AZ seem to be docked, these actually correspond to a superposition of different tethering states in fully hydrated specimens, and therefore to vesicles with different states of assembly of the fusion machinery. Different candidates such as syntaxin [27], the tripartite Munc13/RIM/Rab3 complex [29] or bassoon [30] have been proposed to form synaptic vesicle tethers, but further structural research is necessary to clarify the exact role of these molecules and their arrangement at the AZ.

Following exocytosis, new synaptic vesicles are formed by membrane retrieval mechanisms. Our tomograms provide glimpses of these endocytic phenomena, such as clathrin-coated vesicles or endosomes at different maturity states, and allow the study of the endocytic machinery in native conditions (Fig. 3a–c).

The synaptic cleft and the PSD
The cryo-ET reconstruction of synaptic adhesion complexes of the mammalian central nervous system in their native state showed that they are extensively laterally connected and that they have non-trivial topology [14]. A layer of increased density was observed in the central region of the cleft, consistent with some earlier reports describing an ‘intercellular plaque’ [31], which appeared similar to the well-known basal lamina of the (significantly wider) neuromuscular junction. However, the 3D analysis of this density in frozen-hydrated synapses showed that it had topology of a net, rather than that of a sheet. A similar electron-dense midline was also observed in cryo-ET of native epidermal desmosomes [32]. While the desmosomal
adhesion complexes are formed exclusively of desmosomal cadherins and display a high degree of regularity, the synaptic adhesion complexes appear more disordered, probably due to their heterogeneous composition. Subtomogram averaging of desmosomal complexes (Fig. 3 of [32]) yields a structure that appears to have topology of a net (due to structural interactions between cadhedrins in regions close to the membranes in addition to the complex lateral interactions at the midline), but the topology of the non-averaged complexes remains unclear.

A very complex structural organization is typically observed in the PSD of frozen-hydrated synapses. Upon closer inspection, a dense network of filamentous structures oriented perpendicular and parallel to the synaptic cleft can be discerned, both in the synaptosomes (Fig. 3d) and in cultured neurons [13], in agreement with a previous study on high-pressure frozen, dehydrated synapses [33]. We expect that the combination of advanced image-processing methods that aim to enhance repetitive structures (local means filtering), segment main constituents of the PSD (e.g. watershed and connectivity-based segmentation, see the following section) and detect filamentous structures (scaling index-based segmentation [34]) will help to further clarify the functional organization of the molecular components of the PSD.

**Image processing**

The biological complexity and low SNR often make the interpretation of cryo-tomograms a challenging task. In our laboratory, we have implemented an image-processing workflow specifically tuned to extract structural information from neuronal samples (Fig. 4). First, tomograms are denoised by anisotropic nonlinear diffusion (AND), an algorithm adapted for cryo-ET [35], resulting in a reduction of noise and enhancement of the features of interest.

Data interpretation usually begins by membrane segmentation. Both internal and plasma membranes tend to have a high contrast that allow their manual segmentation, possibly with the help of routines that streamline this time-consuming step for some specialized cases. Visual detection of finer, non-uniform structures, such as connectors linking synaptic vesicles, is difficult in 3D and prone to errors. We developed an automated segmentation method to detect such structures in an objective, reproducible and comprehensive way. This method is applicable to structures that contact one or more already segmented features (e.g. membranes) and combines thresholding and connectivity. In short, a region of interest is thresholded and only the clusters of connected voxels that contact the specified membranes(s) are retained [12,13].

The choice of threshold is obviously very important. A threshold can be chosen by visual

---

**Fig. 3.** Morphology of clathrin-coated vesicles, endosomes, PSD and synaptic cleft in frozen-hydrated synaptosomes. (a) Clathrin-coated vesicles. (b) Tomographic slice (top) and cut-open manual segmentation (bottom) of an early endosome having an invagination. (c) Tomographic slice (top) and manual segmentation (bottom) of a late endosome (multi-vesicular body) containing several vesicles. (d) Tomographic slice with overlaying manual segmentation showing synaptic adhesion complexes (green), PSD (orange) and postsynaptic actin filaments (brown). Insets: raw tomographic data at different z slices. Scale bar: 100 nm.
inspection, but this method is subjective and, moreover, an optimal threshold may not exist at all. Our approach for the segmentation of the molecular complexes present in the synaptic cleft was to segment these complexes at a wide range of different threshold values. The subsequent analysis at all thresholds showed that a chosen feature (Euler characteristic) was only weakly dependent on a particular threshold [13]. A more sophisticated method combining the watershed transform [36] and connectivity-based segmentation was employed for the segmentation of the connectors and tethers of the presynaptic terminal [12]. This approach allowed us to strongly reduce the influence of variable background values.

Having detected the structures of interest, their gray-scale levels, morphological features and location and relationship to other structures are analyzed for each tomogram. In cases where tomograms were obtained at different conditions (such as under different pharmacological treatments), the results from the same conditions are grouped and the differences between the conditions are statistically analyzed. All automated segmentation and analysis steps are performed using custom-made software written in Python.

In sum, the ability to perform analysis at this scale, which we hope to advance towards a usability level of standard biochemical assays, rests on two premises: optimal sample preservation, which makes possible the detection of fine structures, and automated feature detection to allow comprehensive and detailed analysis.

**Multi-scale imaging of intact neurons**

**Ultrastructure and beyond**

Vitrified neuronal cultures offer the possibility of studying the architecture of neuronal complexes in situ and correlating it with functional information obtained by light microscopy (LM). Upon plunge-freezing, the thickness of the vitrified layer at cell bodies and their immediate surroundings would require thinning procedures. On the other hand, vitrified neuronal processes (axons and dendrites) are often below 0.5 µm in thickness, allowing direct cryo-ET investigation.

During early developmental stages, only few processes emanate from cultured neuronal cells, but these processes become predominant at later stages. Cytoskeletal elements are very prominent in neuronal processes. Microtubules are found in all
dendrites and axons, ranging in number from only one in the thinnest axons to more than 10 in larger processes. Cryo-tomograms of neuronal processes showed that microtubules contain lumenal densities, organized in discrete globular particles [15]. Figure 5 shows the morphology of a typical dendrite containing a dendritic laminar body, an organelle found in the proximity of dendrodendritic gap junctions and involved in their synthesis [37]. Tubules and sacks of the smooth ER are typically found winding around microtubules in both dendrites and axons. Mitochondria are often observed, as well as the elements of the secretory system, from early to late endosomes such as multivesicular bodies.

Cultures of mature neurons offer the possibility to investigate synaptic organization. However, mature cultures, even if grown at low density, develop a dense network of processes that often completely covers the EM-grid support film. As a consequence, a large part of the grid surface is covered with thick ice, exacerbating the difficulty of searching for features of interest and tilt series acquisition, and consequently decreasing the yield. Nonetheless, characteristic presynaptic features, such as clusters of synaptic vesicles and tubulovesicular membranous compartments, can be observed in regions of thin ice. Moreover, elements of synaptic molecular complexes, such as distinct filaments within the PSD, and structures protruding from synaptic vesicle membrane were visualized in their natural, cellular environment [13]. Currently, cryo-ET of intact neurons is approaching the level where it can be used to verify structural observations obtained in synaptosomes and put them in a wider, cellular context, bypassing the technical difficulties associated with vitrified slice cultures.

**Correlative LM and cryo-ET**
The large array of fluorescent probes currently available and the widespread use of fluorescence...
microscopy have provided insights into the function and spatial distribution of a wealth of molecules. Resolving their precise localization and supramolecular architecture should help us understand the principles underlying a large number of cellular processes. Correlative microscopy is a hybrid method that allows imaging of the same feature over multiple length scales, combining LM with the ability to obtain high-resolution information in EM [38]. The correlative LM and cryo-ET approach allows the minimization of sample irradiation during the search for features of interest in vitrified neuronal cultures, a process usually hindered by the dense network of neuronal processes and their sensitivity to the electron irradiation.

Dedicated finder grids provide basic orientation and are of significant help for correlation. In developing neuronal cultures where processes are sparse, correlation purely based on visual cues can be achieved [15]. Aligned and superimposed grid scans acquired in the LM and EM are needed in more crowded samples [17]. In dense cultures, the higher reliability and precision of a purely computational approach are required to locate features of interest, such as synapses [13]. Visually detectable landmarks in the LM and the EM, e.g. grid bars, are used to establish a coordinate transform between the pixel-based coordinate system in the LM images and the physical coordinates of the EM goniometric stage. Afterwards, the transformation can be used to retrieve the EM stage positions of features of interest (e.g. fluorescent spots).

Our correlative procedure is schematically depicted in Fig. 6. We commonly culture neurons on gold EM finder grids covered with a holey carbon film (such as Quantifoil™ R2/2), as they offer enough surface for neurons to adhere on, allow blotting from the face of the grid opposite of the one where neurons are located and can aid the correlation step. The features of interest do not need to be located on the holes of the carbon film, as tomograms can also be recorded over carbon (typically with lower SNR). After being fluorescently labeled and plunge-frozen, neurons are imaged in LM. The new generation of cryo-holders for LM, suited for both trans- and epi-illumination, has improved stability and low contamination rate, allowing continuous imaging over few hours [11]. Vitrified cells are caught in a snapshot and therefore can be imaged in exactly the same state in LM under cryo conditions (<−150°C) and in cryo-ET. This is especially important when dynamic events

![Fig. 6. Workflow of cryo-ET of neuronal cultures with correlative microscopy.](image-url)
such as vesicle release or transport processes are studied. On the other hand, the currently available cryo-LM holders are restricted to long working distance (above 2 mm) air objectives and cannot reach the resolution obtained at room or physiological temperatures. The importance of a careful morpho-
logical assessment of vitrified cells in both LM and EM was underscored by a recent study, showing that around 30% of mammalian kidney cells had a compromised plasma membrane during the blotting procedure [39]. However, only damage to regions of the cell in the vicinity of the spot of interest is relevant, as the high speed of the freezing procedure prevents long-range propagation of damage.

A fair amount of grid handling and transfer steps necessary for correlative microscopy carry the risk of grid deformation and damage. The gold grids typically used for cell cultures are particularly malleable, resulting in reduced yield and lower precision of the correlation. This problem was alleviated by the introduction of the AutoGrid™ sample holder (FEI, The Netherlands), a support ring that drastically improves frozen grid handling and preservation, and by avoiding excessive LM before vitrification and relying on LM under cryo conditions instead.

An example of the correlative approach used in our laboratory is given in Fig. 7, where a synaptic bouton fluorescently labeled with both endocytotic marker (FM 1-43) and an antibody against a synaptic protein (synaptotagmin) was located and recorded in cryo-ET. Although the endocytotic marker provides a stronger signal, the antibody labeling is more precise and their overlay reliably indicates sites of synaptic vesicle exo/endocytosis. Thanks to the aforementioned developments, we now typically obtain correlation precision of 1–2 μm.

Future directions

Our understanding of synaptic structure and function has increased drastically through complementary approaches in the last decades, but some fundamental questions remain unanswered. Specifically, determining the exact location of key
molecules and the composition of macromolecular complexes may allow a much more detailed and comprehensive view of the neuron. Cryo-ET is emerging as a powerful tool in clarifying some of these issues by revealing the molecular architecture of neurons in native conditions. The identity of some of the proteins present in cryo-tomograms is likely to be determined directly based on their structural signatures by the already established technique of pattern matching [40]. Subtomogram averaging routines have the potential to increase the resolution of structures of sufficiently abundant larger molecules or complexes found in cryo-tomograms. Coupled with carefully designed genetic and pharmacological manipulations, this approach could lead to the determination of the composition and architecture of such complexes. These techniques may profit from emerging innovations in instrumentation, which are expected to improve resolution and to reduce the influence of noise. Last but not least, hybrid approaches such as correlative microscopy can bring an additional functional dimension to cryo-ET, and new thinning procedures such as focused ion beam milling may increase the range of specimens amenable to cryo-ET. Therefore, we expect that in the next few years cryo-ET will bring a fresh perspective to the cell biology of the neuron.

References


