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Three-Dimensional Imaging of *In Situ* Specimens with Low-Dose Electron Tomography to Analyze Protein Conformation

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Abstract: We describe a novel three-dimensional (3-D) imaging tool for analysis of protein conformation of *in situ* samples. Sidec™ (Sidec Technologies AB, Stockholm, Sweden) electron tomography (SET) uses low-dose electron tomography and a refinement algorithm to reconstruct individual proteins and macromolecular complexes. The approach has successfully reconstructed therapeutic proteins in solution. In this study, we investigate the use of SET to visualize ion channels in cells and tissue samples. SET successfully resolved the volume and structural features of the target complex, showing that it was a tetrameric channel with a central pore. The technology could distinguish and provide 3–D images of the intra- and extracellular domains in the ion channel. In addition, SET was able to show that the channel associates in the form of a tetramer with the four subunits preorganized into dimers. While additional studies using smaller antibody markers are needed to resolve the subunit assembly further, this study demonstrates that SET is a valuable tool for visualization of *in situ* specimens and can provide important information on the subunit assembly of these macromolecular complexes, and thereby aid in the screening assay process in drug development.

Introduction

Ion channels are interesting drug targets as several common diseases are attributable to ion channel dysfunction, including cardiac arrhythmias, diabetes, hypertension, angina pectoris, and epilepsy. Recent breakthroughs, including the development of genomic technologies, implementation of structure-based drug design, and increased understanding of the composition of heteromeric ion channels, can revolutionize the ion channel assay systems.[1] However, a major challenge in ion channel research has been an efficient method to visualize the subunit assembly and structural dynamics of these macromolecular targets when they interact with drug compounds. This study investigates the ability of SET to provide information on the protein conformations of ion channels of *in situ* specimens.

SET is an innovative imaging tool that reconstructs 3-D images of proteins and macromolecular complexes of *in situ* and *in vitro* samples. ET employs transmission EM to take multiple tilted images of an object. These images, or micrographs, are then assembled into 3-D images using reconstruction algorithms (Fig. 1). ET is a general method[2–5] that can be applied to studies of macromolecular complex assemblies without any requirement for crystallization.[6] The novelty of SET is that it uses an algorithm (Sidec COMET [a patented mathematical procedure for optimizing the usage of information from physical measurements]) that is based on constrained maximum entropy principles. The main merit of using Sidec COMET is that it deconvolves the blurring effects of the contrast transfer function.[7] Moreover, Sidec COMET also reduces the effects of measurement errors on the 3-D reconstruction, thus improving the fidelity of

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ABBREVIATIONS: 2-D, two-dimensional; 3-D, three-dimensional; COMET, COnstrained Maximum Entropy Tomography; DRG, dorsal root ganglia; EM, electron microscopy; ET, electron tomography; PBS, phosphate-buffered saline; RIN, rat insulinoma; SET, Sidec™ (Sidec Technologies, Stockholm, Sweden) electron tomography

3-D reconstructions and filtering much of the background noise in electron micrographs. A corollary therefore is that low-dose radiation is sufficient during the image capture stage for high-resolution protein reconstructions. By combining low-dose cryo-ET with the Sidec COMET algorithm, SET can reconstruct 3-D high-resolution images of proteins in their native conformational states.

SET is a well-established method for analysis of therapeutic proteins in solution.[8] In one application, SET was used to visualize the epitope and to record images of protein conformations as antibody–antigen molecules interact. Samples of a single-chain antibody that binds to human complement factor C5 were supplied as proteins in solution by BioInvent International (Lund, Sweden) and analyzed by Sidec (Stockholm, Sweden). Using SET, it was possible to observe different protein conformations as the antibody bound to the antigen and the size of the area involved in binding,[9] thus providing valuable information on the conformational dynamics of the antibody–antigen complex. SET was also able to accurately reconstruct the position of the antigen epitope. Theoretically, the technique could also be used to test two antibodies against the same antigen to obtain data on competitive interactions between antibodies. This information can, in turn, yield insights into selection of the most appropriate antibody from a large library of antibodies for a particular antigen.

In this study, we report the use of SET to visualize protein conformations of *in situ* ion channel specimens. Four isoforms of the pore-forming subunits for the ion channel of interest have been observed. Each subunit is thought to consist of two transmembrane domains with a large extracellular protein component, whereas the channel itself is thought to be a multimeric assembly of one or more sub-

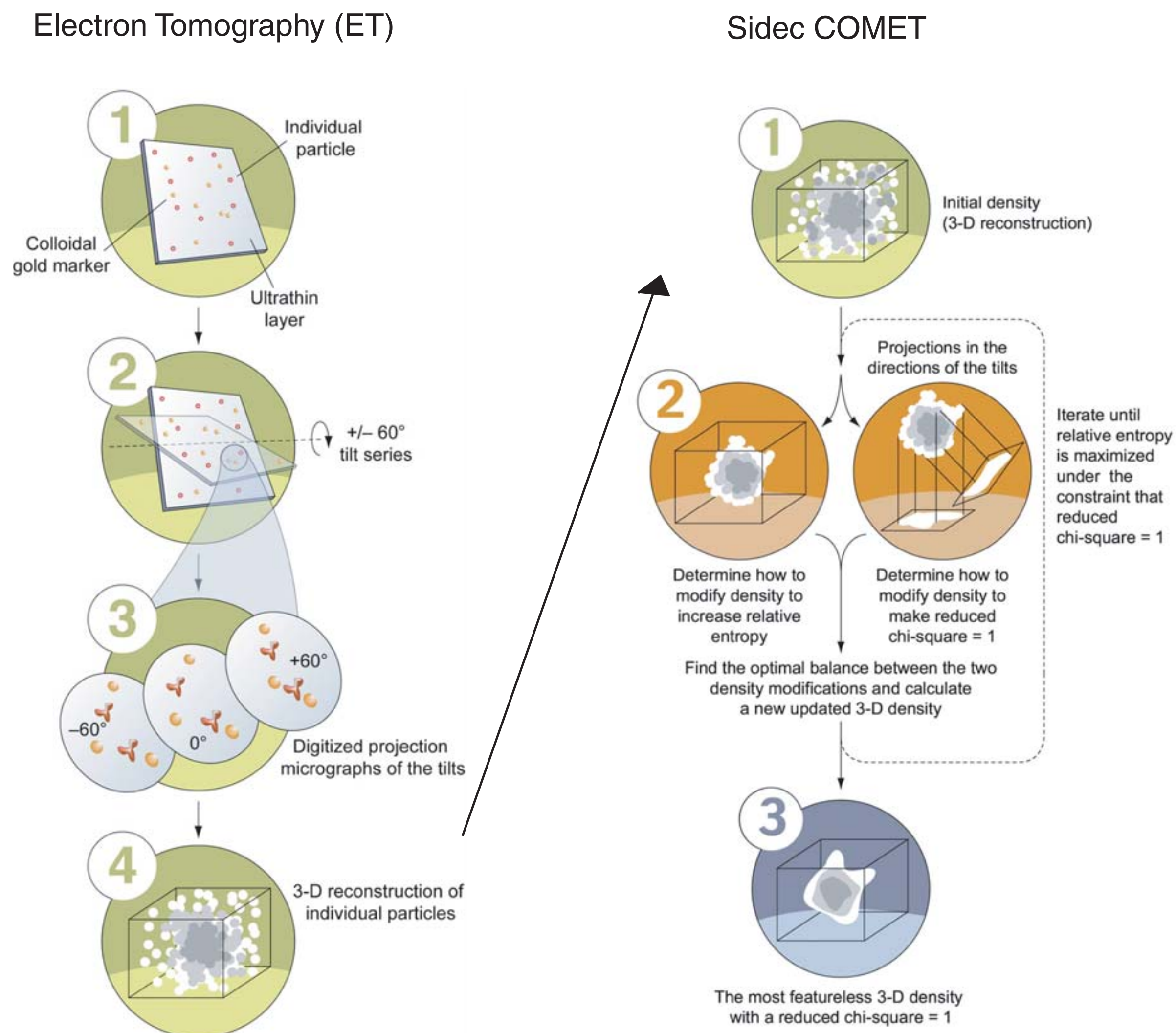

FIG. 1. Schematic of the SET process. Standard ET uses transmission EM to take a tilt-series of micrographs that are then assembled into 3-D images at a resolution of 50–80 Å, revealing cellular features. The Sidec COMET algorithm iteratively refines the image until maximum entropy is obtained to achieve a 3-D reconstruction of the molecules of interest at a resolution of 20–30 Å.

unit isoforms. However, conclusive evidence for the subunit assembly and stoichiometry of the ion channel is lacking. Since the pharmacological properties of ion channels are inextricably linked to their stoichiometry and because such information is presently not available on a routine basis, SET was employed to analyze the assembly of the ion channel and to provide information on protein conformations of the macromolecular complex. Samples were supplied by AstraZeneca (Södertälje, Sweden), and sample preparation and SET analyses were performed at Sidec. SET originates from research[4] conducted at the Karolinska Institute in Stockholm, and is commercially available through Sidec Molecular Imaging, a contract research facility provided by Sidec Technologies.

Materials and Methods

Samples

Three RIN cell line samples and one rat DRG tissue sample were provided by AstraZeneca. The cell line samples consisted of (1) RIN cells with a stable expression of one protein (ion channel subunit x), (2) RIN cells with a stable coexpression of two different proteins (ion channel subunits x and y), and (3) non-transfected RIN control cells. The RIN control cells were used only for 2-D EM experiments to evaluate the antibodies used in the study.

Polyclonal rabbit antibodies towards the two isoforms of interest were provided by AstraZeneca. The antibodies had been affinity-purified and used for cell assays (fluorescence-activated cell sorting), but no immunohistochemistry experiments had been performed with these antibodies. An additional polyclonal guinea pig antibody towards one isoform was obtained from Neuromics Inc. (Minneapolis, MN) for double-labeling experiments. This antibody had been affinity-purified and tested for immunohistochemistry experiments.

Sample preparation

To ensure structural and functional integrity of the proteins, RIN cells and DRG tissue samples were chemically fixed.

Fixation of cells. The RIN cells were fixed in 4% formaldehyde, 0.1% glutaraldehyde, 0.05 M sucrose, and 0.1 *M* phosphate buffer (pH 7.4) for 5 min. The fixative was added at room temperature to the cultivation flask. The cells were harvested, and the resulting cell suspension was centrifuged at 1,300 rpm for 10 min. The fixative was removed, yielding total fixation times of approximately 20 min for the three different cell cultures. Cell pellets were washed in 1× PBS and immersed in 2.3 *M* sucrose in PBS buffer for 60 min. Thereafter, the sample was mounted as microdroplets of dense cell suspension in sucrose on cryo-ultramicrotome specimen holders and immediately frozen in liquid nitrogen. Specimens were stored in liquid nitrogen until sectioned.

Fixation of DRG tissue. DRG capsules were dissected from the rat following perfusion fixation. The rat was sedated, its abdominal cavity was opened, and a needle was inserted in the abdominal aorta. An incision was made in the inferior vena cava and the perfusion pump started. The vascular bed was flushed with PBS at room temperature at a flow rate of 15 ml/min for about 2 min to clear the blood vessels from cells. Thereafter, fixative (4% formaldehyde, 0.1% glutaraldehyde, 0.05 *M* sucrose, and 0.1 *M* phosphate buffer, pH 7.4) was added at a flow rate of 15 ml/min for approximately 8 min. The DRG capsules were dissected from the rat after an additional 7 min and immersed in fixative to yield total fixation times of 20 min, 40 min, and 60 min, respectively. Subsequent analyses were conducted using only samples from the 20-min fixation time.

The DRG capsules were washed in a 1% formaldehyde in PBS buffer. Sample blocks from the inner parts of the capsules were selected and trimmed to ~0.5 mm^3 manually under a stereomicroscope. The blocks were placed in 2.3 *M* sucrose in PBS buffer for 60 min. Tissue blocks were mounted on cryo-ultramicrotome specimen holders and immediately frozen in liquid nitrogen. Specimens were stored in liquid nitrogen until sectioned.

Cryosectioning. Cells and tissue samples were cryosectioned on a DiAtome cryo-immuno diamond knife in an Ultracut UCT cryo-ultramicrotome with FCS chamber equipped with a DiAtome Static Line 11 antistatic device (all from Leica Mikrosysteme GmbH, Vienna, Austria). The outer surface of the specimen blocks were initially trimmed to ~0.4–0.3 mm^2 at −80°C in increments of 100 nm. Toluidine blue-stained 200-nm-thick sections of the tissue samples were visually inspected in a light microscope, and the specimen was trimmed according to where the most DRG cell bodies were found. Subsequently, ultrathin sections (75–90 nm) were sectioned at −105°C, retrieved by a 2.3 *M* sucrose droplet, thawed, and applied to an EM sample-holder grid. The grids were placed in cold PBS for up to 1 h before immunolabeling.

Immunolabeling. Nonspecific binding sites were blocked by incubating the sections on grids, with the section-side facedown, in 0.1 *M* glycine in PBS for 5 × 2 min, followed by 10% fetal calf serum in PBS for 20–40 min. Sections were incubated with primary antibody in an incubation buffer (1:1 molar ratio of 0.1 *M* glycine in PBS and 10% fetal calf serum) for 60 min. Initial 2-D EM experiments showed that the binding of the guinea pig antibody towards one isoform was equal to or better than that of the polyclonal rabbit antibody. Thus, a com-

bination of rabbit polyclonal antibodies (AstraZeneca) in 20 μg/ml concentrations and guinea pig polyclonal antibodies (Neuromics Inc.) in a 1:100 dilution were used as primary antibodies for the double-labeling experiments. Double-labeling was used on the cotransfected cell lines and on the tissue specimens to test for heterogeneous channels. Specimens were washed in PBS (4×2.5 min) and incubated with gold markers conjugated with the secondary antibody in an incubation buffer for 35–60 min. Colloidal gold (6-nm) goat anti-rabbit IgG and colloidal (4 nm) gold donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in 1:50 dilutions were used as secondary antibodies for the 3-D ET single- and double-labeling experiments. Colloidal gold (12-nm) goat anti-rabbit IgG (Jackson ImmunoResearch) and colloidal gold (10-nm) goat anti-guinea pig IgG antibodies (British BioCell International, Cardiff, UK) in 1:50 dilutions were used as secondary antibodies for the 2-D EM single-labeling experiments. Sections were washed in PBS (4×2.5 min) and distilled H_2O (6×1 min) before contrasting with 2% uranyl acetate in distilled H_2O for 3–4 min. Finally, specimen sections were embedded in polyvinyl alcohol to dehydrate the samples before EM.

EM. Formvar-coated electron microscope grids (Cu, 300×75 mesh, Agar Scientific, Stansted, UK) were glow-discharged in an evaporator (model E306, Edwards High Vacuum, Crawley, UK). Samples for the 2-D EM experiments were applied directly to these grids. Grids for the 3-D ET experiments were further treated with 10-nm colloidal gold labeled protein A markers (Amersham Biosciences, Amersham, UK) that were inserted in a double layer of carbon. The gold markers were subsequently used for alignment of the tilt-series of electron micrographs.

The grids were inserted and analyzed in a Philips (Eindhoven, The Netherlands) CEM200 FEG transmission electron microscope, run at 4.48 kV extraction and 200 kV acceleration voltages. Data were collected at room temperature. An automatic low-dose tilt-series of images at 1°-tilt or 2°-tilt intervals in the range $\pm 60°$ to $\pm 65°$ were recorded with a slow-scan camera ($2{,}048 \times 2{,}048$ CCD chip, pixel size 14 mm; TVIPS GmbH, Gauting, Germany) using EMMENU software. Images were captured at a magnification of $24{,}900 \times$ yielding a final pixel size of 5.63 Å. The accumulated dose on the exposed area of the grid was 15–20 $e^-/Å^2$, which is considered low enough to preserve the atomic structure of biological macromolecules.

Macromolecular structure analysis. The conjugated gold markers (6-nm colloidal gold goat anti-rabbit IgG and 4-nm colloidal gold donkey anti-rabbit IgG antibodies) were used to locate the proteins of interest in the 3-D analysis. Gold markers were localized and measured, and the image contrast was adjusted to a value corresponding to that of protein density. All proteins including the primary and secondary antibodies, and the ion channel were evaluated with respect to size and shape. The colloidal gold particles applied to the grid were used to align the micrographs and to determine the overall geometry of the tilt-series. The average alignment error was between 4 and 8 Å. A set of 3-D reconstructions were computed for each aligned tilt-series. The x and y midpoint of each reconstruction was selected with respect to the x and y positions of the secondary antibody gold markers found in the 0° tilt-series image.

The 3-D tomographic reconstructions were performed using SET. Most 3-D volumes were $256 \times 256 \times 256$ or $360 \times 360 \times 360$ pixels in size (1 pixel = 5.63 Å). The volumes (tomograms) were reconstructed using conventional filtered back-projection and then refined using the COMET procedure. Sidec COMET is an algorithm that improves the signal-to-noise ratio of 3-D reconstructions by modifying the density of each molecule so that the relative entropy is increased and simultaneously the fit to the micrographs, in terms of least squares, is increased. The reconstruction is thus the most featureless 3-D density that fits the projection data. The Sidec COMET refinement of each tomogram was run for 10 iterations. Reconstructed volumes were visualized using BOB software (Ken Chin-Purchell, University of Minnesota, Minneapolis, MN).

Two tilt-series were recorded on the single-labeled RIN cells that expressed the one protein (ion channel subunit x). From these two tilt-series, 29 3-D volumes were reconstructed and analyzed individually. Three tilt-series were recorded on the single-labeled RIN cells that co-expressed the two different proteins (ion channel subunits x and y, antibodies towards subunit x), and 19 3-D volumes reconstructed and analyzed separately. Eight tilt-series were also recorded on co-transfected RIN cell sections that were double-labeled towards subunits x and y. From these eight series, 58 3-D volumes were reconstructed and analyzed individually. In addition, 10 tilt-series were recorded on double-labeled rat DRG tissue sections with antibodies towards subunits x and y. From these 10 tilt-series, 96 3-D volumes were reconstructed and analyzed individually.

Results and Discussion

2-D EM to evaluate antibody binding

The 2-D EM experiments of single- and doubled-labeled specimens were performed initially to judge the specificity and affinity of the antibody markers used in the study (Fig. 2). The 2-D analysis of the non-transfected RIN control cells showed no background labeling of primary or secondary antibodies. The single-labeled RIN cells indicated that the one isoform was localized mainly

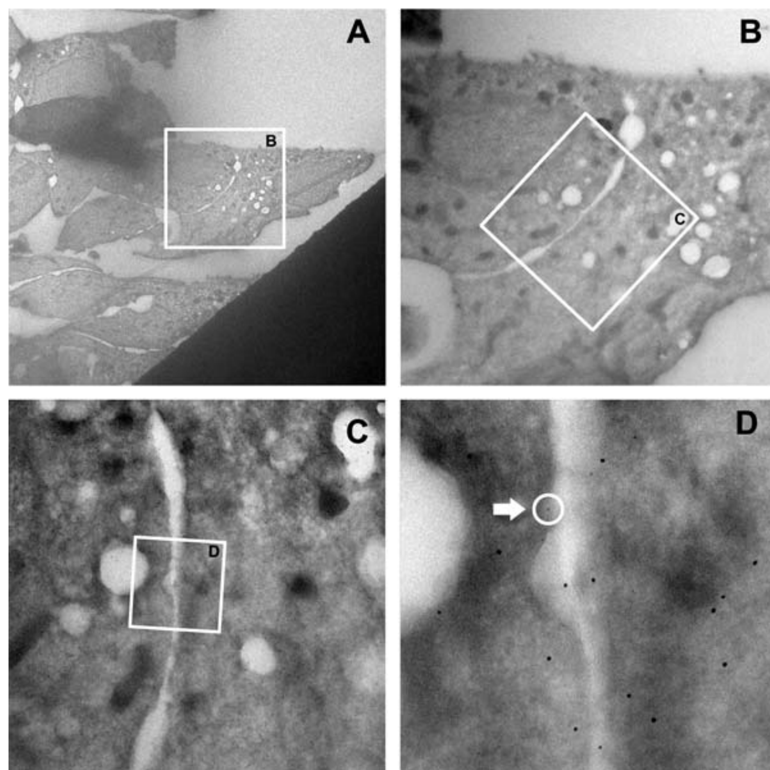

FIG. 2. Close-up of a sample illustrating the level of magnification at which an SET experiment is performed. One of the RIN cell sections used in the study is shown at (**A**) 500×, (**B**) 1,500×, (**C**) 3,800×, and (**D**) 15,000× magnification. In the micrograph in (A) several cells can be seen. This magnification (500×) was used to choose an area where close cell–cell contact was observed. (B) Increased magnification of the area chosen in (A). A portion of two RIN cells are seen in this micrograph, one with its nucleus to the left and the other cell with part of its nucleus to the right. The white curved line in the center of the image is the extracellular space. (C) Zoomed-in micrograph illustrating the two plasma membranes shown in (B) in further detail. (D) Enhanced magnification (15,000×) showing the final SET experimental area. The black dots are the 10-nm colloidal golds used for alignment of the tilt-series (see Materials and Methods). The individual proteins are not visible in the 2-D micrograph, but appear only after 3-D reconstruction of the tilt-series experiment. One of the 6-nm marker golds found in the plasma membrane of one of the RIN cells is indicated by an arrow and circled. This marker gold is linked to the ion channel via secondary and primary antibodies. The position of this marker gold has been used as a center point for the 3-D reconstruction. Depending on how many marker golds are found in the sample, several 3-D results can be generated from the SET experimental area.

in the plasma membrane, while the other protein was predominantly found intracellularly. The 2-D analysis of double-labeled RIN cell sections confirmed these differences in the distribution of the two isoforms. Moreover, the 2-D experiments with the RIN cells suggested that the one isoform is approximately twice as common as the other protein. In contrast, the 2-D analysis of the DRG tissue sections indicated that the more abundant isoform was in fact about 10 times more frequent than the other protein.

3-D visualization of the ion channel

SET resolved the 3-D structure of the *in situ* ion channel in cell and tissue specimens. Results showed that the ion channel associates in the form of a tetramer with a central pore that spans approximately 15–17 nm in height and 9–10 nm in width (Fig. 3). SET accurately reconstructed the protein conformations of the extracellular and intracellular components of the channel. The extracellular part of the ion channel resembles the crown of a tooth (see Fig. 3A), while the dimer units that make up the tetrameric channel are juxtaposed with lobes extending longitudinally. Generally, it was harder to establish ion channel boundaries for the DRG tissue specimens than for the RIN cell samples because of close contact between the channel proteins and associated partner proteins. Nevertheless, the technique was able to resolve ion channels in tissue specimens and proved that SET can be used for 3-D reconstruction of *in situ* ion channels, thus making it possible to study protein conformations as well as subunit composition of macromolecules in their biological context. By visualizing ion channels and their associated extra- and intracellular proteins while they are embedded in the membrane, whole complexes can be characterized in their biological surroundings, which can provide valuable insights into the mechanisms of interaction and protein dynamics in ion channels. This type of information can, in turn, greatly improve confidence in early drug discovery stages.

Ion channel formation

In addition to fully associated ion channels, SET was able to show dual dimers presumably in the process of forming an ion channel. The shape and size of the monomeric units that constitute the dimers could be resolved, and their extracellular and intracellular components could be identified. The dimers are about 15–17 nm in height and 13–14 nm in extracellular width and are composed of monomers that resemble chicken drumsticks. SET reconstructions showed how the dimers are located closely in space and are bridged by a protein com-

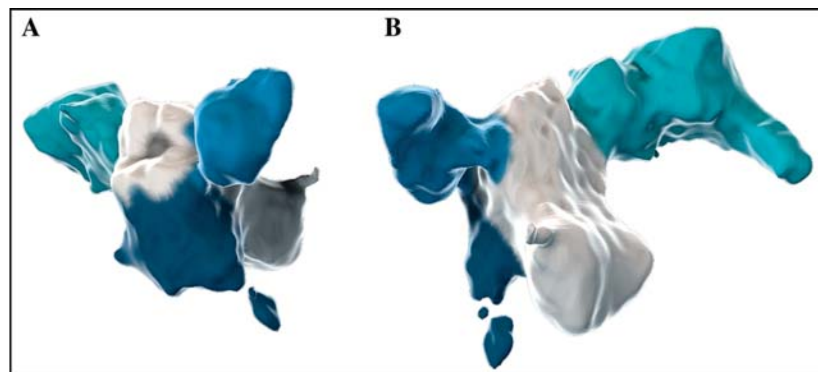

FIG. 3. Ion channel expressed on the plasma membrane of RIN cells. Analysis of the size and structure of the macromolecular complex indicates that the ion channel has a tetrameric structure spanning approximately 15–17 nm in height and 9–10 nm in width. The channel is pictured in white with extracellular components pointing upwards and intracellular components downwards in (**A**). The orientation of the tetramer could be inferred as the polyclonal antibodies were raised against a peptide in the extracellular domains of ion channel subunit x. The lipid bilayer that extends over the neck region of the protein complex [most readily seen in (**B**)] is not visualized because of the staining method used, but appears as an empty space in the 3-D reconstructions. (**A**) This view clearly depicts the extracellular side of the ion channel that resembles the crown of a tooth with a central pore opening marked in gray. Primary antibodies bind to the channel from opposite sides of the extracellular domains. One primary antibody, unmarked by secondary antibodies, binds to the tetramer from the right, with the Fc domain (light blue) and Fabs (dark blue) clearly evident in the figure. The two Fabs could not be distinguished because of binding too closely spaced epitopes. Another primary antibody (green) binds to the channel from the left, and is identified by secondary antibodies conjugated to 6-nm colloidal gold particles (excluded from the figure). (**B**) Clockwise rotation of the ion channel. This view highlights the plasma membrane and intracellular side of the ion channel. The channel is preorganized into dual dimers, which span the channel longitudinally and are fused at the inward curvature. The plasma membrane is positioned over the neck region of the channel. Images are generated using the program 4D Cinema (Maxon, Friedrichsdorf, Germany).

plex, presumably chaperone proteins (Fig. 4). These complexes appear to be intracellular, presumably in the endoplasmic reticulum, and are likely to represent intermediate substructures in the biogenesis of ion channels. Thus, SET was also able to establish that the tetrameric ion channel is pre-organized as dual dimers.

Potential heterogeneity

One possible case of double-labeling was found in rat DRG tissue that tends to indicate a heterogeneous ion channel. Tight binding of the antibodies and interacting partners in the molecular complex confounded interpretation of the actual channel and made it impossible to unambiguously establish heterogeneity. Nevertheless, double-labeling suggests a potential heterogeneous ion channel and calls for further experiments using smaller, more targeted antibodies.

Conclusions

In this study, SET was able to accurately reconstruct 3-D images of *in situ* ion channels from cell and tissue samples. The technology showed protein conformational states of fully associated channels and proteins in the process of forming an ion channel in three dimensions, thus providing valuable insights into the life cycle of these macromolecular complexes. Understanding protein stoichiometry and subunit composition *in situ* is particularly important in the target definition process, being crucial information in setting up a relevant screening assay. The results of this study demonstrate that SET is a valuable tool to visualize protein conformations of *in situ* samples, thus affording crucial information about ion channels that can enable improved assay screening systems in drug development.

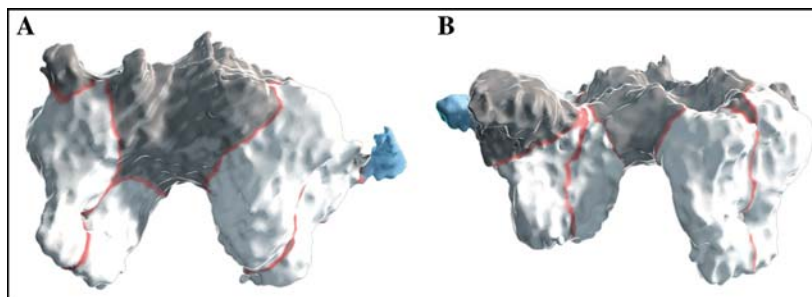

FIG. 4. Ion channel dimer subunits in rat DRGs. The dimers are about 15–17 nm in height and 13–14 nm in extracellular width. The extracellular side of the dimers faces upwards in this figure and the intracellular side downwards. Only a small part of the primary antibody marking subunit x (light blue) is included in the figure. This antibody binds to a 6-nm colloidal gold via secondary antibodies (excluded from the figure). The separate monomeric units (white) constituting the dimers are outlined in red. (**A**) Dimer complex seen from the side. The individual monomeric units are clearly seen, with features resembling that of a chicken drumstick. (**B**) 180° rotation of the complex shown in (**A**). Images are generated using the program 4D Cinema (Maxon, Germany).

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