Conical tomography of freeze-fracture replicas: a method for the study of integral membrane proteins inserted in phospholipid bilayers

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Abstract

We have used conical tomography to study the structure of integral proteins in their phospholipid bilayer environments. Complete conical series were collected from replicas of the water channel aquaporin-0 (AQP0), a 6.6 nm side tetramer with a molecular weight of \(~120\) kDa that was purified and reconstituted in liposomes. The replicas were tilted at 38°, 50° or 55° and rotated by 2.5°, 4°, or 5° increments until completing 360° turns. The elliptical paths of between 6 and 12 freeze-fracture particles aligned the images to a common coordinate system. Using the weighted back projection algorithm, small volumes of the replicas were independently reconstructed to reconstitute the field. Using the Fourier Shell Correlation computed from reconstructions of even and odd projections of the series, we estimated a resolution of 2–3 nm, a value that was close to the thickness of the replica (\(~1.5\) nm). The 3D reconstructions exhibited isotropic resolution along the \(x–y\) plane, which simplified the analysis of particles oriented randomly in the membrane plane. In contrast to reconstructions from single particles imaged using random conical tilt [J. Mol. Biol. 325 (2003) 210], the reconstructions using conical tomography allowed the size and shape of individual particles representing the AQP0 channel to be identified without averaging or imposing symmetry. In conclusion, the reconstruction of freeze-fracture replicas with electron tomography has provided a novel experimental approach for the study of integral proteins inserted in phospholipid bilayers.

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1. Introduction

Electron tomography is a general method for the determination of the three-dimensional (3D) structure of macromolecular assemblies and organelles using the electron microscope. A distinctive property of electron tomography is that the structure of the assemblies and that of protein machines can be investigated in their cellular environments, rather than in solution or in crystalline arrays (Medalia et al., 2002). This property results from the imaging of the same assembly in different directions with respect to the incident electron beam, producing a series of images (the “tilt series”). The use of these tilt series allows for the reconstruction of geometrically unique structures without the need for imposing symmetry or averaging over many molecules. Furthermore, the resolution of the 3D reconstructions calculated with
electron tomography is at least one order of magnitude higher than that achieved by optical methods.

A critical factor limiting the accuracy of electron tomography is the presence of regions without information (the “missing regions”). This limitation is better understood in Fourier space where each micrograph of the tilt series is represented by a central plane oriented orthogonal to the viewing direction (the “central section theorem”). A 3D reconstruction would represent the original structure faithfully only when the tilt series involves a large portion of the Fourier space. Since 90° tilt angles cannot be achieved with the electron microscope, all 3D reconstructions calculated by electron tomography are limited by the presence of missing regions.

In canonical orientation, the missing region of the reconstruction extends around the reciprocal axis $Z^*$, and can be estimated in advance. Adequate sampling around the $Z^*$ axis requires tilting the specimen at the highest possible angle. However, at very high angles ($>70^\circ$), the increase in specimen thickness due to tilting induces multiple scattering, which greatly degrades the quality of the image. On the other hand, limited tilt angles result in 3D maps where the reconstructed structure is elongated in direct space along the $z$-direction (Frank and Radermacher, 1986). Also the sampling around the reciprocal $X^*$–$Y^*$ axes depends on the geometry used to collect the series. The “single-axis” tilt geometry ($\pm 60^\circ$ or more) has a missing volume shaped as a double wedge (Fig. 1A), which influences the structure of macromolecules based on their orientation with respect to the tilt axis (anisotropic $x$–$y$ resolution). This effect has been reported in 3D reconstructions of frozen microtubules whose details “tend to fade from view when they are oriented perpendicular to the tilt axis” (McEwen and Marko, 2001).

In “double-axis” tilt geometry, the specimens are tilted $\pm 60^\circ$ along orthogonal axes (Penczek et al., 1995) and the missing volume is shaped as a pyramid (Fig. 1B). A number of reconstructions calculated from double-axis tilt series clearly indicate a significant improvement in resolution isotropy along the $x$–$y$ plane (Mastronarde, 1997). In “conical” tilt, the geometry investigated in this paper, the specimen is first tilted to the maximum angle and then rotated in small increments until completing a 360° turn. The missing volume along the reciprocal $Z^*$ axis is shaped as a cone, which greatly reduces the anisotropy along the $x$–$y$ plane since each central section shares a different line with every other section of the series (Fig. 1C). Conical tilt has been studied theoretically due to its relationship to the random conical tilt method for imaging particles that exhibit preferential orientation (Radermacher, 1988; Radermacher et al., 1987). However, apart from an initial test (Radermacher and Hoppe, 1978), this method has not been used to reconstruct biological specimens, due to the lack of stages capable of tilting at high angles while still permitting accurate control over the rotation.

We have studied freeze-fracture replicas of the water channel aquaporin-0 (AQP0) reconstituted in phospholipid bilayers and imaged using random conical tilt (Zampighi et al., 2003, 2004). The images of the particles...
2. Materials and methods

2.1. Preparation of the specimen

The purification of AQP0 from the lenses of bovine eyes as well as its reconstitution in phospholipid membranes has been described previously (König et al., 1997; Turk et al., 2000). Aliquots of liposomes were attached to a glass surface, covered with a small piece of copper and rapidly frozen by immersion in liquid nitrogen temperature and 1 mbar partial pressure. The samples were fractured in a JOEL freeze-fracture apparatus at liquid nitrogen temperature and 1 mbar partial pressure. The cleaved surfaces were shadowed first with a 2 Å thick layer of carbon (to avoid decoration) and then in all directions (multi-axis) with platinum-carbon (Zampighi et al., 2003). The replicas were mounted on substrates with ~1 μm diameter holes (“holey” grids), and the regions spanning the holes were used for imaging to eliminate the contribution of the support film to the thickness of the specimen. Ten nanometer diameter gold particles were used as markers to facilitate the identification of the region being imaged.

2.2. Electron microscopy

The conical series were collected in the Gatan 650 Single Tilt Rotate Holder (designed by R. Zolkowski, Gatan). The holder was used in a Philips CM12 and a FEI Tecnai 12 electron microscope operated at 120 kV. To tilt the replica, we used the controls of the goniometer and to rotate it a set of separate controls provided by Gatan (the Accutroller). The height along the Z-axis was made to be the same for all the series to guarantee eucentricity. Rotation resulted in displacements of the area of interest, which were then manually re-centered using the controls of the Compu-stage.

The Philips CM12 permitted only a 38° tilt since at higher tilt angles the Gatan 650 holder touched the objective pole piece (a “pole hit”). To increase the tilt angle, the Gatan 650 Single Tilt Rotate Holder was modified to fit the Tecnai 12 Twin Electron Microscope. Using this configuration, we collected conical series tilted at 50° and 55° with rotation increments of 2.5°, 4°, and 5°. The 55° tilt limit relates to the density of intra-membrane particles in the liposome. At ~1000 particles/μm², tilt angles larger than 55° project neighboring particles together and interfere with the tracing of their elliptical paths required for image alignment.

Our hardware combination exhibited two limitations for collecting complete 360° series: first, the region of interest must not be farther than ~100 μm away from the optical center of the microscope. Construction of a new stage and development of simple manipulations during imaging have since resolved the problem. Second, the region must be at the center of the square to avoid interference with the grid bars. Both limitations curtailed the number of regions available for reconstruction.

The replicas were imaged using minimum dose conditions. The search mode was set at 2700× magnification to decrease the electron dose on the region of interest. The focus was performed in an area oriented at 180° and located 3 μm away from the area of interest to guarantee a defocus gradient. The images were collected using a Gatan 2k × 2k CCD camera at 30000× magnification.

2.3. Conical tomography

In conical tomography, the specimen is tilted by an angle ϑ, say along Y, and then rotated in incremental steps by ϕ (Fig. 2A). According to Eqs. (1a) and (1b), every point (at the coordinates P, P1, P2) during rotation describes a circle in the specimen plane and an ellipse on the image plane:

\[
\begin{align}
\begin{cases}
x = p_1 \cos(\vartheta) \cos(\phi) + p_2 \cos(\vartheta) \sin(\phi) + p_3 \sin(\vartheta), \\
y = p_1 \sin(\vartheta) + p_2 \cos(\phi).
\end{cases}
\end{align}
\]

(1a)

Or equivalently:

\[
\begin{align}
\begin{cases}
x = r \cos(\vartheta) \cos(\phi + \delta) + p_2 \sin(\vartheta), \\
y = r \sin(\phi + \delta),
\end{cases}
\end{align}
\]

(1b)
the experimental parameters are evaluated as follows:
\[ \vartheta = \cos^{-1}\left(\frac{a}{b}\right); \quad p_x = b\cos(\delta); \quad p_y = b\sin(\delta); \quad p_z = \frac{x_0}{\sin(\vartheta)}. \]

Therefore, the trajectories of the ellipses allow accurate alignment of the images comprising the conical tilt series.

The stack of Fourier sections from images collected by conical tomography form a hyperboloid at one sheet (Lanzavecchia and Bellon, 1996). Within the hyperboloid (Fig. 1C), the empty central, double cone encompasses the portion of reciprocal space where information is missing due to limited tilt along the Z-axis. If the projections are equally spaced with respect to the azimuth, the 3D reconstruction can be computed with “ad hoc” algorithms: weighted back projection (WBP) with \( r^* \) weights (Radermacher, 1988) or a direct Fourier method (Lanzavecchia et al., 1993). Since the conical series exhibited unequal spaced projections, algorithms developed for general geometry were used for reconstruction (Harauz and van Heel, 1986; Lanzavecchia et al., 1999; Radermacher et al., 1987).

2.4. Alignment of projections

To bring the \( n \) images of the series into a common reference system, we need: (a) the tilt angle \( \vartheta \), (b) the angle \( \gamma \) defining the direction of the tilt axis, (c) the \( n \) azimuth angles \( \phi_j \) (\( j = 1,2,\ldots,n \)), and (d) the \( n \) relative shifts \( \Delta_j = (\Delta x_j, \Delta y_j) \). We also assumed that no in-plane rotations occurred among the \( n \) images of the series. The values of the tilt and azimuth angles were known in advance and only refined a posteriori. The values of the \( n \) relative shifts were large because the re-centering of the region of interest was done manually, which made the trajectory of fiduciary markers resemble Brownian motion rather than the predicted ellipses (not shown).

We corrected for the \( n \) relative shifts by first choosing a common point and then shifting the images of the series along the \( X-Y \) plane to make this point the actual center. The particles used as centers were selected to minimize large shifts since they greatly reduce the field of the final reconstruction. After centering, intra-membrane particles (\( m = 6-12 \)) were selected as markers and their coordinates \( (x_{ij}, y_{ij}) \) recorded for every image of the series. The \( i = 1, \ldots, m \) coordinate indicates the fiduciary particle while the \( j = 1, \ldots n \) coordinate indicates the image of the series to which it belongs. The particles now described elliptical trajectories oriented parallel to the tilt axis (Fig. 2B) and the ratio between the two axes was equal to \( \cos(\vartheta) \). To evaluate the parameters, every ellipse (\( i \)) was brought in canonical orientation: first the center of mass was subtracted from the coordinates:
\[ x'_{ij} = x_{ij} - \frac{1}{n} \sum_{j} x_{ij}, \quad y'_{ij} = y_{ij} - \frac{1}{n} \sum_{j} y_{ij}, \]

then the quadratic form \( A_{0} \) of the set was computed:

\[
A_{0} = \begin{bmatrix}
\sum_{j} (x'_{ij} \cdot x'_{ij}) - \sum_{j} (x'_{ij} \cdot y'_{ij}) \\
\sum_{j} (y'_{ij} \cdot x'_{ij}) - \sum_{j} (y'_{ij} \cdot y'_{ij})
\end{bmatrix}.
\]

The length of the axes of the ellipses \((a, b)\) correspond to the eigenvalues of \( A_{0} \), \( \lambda_1 \) and \( \lambda_2 \) while the orientation of the axes corresponds to the eigenvectors, \( u_1, u_2 \):

\[
a = \sqrt{\lambda_1}, \quad b = \sqrt{\lambda_2}, \quad \theta = \cos^{-1} \left( \frac{b}{a} \right),
\]

\[
\gamma = \tan^{-1} \left( \frac{u_{1z}}{u_{1x}} \right) = \tan^{-1} \left( \frac{u_{2z}}{u_{2x}} \right) - \frac{\pi}{2}.
\]

In this manner, we obtained the lengths of the axes of the ellipses \((a, b)\) and a value of \( \theta \) and \( \gamma \) for each particle traced. The final values \( \theta \) and \( \gamma \) were averages and the lengths of the axes were rotated by \( \gamma \) to bring the tilt axis in coincidence with the \( Y \)-axis (Fig. 2C).

Further parameters were obtained by comparing the experimentally rotated coordinates \((x_{ij}, y_{ij})\) to the theoretical coordinates of the projections of points \( P_i = (P_{ix}, P_{iy}, P_{iz}) \) calculated with Eqs. (1a) and (1b) (Fig. 2D). To do this, the three coordinates were estimated with Eq. (3) after evaluating the angle \( \delta \) for every ellipse \( \ell \).

Each angle \( \delta_i \) was evaluated by minimizing the quantity:

\[
\sum_{j} [(x_{ij} - a_i \cos(\phi_j + \delta_i))^2 + (y_{ij} - b_i \sin(\phi_j + \delta_i))^2]
\]

with \( \phi_j = \frac{2\pi j}{n} \) as the first approximation.

Now the \( n \) shifts are computed as:

\[
\Delta x_j = \frac{1}{m} \sum_{i} (x_{ij} - \bar{x}_{ij}), \quad \Delta y_j = \frac{1}{m} \sum_{i} (y_{ij} - \bar{y}_{ij}),
\]

(4)

where \( \bar{x}_{ij} = a_i \cos(\phi_j + \delta_i), \bar{y}_{ij} = b_i \cos(\phi_j + \delta_i) \).

At this point all the maps were shifted and refined using Eq. (4). For each point \((x_{ij}, y_{ij})\) a theoretical value of \( \phi \) was computed according to the parameters \( a, b, \) and \( \delta \) of the corresponding ellipse: \( \phi_{ij} = \cos^{-1} \left( \frac{y_{ij}}{x_{ij}} \right) \). Then each angular step was refined as \( \phi_j = \frac{1}{m} \sum_{i} \phi_{ij} \).

The process of alignment, iterated several times using the new parameters, was as follows: The shifts were applied and the center of mass and the quadratic form of each ellipse was computed; the new angular steps \( \phi_j \) were used to compute the \( \delta \) angles; these values were used to estimate new shifts that were used to obtain new angular steps.

After estimating the 3D coordinates of the \( m \) reference markers, we computed the root mean square deviation of the experimental projections with respect to the theoretical values. In each cycle, a decrease of this deviation followed the application of the shifts and the refinement of the angular steps. The process converged quickly and the final deviation did not change appreciably after the second or the third cycle.

2.5. 3D reconstruction and resolution

The alignment process provided the Euler angles \((\phi, \theta, \gamma)\) and the origin for all the images in the conical series. The 3D reconstruction was performed with an algorithm for general geometry, because the azimuth angles were not equally spaced as assumed in the first approximation. We adopted the weighted back projection (WBP) algorithm (Radermacher, 1992), because it allows for the selection of a final volume. Since the number \((n = 74, 90, \) and 144) and size of the image \((1k \times 1k \) or \(2k \times 2k)\) varied, the volumes of the reconstructions were also different. In the case of \(1k \times 1k\) images and flat samples the volume was 768 × 768 × 128 while in the \(2k \times 2k\) images it was up to 1152 × 1152 × 128 voxels.

We took two approaches to calculate the 3D reconstructions: (a) from the largest area present in all images of the series, and (b) using a number of small areas that were subsequently pasted together in a single volume. The 3D reconstructions obtained with both strategies were restored by iterative cycles of projections onto convex sets (POCS; Carazo, 1992) based on the positivity constraint.

We tested the influence of the size of the image by reconstructing a 64 × 64 × 64 voxels wide section of the central part of the volume from full image projections (strategy a) or from their central regions (strategy b). This was also true for all portions of the volume, provided that the corresponding set of projections was properly extracted from the large maps. After aligning the series according to the orientation parameters \( \gamma \) and \( \Delta_{\ell} \), Eq. (1a) gave us the projection coordinates of a generic point \( P \) of the volume. A gallery of small projections was created. The small volume comprising the point \( P \) was reconstructed using the same angular value \((\phi, \theta, \) \( \gamma)\) used for the reconstruction with strategy (a). This strategy improved the crispiness of features in the reconstructed volume.

We also tested the resolution of the reconstructions experimentally, by calculating independent reconstructions from odd and even images of the series. Since the two sets of images possess identical missing regions, the resolution achieved in neighboring regions of the reconstructed volume was estimated by Fourier shell correlation (FSC; van Heel, 1987).

2.6. Visualization

We computed surface and volume rendering views of the 3D reconstruction consisting of arrays of up to
1024 × 1204 × 128 pixels with Amira (TGS). The reconstruction of the metal replica, the “mould,” was used to estimate the volume under the replica (the “imprint”) (Lanzavecchia et al., 1998; Zampighi et al., 2003). Due to the thinness of the replica (1.2–1.5 nm), masking regions above and below the mould suppressed part of the noise. For a better visualization and for a more accurate computation of the imprint, the array was first threshold at a high value to select only the voxels belonging to the mould. Also, the threshold array was convoluted with a Gaussian function to create a smooth mask, which was then applied to the original array. Masking was used only for visualization and the computation of the FSC was always performed in unmasked arrays.

2.7. Imprint computation

To visualize the volume of the external and cytoplasmic domains of the AQP0 channels, we computed the imprints from the lower surface of the replica. Replicas appeared as grainy surfaces with small holes that disrupted the continuity of the surface of the reconstructed volume. To cope with this problem, we devised a strategy whereby a relief map was created for a given threshold by projecting the volume along Z and writing in each point XY the Z coordinate of the lowest voxel above the thresholds. In the holes, an iterative process of region growing assigned values based on the values of neighbouring pixels (Rosenfeld and Kak, 1982). Finally, the map was used to calculate the imprint (Fig. 9).

3. Results

Purified AQP0 reconstituted in phospholipid bilayers fractured down the middle exposing complementary fracture faces to the metal shadowing. The center of the phospholipid bilayer appeared as a flat, featureless surface with intra-membrane particles ~9 nm in diameter. These particles corresponded to the external and the cytoplasmic domains of the AQP0 channel since reconstitution in liposomes is random (Fig. 3).

We reconstructed seven series tilted at 37°, 50°, and 55° and rotations of 2.5°, 4°, and 5°. Only the results obtained from series collected at 55° and 4° increments are described in detail because they provided the best balance in terms of radiation damage, resolution, dimensions and the ability to perform post-processing. We have reconstructed either all of the replica’s useful area (~1 μm²) at a pixel size of 0.9 nm, or a smaller portion at the pixel size of the original images (0.46 nm). In these conical series, we used the elliptical path of 12 intra-membrane particles for alignment that was checked by correlation methods. The 3D reconstructions were then studied by projecting the reconstructed volume both parallel and perpendicular to the Z-axis (Figs. 4 and 5), by iso-surface and volume rendering (Fig. 7), and by estimating the volume under the replica (the “imprint,” Figs. 8 and 9).

3.1. Resolution

We compared the resolution of the 3D reconstructions computed from the entire field (strategy a) and from pasting small volumes reconstructed indepen-
The reconstructions were first projected in 2D to recreate the un-tilted view of the replica (Fig. 4). At first impression, the projections calculated from reconstructions using the two strategies appeared to differ principally in contrast (compare Figs. 4A and B). However, the overall shape of the particles representing the ~120 kDa channel assembly was better defined in projections calculated using strategy b (compare the two insets of Fig. 4). This observation was confirmed by higher frequency components in the spectra of projections calculated by strategy b (not shown). Since pasting small volumes calculated independently (strategy b) provided better 3D results, all subsequent projections and maps were calculated using this strategy.

To estimate the resolution of the 3D reconstructions, we separated odd and even images of the conical series and reconstructed them independently. The 3D maps were subdivided in arrays of 9 × 9 squares (Table 1) and in each square FSC was calculated using the 5 times criterion (Radermacher, 1988). The numbers located in each square indicate the points where the FSC curve intersected the noise level (Table 1). Also, FSC was computed before (Table 1(A)) and after (Table 1(B)) the application of the POCS filter. As expected, the application of the filter improved the resolution of the reconstruction up to ~2 nm. Curiously, the regions exhibiting the best resolution values were not always at the center of the field, and one side or one corner consistently exhibited higher values. Additionally, one would expect that the resolution of the reconstructions calculated with the entire series (90 images) should be lower than 2 nm. Yet since no CTF correction was applied we choose the more conservative approach and claim that the resolution was the value calculated using half of the images (2–3 nm).
3.2. Analysis of the 3D reconstruction

The reconstructions contained information about the size, shape, and molecular volume of the particles representing the cytoplasm and external domains of the AQP0 channels and were studied by sectioning their volume parallel and orthogonal to the \( z \)-axis (Fig. 5A). Sections cut parallel to the \( z \)-axis showed the replica as a thin electron-lucent line \( \sim 2 \text{ nm} \) in thickness (Fig. 5B) and the particles as small cup-shaped elevations in the fractured bilayer (arrows, Fig. 5B). Restoration using the POCS filter improved the contrast of the replica, reduced the elongation resulting from the limited tilt angle (55°) and improved the sharpness of the elevations corresponding to the particles (Fig. 5C).

In sections cut orthogonal to the \( z \)-axis, the particles representing the cytoplasmic and external domains of the protein were shaped as annuli comprised of electron-lucent rims also \( \sim 2 \text{ nm} \) in thickness surrounding electron-dense cavities \( \sim 6 \text{ nm} \) in diameter (Fig. 5D). Restoration using the POCS filter improved the contrast of the replica, reduced the elongation resulting from the limited tilt angle (55°) and improved the sharpness of the elevations corresponding to the particles (Fig. 5C).

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Table 1

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The resolution of the reconstruction was assessed by FSC from reconstructions calculated from even and odd projections of the series. The computed FSC from the volumes extracted from both reconstructions was used to create maps describing how the resolution’s value varied along a field \( \sim 500 \text{ nm} \) in side (values in Angstrom units). (A) The resolution’s values without restoration (missing cone excluded from FSC computation). (B) The distribution after POCS restoration (all reciprocal volume included in FSC computation).

Fig. 6. Independent reconstructions calculated from even (A) and odd (B) images of the series. The similarity of the reconstructions permitted the use of FSC methods to estimate the resolution of the tomogram. Scale bar: 20 nm.
tistinguishable as small elevations protruding from the flatter surface representing the center of the phospholipid bilayer (Fig. 7C). Since the bottom surface of the replica was in direct contact with the cleaved membrane, the small, cup-shaped depressions of the particles represent the molecular envelope of the channels (Fig. 7D).

To simplify the study of these envelopes, we computed the volume (the “imprint”) under the entire field and under individual cup-shaped depressions corresponding to the particles (Figs. 8 and 9). The imprint of the entire field appeared as an irregular surface with elevations and depressions resembling a lunar landscape (Fig. 8). (Elevations that appeared as ribbons crossing the imprint for long distances in Fig. 8 mostly correspond to folds of the liposome.) The flatter areas representing the middle of the phospholipid bilayer were also irregular due to the absence of averaging and the method used to compute the imprints (see Section 2). The particles representing the AQP0 protein appeared as small elevations (colored areas, Fig. 8) with identical distribution to the annuli seen by pro-

![Fig. 7. Representation of the reconstructed replica by iso-surfacing methods. The region seen in (A)-(C) is 200 × 200nm². (A) The top surface of the reconstructed replica. The intra-membrane particles appear as smooth elevations on the flat surface representing the phospholipid bilayer. (B) The bottom surface of the reconstructed replica where the particles appear as depressions. The rectangle encloses two adjacent depressions. (C) A side view to emphasize the thinness of the replica. (D) The two depressions inside the rectangle in B at higher magnification.](image)

![Fig. 8. Visualization of the volume under the replica (the “imprint”). The irregularity of the surface arises from the distribution of metal grains. The particles representing the external and cytoplasmic domains of the channel were labelled red. Area size: 200 × 200 nm².](image)

![Fig. 9. Segmentation of intra-membrane particles. The imprints of three freeze-fracture particles were extracted from the reconstructed replica. In all three cases, the plane colour yellow indicates the location of the middle of the phospholipid bilayer. The height of the particle measured ~3 nm. The imprints have irregular shapes since they arise from individual particles without averaging or imposing symmetry. The imprints exhibited the size, overall shape and molecular volume of models derived by averaging and imposing symmetry. Area of the plane: 12 × 12 nm².](image)
jecting the volume of the reconstruction parallel to the z-axis (Figs. 5D and E).

The imprints of individual particles were segmented from the 3D maps and their volume rendered for visualization (Fig. 9). Each particle now represented the volume contained within the cup-shaped depression at the bottom surface of the replica. The particles measured \( ~6 \text{ nm in diameter, 3–3.5 nm in height and occupied } \sim80–90 \text{ nm}^3 \text{ in volume} \). These dimensions were similar to those calculated from 3D reconstructions of single particles after averaging and imposing symmetry (Zampighi et al., 2003, 2004).

4. Discussion

4.1. Freeze-fracture replicas and conical tomography

Early attempts to gather 3D information from specimens prepared by either unidirectional or rotary shadowing used surface relief techniques (Baumeister et al., 1986; Smith and Kistler, 1977; Smith and Ivanov, 1980; Walz et al., 1996), which work optimally only when the replica approaches the ideal situation of being a homogeneous metal film. Consequently, the best results have been obtained by applying the algorithm to the average unit cell of proteins arranged in 2D lattices. An alternative approach is to study specimens prepared by multi-axis shadowing where the replica approaches the ideal of being a homogeneous layer of metal. Reconstruction of the intra-membrane particles of “individual” integral proteins using averaging methods produced models where the X-ray structure of the closely related AQP1 (Sui et al., 2001) was accurately docked in the envelope of the particle (Zampighi et al., 2003, 2004). Unfortunately, this approach cannot be applied to biological membranes because their extensive protein heterogeneity invalidates the assumption that all particles are views of the same protein. Therefore, we used conical tomography to reconstruct the entire replica in an effort to study the size, shape, and molecular volume of the proteins in biological membranes.

The reconstruction of freeze-fracture replicas by conical tomography has notable advantages. First, the resolution of the 3D maps is high (2–3 nm) due in large part to the fact that the thickness of the replica, a critical factor in determining the resolution of electron tomograms, is only \( \sim1.5 \text{ nm} \). Second, the reconstructions have isotropic resolution along the \( X-Y \) plane. This is an important advantage when studying biological membranes since integral proteins exhibit the same orientation perpendicular to the membrane plane, but are rotated at random around this axis. Such orientations in the bilayer made isotropic resolution mandatory for any analysis involving the comparison of the different integral proteins in biological membranes. Third, since odd and even images of the same conical series produced very similar 3D reconstructions (Fig. 6), resolution is estimated by FSC methods. Fourth, the use of intra-membrane particles permits great flexibility in selecting the number and location of markers (6–12) needed for image alignment. Fifth, the upper and lower surfaces of the replicas can be analyzed independently. Since the replica’s lower surface is in direct contact with the fractured surface, factors such as unevenness of its thickness or contamination during metal evaporation do not interfere with the interpretation of the reconstruction. Finally, the inherent irregularities of the surface induced by the fracturing process are not as serious a limitation as they are in the surface relief approach.

A disadvantage of conical tomography is that all the images of the series must be collected at the maximum tilt angle. This means that the thickness of the specimen will always influence the quality of the reconstruction. In our studies the density of intra-membrane particles (~1000/\( \mu \text{m}^2 \)) also limited the tilt angle to 55° since, at higher angles, the particles projected into each other and interfered with the tracing of the elliptical paths needed for image alignment. In thin sections cut from plastic embedded or ice-embedded tissues, this limitation does not apply and the magnitude of the tilt angle will depend only on the specimen’s thickness, a problem that can be dealt with by imaging at higher accelerating voltages. Finally, since the principal information in replicas is the local variations in height, additional computational steps are needed to estimate the molecular volume of the particles representing the different proteins (Figs. 8 and 9).

In conclusion, the isotropic resolution in the \( X-Y \) plane allows us to characterize the size, shape, and volume of small (\~120 kDa) proteins in the phospholipid bilayer environment.

4.2. Comparison with standard tomography

The analysis of the extension of the missing regions shows that single-tilt, double-tilt, and conical tilt are almost equivalent at their experimental limit. For example, a single-axis series collected at 70° tilt collects the same percentage of the Fourier space (~78%) as a double-axis series at 60° tilt and a conical series at 55° (~80%). However, in conical tilt tomography, each central section shares a different line with every other section of the series (Fig. 1C) improving image alignment and the consistency of the final reconstruction.

The resolution of complete tomographic maps (tilt = ±90°) depends also on the number (\( N \)) of projections comprising the series (Crowther et al., 1970). In the case of sections or slabs, where \( D \) is substituted by the thickness (\( T \)) divided by \( \cos(\text{max tilt}) \), the relation between resolution and number of images is: \( R = \pi T/ \)
predict that achieving 2 nm resolutions in the electron microscopy field support this conclusion (McEwen and Marko, 2001; McEwen et al., 2002) and it also applies to clinical tomography (Lewitt, 1983).

An important question is how to determine the number of images a priori to avoid under-sampling. Previous studies with Radon methods applied to both clinical tomography (Bellon and Lanzavecchia, 1997) and electron microscopy (Lanzavecchia et al., 1999) shows that the number of views required in a 360° turn is about 2 times the number of pixels required in the radial direction. Using this relationship as a “rule of thumb,” we predict that achieving 2 nm resolutions in the X–Y plane (1 nm sampling step) of a 100 nm thick object requires 100 projections at 180° (assuming perfect alignment and an optimum S/N ratio). In conical geometry this means 200 projections, which is more that what is normally done even for dual axis-tomography. However, the Fourier space is sampled twice, which can decrease the number of projections with angular sampling as is done in clinical tomography. Further test of this possibility would require reconstructions of thicker specimens (~50 nm) using conical tomography.

4.3. Resolution

The resolution of conical tomograms was assessed using FSC maps (Table 1) since independent reconstructions can be calculated from odd and even images of a conical series. Analysis of the maps shows that the resolution (3–4 nm) was improved to 2–3 nm by using POCS filters. Moreover, we also observed that the resolution varied without an apparent pattern throughout the tomogram (Table 1), an observation that was confirmed in several reconstructions. A close analysis of the micrographs suggested that this distribution probably originated from the interplay between the defocus gradient induced by tilting (the first zero of the CTF varied around a value of ~2.2 nm) and the alignment strategy, since regions closer to the particles used as markers appeared better aligned than regions farther away. Therefore, the resolution attained with conical tomography is determined principally by the thickness of the layer of metal comprising the replica.

The reconstructions were performed using the WBP algorithm and we obtained better results by pasting together small volumes reconstructed separately (strategy b) than by reconstructing the entire field common to all the images in the series (strategy a). On the other hand, as expected, the FSC values did not improve by the choice of strategy since the correlation values did not change when the amplitude of Fourier coefficients increased. Strategy b did improve the crispness of the details in real space (Fig. 4) but its use depends on the thickness of the specimen, which limits the small volumes pasted together when reconstructing the field.

The choice of strategy would not affect reconstructions using the Fourier methods that take advantages of padding to improve interpolation accuracy (Lewitt, 1983). Yet, a direct comparison with direct Fourier methods is not presently possible because replicas are not finite samples and differences at the peripheries of the field cause artifacts. Using other methods (Sandberg et al., 2003) would provide further insights into this problem.

4.4. Conclusions

We have developed conical electron tomography as a general method to reconstruct biological organelles and macromolecules in their cellular environments. To test the steadfastness of the method, we showed that reconstructions of “individual” freeze-fracture particles described the size, overall shape and molecular volume of the small (6.6 nm side and ~120 kDa) AQP0 water channel in phospholipid bilayers. Since the resolution is isotropic along the reciprocal X–Y plane, this method offers a general experimental solution for the study of channels, receptors and transporters in biological membranes.

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