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Accurate membrane tracing in three-dimensional reconstructions from electron cryotomography data

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Abstract

The connection between the extracellular matrix and the cell is of major importance for mechanotransduction and mechanobiology. Electron cryo-tomography, in principle, enables better than nanometer-resolution analysis of these connections, but restrictions of data collection geometry hamper the accurate extraction of the ventral membrane location from these tomograms, an essential prerequisite for the analysis. Here, we introduce a novel membrane tracing strategy that enables ventral membrane extraction at high fidelity and extraordinary accuracy. The approach is based on detecting the boundary between the inside and the outside of the cell rather than trying to explicitly trace the membrane. Simulation studies show that over 99% of the membrane can be correctly modeled using this principle and the excellent match of visually identifiable membrane stretches with the extracted boundary of experimental data indicates that the accuracy is comparable for actual data.

Keywords

electron tomography; cryo; image processing; membrane detection; feature extraction

1 Introduction

The transmission of forces from the cell to and from its environment critically depends on transmembrane receptors like integrins that link the cell to the extracellular matrix (Hanein and Horwitz, 2012). Electron cryo-tomography (cryo-ET) can provide three-dimensional reconstructions of the region in question at nanometer resolution, in situ, and in their native environment, potentially enabling high-resolution analysis at the single molecule and even structural domain level (Volkmann, 2010). Furthermore, the information can be combined with live cell dynamics through correlative light and electron microscopy imaging (Hanein and Volkmann, 2011).

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Cryo preservation allows imaging of biological material with the electron microscope in their native environment without any chemical fixation, staining or drying (Dubochet et al., 1988). In tomographic data collection, the sample is physically tilted through a range of angles and projection images are recorded for each angle. These projections can then be converted into three-dimensional reconstructions of the underlying density (DeRosier and Klug, 1968). However, technical restrictions of specimen holders and sample geometry only allow tilt angles of $\pm 70^\circ$ at best, acting as an orientation filter referred to as the ‘missing wedge’, a distinct wedge of missing data in Fourier space.

This situation can lead to severe distortions of some features in the density, especially thin structures perpendicular to the missing projections. These distortions are particularly severe for whole cell samples in which the geometry dictates that the majority of the membranes are perpendicular to the Z-axis, the dorsal and ventral membranes, which are affected by the missing-wedge and thus not visually detectable (Figure 1). However, tracing of these membranes would be a prerequisite for in-depth analysis of the connection between the cell and the extracellular matrix. The situation is often further exacerbated by the extraordinarily low signal-to-noise ratio in the cryo-tomograms caused by the need to restrict the electron dose used for imaging.

Previous attempts at membrane extraction have primarily focused on automating the tracing of membranes that are identifiable by eye in the tomograms. These methods can be roughly divided into boundary-based and region-based approaches. Boundary classification tries to define a boundary based on some feature of the boundary itself such as the intensity or intensity gradients. Region-based methods attempt classification of the tomograms into distinct regions that have some common characteristics such as texture or intensity values. In the case of segmenting cryo-tomograms of biological material, the object of interest is often the boundary of the detected region.

However, most of these methods are inherently unsuited for tracing membranes affected by the missing wedge that makes the membrane virtually invisible, generating a need for new approaches to address this important issue. Here, we describe a novel method that allows the extraction of the ventral and dorsal cell membranes from cryo-tomograms with high fidelity.

2 Methods

We introduce a semi-automatic method for membrane tracing that circumvents the problem of missing-wedge artifacts by using an indirect route rather than attempting to detect the membrane directly. Instead, we identify the membrane as the boundary of the inside and the outside of the cell. Our algorithm is semi-automatic in the sense that it includes manual editing steps but there is no manual tracing of membranes. The algorithms have been implemented in our pyCoAn python framework for electron microscopy image processing and are available from the corresponding author upon request.

2.1 Tomographic reconstruction

Experimental tilt series of the leading edges of several cryo-frozen Chinese Hamster Ovary cells were acquired on a Titan Krios (FEI Company) transmission electron microscope

equipped with a field emission gun at 300 KeV, operated at liquid nitrogen temperatures, at magnification of 6500 X, and a defocus of $-14\text{ }\mu\text{m}$. The data was collected using Batch Tomo (FEI Company) with an angular range of $\pm 65^\circ$ and 1.3° step size around an axis perpendicular to the electron beam. The individual tilts were mutually aligned using the procedures implemented in the IMOD package (Kremer et al., 1996). Three-dimensional densities were generated with a voxel size of about 1 nm using the Simultaneous Iterative Reconstruction Technique (SIRT) as implemented in Tomo3D (Agulleiro and Fernandez, 2011). This reconstruction technique tends to show a better distinction between the inside and the outside of the cell than alternative Fourier methods or weighted back-projection methods. All tilt series, including the synthetic ones, were reconstructed using SIRT with 70 iterations. Regions that included cellular material, the cell membrane, and a limited amount of extracellular space, typically measuring about $500 \times 500 \times 200$ pixels, were selected for further processing.

To decrease the amount of noise and to amend the effects of the missing wedge, we postprocessed the reconstructed density in the following way. First, we applied a variant of the nonlocal means filter (Buades et al., 2005) to each Z-slice of the tomograms individually. The parameters for this operation were adjusted to favor the flattening of regions of similar density values over preservation of fine details. This procedure not only suppresses noise in general, it also leads to suppression of the streaking along the Z-axis that is often observed in tomographic reconstructions due to the missing wedge. As a second step, a sharpening operation using an unsharpened mask is applied to emphasize the edge of the cell.

2.2 Boundary detection

After processing, the cell boundary is already quite evident in side views even though there is no connectivity in the XY-plane (Figure 2). To allow tracing of this boundary, we introduced several sequential steps (Figure 3A). First, we segment the entire cellular density as a single segment or a few large segments using the electron-tomography implementation of the watershed transform (Volkman, 2002). This step allows removing segments that are outside the cell by a simple manual editing operation (Figure 3Bi, ii). While this step is critical, it is not overly sensitive to the accidental removal of small amounts of cell density because interpolation operations involved in the subsequent steps are effectively compensating for this effect.

Once segments outside the cell are removed, the inside of the cell is converted into a point cloud with the highest possible density of points (Figure 3Biii). To achieve this, the watershed transform is rerun with very small step size on the isolated cell density, deliberately over-segmenting into small units. These segments are then converted into a point cloud by extracting the center coordinates of each segment. This point cloud forms the basis for the actual membrane extraction. As mentioned before, the cell boundary is quite evident in side views of the cell without showing continuity in the XY plane, making it difficult to extract a boundary directly.

Instead, we use a slicing strategy where we reduce the dimensionality of the boundary delineation to two dimensions (Figure 3Biv). To achieve this, we collapse the coordinates

along the axis that is most closely aligned with the leading edge of the cell (the X axis in our example) every 10 slices (or every 10 nm). That is, all coordinates that have X values between 0 and 10 are mapped to $X = 5$; all coordinates with values between 10 and 20 are mapped to 15 and so on. The effect of this operation is that the slices are dense enough to give a good enough local approximation of the cell boundary at the ventral and the dorsal side of the cell to allow meaningful application of the steps outlined below. At the same time, the distance between the slices is small enough to allow accurate interpolation of the membrane boundary along the slicing direction. At this stage, outliers are quite evident and can be removed easily by a simple editing operation (Figure 3Bv).

Each collapsed slice contains points that are inside the cell as well as at the cell boundary. To isolate the boundary points from the inside points, we calculate the two-dimensional convex hull of each collapsed slice. This operation can be achieved by a simple algorithm that recursively eliminates points that lie inside triangles between two neighboring points and the center of the point cloud until only the convex hull is remaining. The resulting three-dimensional point cloud should then contain only points on the boundary between inside and outside of the cell, albeit very sparse, at least in the ventral and dorsal surfaces (Figure 3Bvi).

2.3 Membrane tracing

Now that we have a cloud of points that is located on the boundary between the inside and the outside, we can go ahead and derive an estimate of the entire boundary using this sparse point set. We are using the Thin Plate Spline interpolation method (Donato and Belongie, 2002) as implemented in R (Ihaka and Gentleman, 1996) to achieve this goal. Thin Plate Splines find a minimally bent smooth surface that passes through all given points. Thin Plate Splines behave more or less like a thin metal plate if it would be bent to go through the control points and thus provide a smooth interpolation between control points on a plane, similar to b-splines give a smooth line for curves (Figure 3Bvii). In order to obtain the approximation for the entire cell boundary, we divide the convex hull representation into three overlapping sets (top, bottom and front, the latter rotated by 90°) that form two-dimensional surfaces with unique $z(x,y)$ mapping. These three point sets are separately used with the Thin Plate Spline algorithm. After rotating the 'front' Thin Plate Spline back by -90° , the three Thin Plate Splines are merged to give the final approximation of the cell boundary or the membrane.

3 Results

To test our method for membrane tracing through boundary detection, we first derived simulated data that capture the main properties of the actual experimental data. This allows us to compare resulting traces with the ground truth and with results from other approaches. We also applied the method to experimental cryotomograms of leading edges of motile cells.

Using experimental data as a guide, we generated a synthetic leading edge of a motile cell (Figure 4). The interior of the cell was emulated by a mixture of pink Gaussian and shot noise, adjusted to give a rough approximation of the texture generally encountered in

experimental cryotomograms of leading edges. This interior was surrounded by a membrane layer that was perturbed with Gaussian noise to match the more bumpy appearance in the experimental data. The outside of the cell was modeled with pure Gaussian noise to emulate the texture of the buffer. The contrast between inside, outside, and the membrane layer was adjusted to approximate the average in-plane contrast between the same features in experimental reconstructions.

To emulate an experimental tilt series, we rotated the synthetic cell density from -65° to $+65^\circ$ with 1.3° steps around the Y-axis and calculated a projection image at each step. We also generated an artificial tilt series for the membrane model alone to demonstrate the artifacts caused by the missing wedge (Figure 1). The synthetic cell reconstruction was then processed using the procedure outlined in the Methods section. To assess performance of the method and to compare with results from other methods (Figure 5), we calculated two performance scores: (i) the percentages of estimated boundary points within i nanometers of the ground truth (P_i) to measure accuracy; and (ii) the percentage of ground-truth boundary points accounted for by the estimated boundary (B) as a measure of completeness. Our methods gave excellent performance values for both accuracy and completeness for our method (Table 1). A representative boundary-based method (Martinez-Sanchez et al., 2013) applied to the same simulated data also performed well in terms of accuracy but accounts only for a small fraction of the boundary while application of the region-based watershed method (Volkman 2002) accounts for the entire boundary but is not very accurate (Table 1).

The main assumption of the method is that the membrane is locally smooth and compliant. Sharp kinks in the membrane, such as those that can be induced by certain molecules binding to the membrane, cannot be well accounted for by our algorithm. The main adjustable parameter of the algorithm is the thickness of the data slice that is used to generate the convex hull slices. This is ideally balanced to take maximum advantage of the local smoothness of the membrane while preserving as much of the actual spatial variations as possible. In all our tests, a thickness of about 10 nm gives excellent results but alterations by 50% do not show any appreciable effect.

Another important factor is the manual intervention during the editing steps. From our tests it is clear that it is advantageous to remove segments when in doubt rather than leaving them in. The reason is that the Thin Plate Spline algorithm is very good at extrapolating missing points while the convex hull can be sensitive to outliers. If the resulting convex-hull representation is too noisy, a regularization parameter can be used to relax the interpolation requirements of the Thin Plate Splines slightly so that the resulting surface does not have to go exactly through the control points. Randomly deleting 5% of the convex hull points before calculating the Thin Plate Splines has no appreciable effect on the end result, indicating the robustness of the Thin Plate Spline approximation. The quality of the approximation can always be gauged by looking at the match between the boundary estimation and regions where the membrane is clearly visible in the reconstruction.

Experimental tilt series were aligned, reconstructed and processed as described in the Methods section. After processing, the side view of the reconstructions showed clear

outlines of the cells in all cases. The cell boundaries were then extracted using our algorithm. While, in this case, a ground truth is not available, the excellent match between the estimated cell boundaries and the membranes in regions where the membranes are clearly identifiable by eye indicates a high level of accuracy (Figure 6).

4 Discussion

Because of the extremely low signal-to-noise ratio of electron tomograms in general and electron cryo-tomograms in particular, automatic or semi-automatic segmentation approaches developed for other imaging domains do not translate well to electron tomography. As a consequence, one of the mainstays of analyzing these tomograms is the manual tracing of feature in their XY planes. This can give a good representation of features in these planes but can generate misinterpretations in other slicing directions (Marsh et al., 2004).

Recent years have seen several attempts to make membrane tracing in cryo-tomograms more automatic. These efforts included boundary-based methods formulated through several different energy functions that are then optimized using some type of local search (Whitaker et al., 2001; Bajaj et al., 2003; Bartesaghi et al., 2005; Nguyen and Ji, 2008). Other boundary detection methods used with electron tomography data include line and orientation filtering (Sandberg and Brega, 2007), template matching (Lebbink et al., 2007) and methods based on differential geometry (Martinez-Sanchez et al., 2013; Martinez-Sanchez et al., 2014). Because all these approaches focus on automatic or semi-automatic tracing of visibly identifiable membrane features in the tomograms, they are ill suited for detection of the ventral membrane in cryo-tomograms of adherent cell edges. Application of a boundary-based method (Martinez-Sanchez et al., 2013) to simulated leading edge tomography data shows that this method only accounts for a very small fraction of the cell boundary (Table 1), namely the visible portion of membrane at the leading edge, and does not generate any estimate for the ventral and dorsal membranes at all (Figure 5A).

Approaches based on region classification are potentially better suited for tracing of the ventral membrane than the boundary-based approaches. Region classification methods using energy functions based on graph cuts (Frangakis and Hegerl, 2002) and on fuzzy sets (Garduño et al., 2008) have been used with mixed success. Another region-based method, the watershed transform, has been applied to detect membranes in electron tomograms with good success when quality and contrast of the tomograms was good (Volkman, 2002; Marsh et al., 2004). However, like the other region-based methods, the watershed transform has difficulties when the texture or intensity inside and outside the boundary is inconsistent as is the case for cryo-tomograms. Application of the watershed transform to simulated data shows that the method can indeed account for the entire cell boundary but suffers from significant inaccuracies predicting less than half of the estimated boundary points close to the actual boundary (Figure 5B).

Recently, an interesting combination of trained boundary texture classification and global shape models has been proposed for membrane tracing in cryo-tomograms (Moussavi et al., 2010). The inclusion of the shape modeling enables estimation of lower and upper boundary

and impressive results on the cell membranes of *Caulobacter crescentus* were presented. However, the method relies on elliptical shape models and closed contours so it is unclear how this method could be adapted to detecting ventral membranes in cryo-tomograms of eukaryotic cells.

In contrast, the method presented here allows efficient and accurate tracing of membranes affected by the missing wedge (Figure 5C) with minimum user intervention. Even though the approach was developed with the explicit purpose of accurately estimating the ventral membrane location at the leading edge of motile cells, it is not restricted to this particular use and should provide a rich tool for membrane segmentation in electron cryo-tomograms in general.

5 Conclusions

We presented a novel way of tracing membranes in electron cryo-tomograms. The approach is based on detecting the boundary between the inside and the outside of the cell. This strategy allows overcoming the missing-wedge artifacts that commonly hamper direct detection of membranes in these data sets. Simulation studies and the excellent match of extracted traces with visible membranes in experimental data indicate extremely high accuracy (more than 99% of traced points within one nanometer of the original membrane layer). This tracing capability sets the stage for nanometer-resolution analysis of the connections between cells and the extracellular matrix.

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highlights

The connection between the ECM and the cell is of major importance.

Electron cryo-tomography provides nanometer-resolution information.

Data collection geometry hampers extraction of membranes from tomograms.

We introduce a novel membrane tracing strategy allowing high fidelity extraction.

Simulations show that over 99% of the membrane can be correctly modeled this way.

Figure 1. Effect of missing wedge. A. Orthogonal slices through a membrane model. B. Orthogonal slices through a tomographic reconstruction with restricted angular range ($\pm 65^\circ$) tilting the model in A around the Y-axis. Note how a large fraction of the membranes perpendicular to the missing projections disappear.

Figure 2.

Reconstruction and post-processing of tomographic reconstructions. A. Side views (YZ) of simulated cell reconstruction. B. After post-processing, recognition of the cell boundary is much improved. C. Original membrane model overlaid on side view in B. D. View tilted by 15° around the Y-axis. Despite the apparent boundary in the side view, there is no connectivity for the top (dorsal) and bottom (ventral) membranes that would allow automatic or manual tracing. Only the leading edge part of the membrane (to the left) can be traced. E. Side view (YZ) of experimental reconstruction. F. Experimental reconstruction after post-processing. G. Final membrane estimate overlaid on side view in F. H. View tilted by 15° around the Y-axis shows that neither dorsal nor ventral membranes can be traced.

Figure 3.

A. Processing flowchart for membrane tracing in electron cryotomograms. Typical timing for each step is shown to the right, roman numerals refer to steps shown in (B). B Depictions of processing steps. Coarse-grained application of the watershed transform (i) allows deletion of density outside the cell (ii). Tomogram segments shown in different colors in YZ side view. After fine-grained watershed application and center extraction from the segments, the density is represented as a point cloud (red dots), shown in XY view from the top (iii). The points are collapsed onto X-slices (iv) to allow determination of the two-dimensional convex hulls for each slice. In side view (YZ), outliers (green dots) are easily identified and eliminated (v). The convex hulls only leaves a sparse set of points on the boundary between inside and outside of the cell (vi). Linear interpolation between the sparse convex hull points (vii, red lines, upper panel) leads to inaccuracies and unnatural kinks in the membrane estimation. Thin Plate Splines (vii, blue trace, lower panel) provide a smooth interpolation between the points taking the entire 3D point set into consideration. C. The Thin Plate Splines (blue dots top, yellow dots front, pink dots bottom splines) provide an excellent approximation of the membrane.

Figure 4.
Orthogonal views of the simulated cell regions.

Figure 5.

Comparison of membrane tracing performance using simulated data. A thin slice along the X-axis of the density is shown (YZ view). The grey area corresponds to the model membrane. A. Boundary-based membrane detection (Martinez-Sanchez et al., 2013). Only a small portion of the membrane (red area) is detected. B. Watershed transform region-based segmentation (Volkman 2002). The entire membrane is estimated (green area) but the accuracy of the estimate is not very high. C. The method described here achieves high accuracy for essentially the entire membrane (blue area).

Figure 6.

Membrane tracing in experimental reconstructions. A. XY slice through one example tomogram. The extracted membrane trace (blue) is overlaid with the visible portion of the leading edge membrane. B. Enlargement of A showing the excellent fit between membrane trace and actual leading edge membrane. C. Surface representation of the same tomogram with membrane overlay. D. YZ and XZ slices through the same tomogram with membrane trace overlaid in blue. E. Slices through two additional experimental example tomograms with extracted membrane traces (blue) overlaid.

Table 1

Performance of membrane tracing methods

	P₁	P₂	P₃	B
Current Method	99.6%	100.0%	100.0%	99.5%
Boundary-Based	99.6%	100.0%	100.0%	7.0%
Region-Based	45.8%	61.2%	70.9%	100.0%