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Efficient Extraction of Macromolecular Complexes from Electron Tomograms Based on Reduced Representation Templates

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Abstract

Electron tomography is the most widely applicable method for obtaining 3D information by electron microscopy. In the field of biology it has been realized that electron tomography is capable of providing a complete, molecular resolution three-dimensional mapping of entire proteoms. However, to realize this goal, information needs to be extracted efficiently from these tomograms. Owing to extremely low signal-to-noise ratios, this task is mostly carried out manually. Standard template matching approaches tend to generate large amounts of false positives. We developed an alternative method for feature extraction in biological electron tomography based on reduced representation templates, approximating the search model by a small number of anchor points used to calculate the scoring function. Using this approach we see a reduction of about 50% false positives with matched-filter approaches to below 5%. At the same time, false negatives stay below 5%, thus essentially matching the performance one would expect from human operators.

Keywords

Electron tomography; Feature extraction; Template matching; Reduced representation; Cellular tomography; Biological systems; Ribosomes; Actin filaments

1 Introduction

Modern biology has now advanced to a stage where structural information about isolated macromolecules and assemblies must be integrated to define higher-order cellular functions. Electron tomography (ET) has become a powerful tool for revealing the molecular architecture of biological cells and tissues [1–5]. The achievable resolution (3–9 nm) is intermediate between that achievable by light microscopy and X-ray crystallography, thus capable of bridging the gap between live-cell imaging and atomic resolution structures. Approaches that can directly visualize cellular organization by defining and analyzing macromolecular arrangements and interactions at high resolution within these tomograms would have a direct and major impact on research efforts in many branches of biology and biomedicine [6–8].

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Electron tomography is the most widely applicable method for obtaining 3D information by electron microscopy [1], [4]. In fact, it is the only method suitable for investigating polymorphic structures such as organelles, cells and tissue. Its principle is based on illuminating the sample from many different directions (usually a tilt series around one or two axes) and to reconstruct it from those projection images. While the principles of electron tomography have been known for decades, its use has gathered momentum only in recent years.

Electron tomography provides data that is - at least in principle - capable of connecting between high-resolution structure and function in living cells by high-resolution mapping of the entire proteome of cells and tissues. However, the lack of algorithms for reliable detection and extraction of structural features and the lack of tools for large scale and automated analysis of the extracted data pose severe barriers to progress in the field. As of today, the tasks of extracting and interpreting information from the highly complex, three-dimensional scenes that make up cellular tomograms are, for the most part, painstakingly carried out manually. Apart from the subjectivity of the process, the time consuming (and tiring) nature of this manual task all but precludes the prospects of the high throughput necessary to take full advantage of the method's potential.

For example, it took over 10 months to manually segment and interpret roughly 1% of the volume of a pancreatic beta cell at high fidelity [9]. Extrapolating this to the time it would take to segment just one entire cell at similar detail comes out to be 83 years, and 4 months. Conducting a meaningful study comparing a number of cells under disease conditions with a control set would literally take thousands of man-years. The need for computational tools to efficiently aid the process and automate the structure recognition, extraction, and interpretation process as much as possible is clearly vital for making these types of studies viable.

2 Material and Methods

The quality of cryo-tomographic reconstructions can be correlated with the electron dose. A total dose of 50–300 $\text{e}^-/\text{\AA}^2$ appears to give reasonable results with a “sweet spot” around 120 $\text{e}^-/\text{\AA}^2$ [10]. Because this dose needs to be spread over the whole data set, the dose for each image needs to be kept low enough as to not exceed a total dose of 120 $\text{e}^-/\text{\AA}^2$. For a $\pm 60^\circ$ double tilt series with a 2° increment, the dose available for a single image is only 1 $\text{e}^-/\text{\AA}^2$, which gives rise to extremely high noise levels in the individual images. The signal in the resulting 3D reconstructions is improved by the dose fractionation effect [11] but the signal-to-noise ratio for these tomograms is still well below 1 (often only 0.01–0.1). Together with complications from missing data and the electron microscope's contrast transfer, this makes noise from many other image-possessing disciplines look like “Girlie Noise” (Stephan Nickell, personal communication). Owing to the immense technical difficulties of cryo-sample sectioning [12], “conventional” electron tomography, which involves staining and plastic embedding, is often preferred in practice for samples that require sectioning [13]. While the signal-to-noise ratio is improved in these samples as compared to cryo-samples, the resulting images and reconstructions still tend to be rather noisy with signal-to-noise ratios usually well below five.

2.1 Challenges

The lack of automatic and/or objective interpretation tools for electron tomograms poses a critical barrier to progress in the field [1, 5, 14]. While there have been substantial efforts during the last few years [15–18], progress has been much slower than in related fields. One of the prime reasons for this is that image and signal processing methods developed for other domains are not straightforward to apply to electron tomography data owing to its special characteristics:

- As a consequence of the damaging effect of the electron beam, which limits the amount of electrons available for image formation, electron tomograms tend to exhibit very high noise levels.
- The geometry of the electron tomography sample holders and the shape of the electron microscopy chamber do not allow tilting more than $\sim 70^\circ$ so about a third of data space is not accessible. This generates a “missing wedge” of data, which is best seen in Fourier space as a wedge-shaped segment that contains no information. This problem can be partially alleviated experimentally by taking a second data set after rotating the sample by 90° around the optical axis, yet some of the data space is still not accessible so that some missing data artifacts will always remain.
- The transfer function of the microscope is not well determined, especially for thick specimens where there can be a significant variance in focus. In addition, the tilting introduces a focus gradient, further obstructing the underlying signal.

Although each of these issues would probably not be completely prohibitive by itself, the combination of the three makes devising automatic and objective interpretation tools a very difficult task.

2.2 Related Work

The idea of detecting and mapping macromolecules in cellular tomograms is not new. In 2000, the first feasibility test for detecting macromolecules in tomographic reconstructions using a correlation-based template-matching approach were presented [19]. This test was later expanded by others [20–22] to demonstrate feasibility in experimental settings. This type of template matching consists of using a ‘matched filter’ which can be shown to be a Bayesian classifier (minimizing the probability of identification errors), as long as the template and the target are nearly identical and the noise is independent and identically distributed, Gaussian, and additive [23]. These conditions are not very well met for electron tomographic reconstructions: the noise is spatially correlated by the reconstruction process and the point spread function; the tails of the noise distribution are often quite heavy, especially in stained samples [24], making the noise distribution distinctly non-Gaussian; and the uncertainty in the magnification, the potential mix of conformations, and/or the presence of stain make it difficult to obtain sufficiently accurate templates. As a consequence, many false hits are generated by this method in areas of high density such as membranes or dense vesicles (see Figure 2A, also reference [21]).

2.3 Reduced Representation Templates

To address these challenges, we are currently developing an alternative method for feature recognition in tissue volumes, which is based on reduced representation templates. Reduced representations approximate the target by a small number of anchor points. These anchor points are then used to calculate the scoring function within the search volume. This strategy makes the approach robust against noise and against local variations such as conformational pleiomorphism. The first step of the procedure consists of the construction of suitable representations that are to be used as templates for pattern matching. This can be either done from reprojections of existing models (if available) or directly from the data. In principle, the direct use of data is preferable to the use of models because this strategy eliminates model bias and avoids possible problems connected to differences in scale or resolution.

Macromolecules: The simplest possible representation of a density distribution is a set of points that describe the position of bright and dark spots within the motif. More complex representations that involve ranking of points with varying gray-scale can also be constructed. The speed of calculating a particular scoring function in real-space depends on the number of contributing anchor points. The fewer anchor points are used in the calculation, the faster the calculation will be finished. In addition, the idealization of the density pattern to a collection of bright and dark spots tends to be more robust than a fully detailed template in respect to minor distortions due to noise.

With the reduced representation concept, arbitrary real-space scoring functions can be used without loss of generality while still taking advantage of the corresponding speed gain. In practice, a simple expression of the form

$$score = \max_{angle} \left[\sum (D(p_{outside}) - D(p_{inside})) \right] \quad (1)$$

where $D(p)$ denotes density sampled at anchor point p , achieves high quality results at maximum speed. Here, the sum is calculated for each Euler angle of the template the maximum is taken as the final score. *Score* will be high if at some angle all anchor points assigned ‘inside’ in the reduced template have relatively high intensities and - simultaneously - if all points assigned ‘outside’ have relatively low intensities.

Filaments—Reduced representation templates can capture arbitrary shapes. However, for the construction of reduced representation templates for filaments within cells, we can take full advantage of the filament symmetry. In fact, our tests indicate that a simple, rotationally symmetrical rod-like template works equally well as templates coding filament symmetry more accurately. The use of a rotationally symmetric template speeds up the search considerably: the template does not need to be rotated along the long axis, and only half of the search space needs to be explored because the template is also two-fold symmetrical perpendicular to its long axis. Another important consideration for filament detection is the length of the template. Optimal response will be at a length that is not too long to miss shorter segments or filament bents, and not too short to be unreasonably affected by noise in the tomogram.

2.4 Score Map Analysis

The second step of our detection approach is an analysis of the peak properties. The rationale behind this idea is that, for isolated macromolecules, a true hit should give a scoring-function peak that is compact and spherical around the correct position. For the 2D case, a step that tests for the sharpness of the peaks significantly enhances performance [26]. For the proof-of-concept 3D studies presented here, we implemented a test for the sphericity Sph of the peak. Here, sphericity is defined as

$$sph = (\pi^{1/3} 6V_{0.5}^{2/3})/S_{0.5} \quad (2)$$

where $V_{0.5}$ is the peak volume at half maximum and $S_{0.5}$ is the surface area at the same cutoff.

For filaments, sphericity is not a useful criterion. Instead, we use a modified scoring function that penalizes high variance along the long axis of the reduced representation template, thus enhancing the signal when brightness is approximately the same along the filament axis. As an additional criterion we use directional coherence. Here the angle that gives the highest sum is recorded for each pixel in the density map and then compared to the angles giving the highest sum for pixels that lie along the direction of the first angle so that

$$ang = \sum (\cos(\varphi_0 - \varphi_i)) \quad (3)$$

with φ_i denoting the angle at the pixel i along the axis defined by φ_0 at the central pixel.

Once filaments are detected, the score map need to be converted into parametric filament traces for further analysis. The traditional approach to achieve this task is skeletonization. However, the high noise level and the distortions due to the missing wedge in the tomograms make this approach ill-suited for extracting filament traces from score maps originating from electron tomograms.

We implemented an alternative approach that combines hierarchical watershed segmentation of the score map with classification of the segments based on the eigenvalues of the inertia tensor to identify filament-like segments. Briefly, a modified watershed transform specifically developed for electron microscopy data [27] is run directly on the score map using a conservative step size that allows separation of neighboring filament traces as well as branching filaments at the expense of slight over segmentation. The eigenvalues of the inertia tensor are then calculated for each segment. At this stage, false hits such as sheets (membranes) or star-like structures (highly intense compact contaminations) can be eliminated based on the ratios of the eigenvalues. Next, the centers of mass of slices perpendicular to the longest principal axis are determined for each segment to provide the corresponding traces. Traces are linked according to an analysis of the density between them and the directionality of the traces. This approach is significantly more robust in the presence of noise and gives much better defined traces than skeletonization approaches (Figure 2).

3 Results and Discussion

The reduced-representation template strategy presented here provides an approach robust against noise and against local variations such as those expected from thickness variations common in 3D biological tomograms. We recently completed two proof-of-concept applications of this algorithm (i) for detecting ribosomes in electron tomograms of high-pressure-frozen plastic embedded mammalian cell sections (Figure 1) and (ii) to trace actin filaments in reconstituted systems (Figure 2).

A study comparing performance of various algorithms for picking particles from two-dimensional cryo-micrographs showed that a 2D version of the reduced template matching approach [26] yields comparable or better results than methods using matched filters even if near-correct templates can be constructed for the matched filters [30]. Results for the implementation of a 3D version described here, using hand-annotated data, show very encouraging results for the detection of macromolecular assemblies (Figure 1) as well as for the extraction of filament traces (Figure 2).

The test for macromolecule detection was done using a hand-annotated tomogram of an embedded and stained beta cell section. Ribosomes were manually picked to provide a ‘gold standard’ for assignment of false positives and false negatives when using computational procedures for ribosome detection. Hits produced using a correlation-based matched filter approach [22] using a low-pass filtered high-resolution single-particle reconstruction [25] as a template produces 49.3% false positives. Application of the reduced-representation template approach produced 18.5% false positives. The reduced representation used consists of a sphere of 20 inside anchor points surrounded by a shell of 18 outside anchor points. The fact that the template is spherically symmetric, allowed a further speed-up of the calculation, which can be done within a fraction of the time required for the matched filter approach. After analysis of peak sphericity and filtering accordingly, only 3.2% of the original 18.5% false positives remain. With a percentage for missed targets of 2.6%, this is essentially the type of performance one would expect from human operators.

As an independent test of whether the extracted motifs correspond to ribosomes and not arbitrary blobs of density, we mutually aligned and averaged the extracted motifs and compared the average with the known high-resolution structure of the ribosome [25]. The analysis shows that the average and the known ribosome structure are indistinguishable at a resolution of about 6.5 nm, which is about the resolution expected for tomograms of embedded, stained sections, thus providing a strong indication that the extracted motifs are indeed ribosomes.

For testing filament detection performance, we used two sets of hand-annotated tomograms. The first consisted of tomograms of reconstituted Arp2/3 complex mediated actin networks [28], similar to those encountered at the leading edge of motile cells [31]. This is a very good test case because of the high background of particles (unbound Arp2/3 complexes), which allows testing performance in the presence of particles that potentially throw off the detection. Furthermore, Arp2/3 induces branch junctions that may also degrade performance. The second set consisted of tomograms of actin arrays cross-linked by vinculin

[29], mimicking dense actin bundles similar to those encountered in stress fibers and other actin-based structures in the cell. In both cases, the performance of the detector reaches a false positive rate below 5% with the false negative rate staying below 5%, again essentially matching the performance expected for human expert operators.

4 Conclusions

Automatic feature detection for macromolecules in biological 3D tomographic data is an important and unsolved problem. This paper introduces a new approach based on the use of reduced representations and analysis of the resulting score map. The percentage of false positive for the proof-of-concept data presented here drops dramatically from about 50% to under 5% if compared to the matched filter approach.

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Fig. 1.

Feasibility test of our detection algorithm applied to ribosome detection in a tomogram of an embedded and stained beta cell section. Hits were classified manually into true hits (red) false membrane hits (cyan), false hits in dense vesicles (green) and other false hits (blue). **A.** Hits produced using a correlation-based matched filter approach [22] using a low-pass filtered high-resolution single-particle reconstruction [25] as a template (yellow surface in E and F). This approach produces 49.3% false hits. (bottom) Enlarged area. **B.** Hits produced using the reduced representation template shown in D. 18.5% false hits. **C.** Hits after analysis of peak sphericity on the hits in B. Only 3.2% false hits remain. **D.** Reduced representation used for template matching in B. The reduced representation template consists of a sphere of 20 inside anchor points (red) surrounded by a shell of 18 outside anchor points (grey). The template used in A is overlaid as a transparent surface for reference. **E, F.** Two orthogonal views of the ribosome template used in A (yellow) and an average of 256 motifs extracted as in C (grey), calculated according to the procedure laid out in the text. The resolution estimate for the average is 62Å using the 0.5 cutoff of the Fourier shell correlation between the yellow and the gray densities. **G.** Slice through the template density used for the test in A. **H.** Slice through one of the extracted ribosomes in a similar orientation.

Fig. 2. Actin filament detection using reduced representation templates **A.** Slice through a tomogram of reconstituted Arp2/3 mediated actin networks [28] **B.** Filament detection performance (score map) using reduced representation templates as outlined in the text. Note the lack of interference from the heavy background. **C.** Filament detection in actin arrays cross-linked by vinculin [29]. Original data (top), and score map with overlaid filament traces (in red) are shown. Despite of the heavy cross-linking, the traces are extracted with high accuracy.