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Putting structure into context: fitting of atomic models into electron microscopic and electron tomographic reconstructions

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Abstract

A complete understanding of complex dynamic cellular processes such as cell migration or cell adhesion requires the integration of atomic level structural information into the larger cellular context. While direct atomic-level information at the cellular level remains inaccessible, electron microscopy, electron tomography and their associated computational image processing approaches have now matured to a point where sub-cellular structures can be imaged in three dimensions at the nanometer scale. Atomic-resolution information obtained by other means can be combined with this data to obtain three-dimensional models of large macromolecular assemblies in their cellular context. This article summarizes some recent advances in this field.

Introduction

Recognition and cooperative interaction among molecules in large assemblies are fundamental for dynamic processes in living cells. Understanding of how these assemblies work often requires structural information at the atomic level. Nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography are well-established approaches for obtaining atomic structures of individual molecules and domains. However, atomic structures of large macromolecular assemblies remain more difficult to obtain with these methods. These complexes can be too large to be amenable to NMR and often exhibit a large degree of flexibility that hampers crystallization attempts.

Electron microscopy has been a powerful tool for investigating biological structures for several decades now, but only recently steps towards achieving its full potential have begun to come to fruition. High-resolution electron microscopy studies of purified macromolecules are starting to rival X-ray crystallography in the resolution achievable and it is now possible to image frozen hydrated cells and tissue sections at close-to-native conditions at resolutions that allow identification and analysis of molecular components [1,2]. Technical advances in electron microscopy equipment, in methods of specimen preparation, and in computational image reconstruction methods have all been essential in enabling the remarkable progress we have witnessed in the last few years. It is now possible to achieve resolutions of 0.5 nm or better not only from two-dimensional crystals [3] or helically symmetrical objects [4], but also from icosahedral virus particles [5] and even from smaller, less symmetric particles [6**,7]. Electron tomography, the most widely applicable method for obtaining three-

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dimensional information by electron microscopy, can now be combined efficiently with localization and dynamics data from light microscopy [8,9*] and potentially allows investigation of entire mammalian cells at molecular resolution [10], paving the way for structure-based systems biology [11].

Since the potential of the method has been realized, more and more methods are emerging that target efficient incorporation of atomic-level information into reconstructions derived by electron microscopy. Here, we will describe recent advances and open challenges in this field with an emphasis on assembly reconstructions at intermediate resolution (1–3 nm) and tomographic reconstructions of eukaryotic cells as they relate to the actin cytoskeleton, a major determinant of dynamic cellular processes such as directed cell migration and focal adhesion dynamics.

Reconstructions of isolated cytoskeletal assemblies

Many biological assemblies occur naturally in helical form, particularly cytoskeleton filaments. These filamentous structures are not usually amenable to crystallization due to their natural tendency to polymerize. Image processing of electron microscopy data can take advantage of the helical symmetry and can, in principle, achieve near-atomic resolution [4,12,13]. However, owing to the intrinsic flexibility of filamentous actin [14], the structure determination of actin-filament assemblies generally does not extend into the subnanometer range. The notable exception to this rule is the recent three-dimensional structure of rabbit skeletal actin filaments, which was determined at a resolution of better than 0.7 nm [15**]. Electron microscopy reconstructions of actin filaments in complex with binding partners most commonly fall into the 1.2–2.5 nm resolution range. Examples of actin filaments with bound domains of cytoskeletal proteins that were recently solved include actin in complex with tropomyosin [16], myosin binding protein C [17,18], α -actinin [19], eps8 [20], drebrin [21], coronin-1A [22], talin [23], and fimbrin [24]. Three-dimensional structures of actin filaments crosslinked by villin [25] and vinculin [26] as well as of arp2/3-mediated actin branches [27] have been determined by electron tomography at resolutions of about 2.5–3.5 nm.

Fitting of atomic models into intermediate resolution density maps

In the 1–3 nm resolution range that is most generally achievable for actin-based cytoskeletal assemblies, it is possible to map individual subunits and thus to understand the general architecture of the assemblies. This intermediate resolution also gives a solid basis for fitting high-resolution structures of smaller entities into the reconstructions. The resulting models are often referred to as 'pseudo-atomic' models to hint at the fact that the accuracy of the atom positioning is of limited resolution. This is an unfortunate and confusing term because the models are built out of actual atoms and the term 'pseudo atom' is often used to denote atom-like representations of entire residues or other groups of atoms in coarse-grained molecular modeling [28], direct phasing approaches [29,30] or nuclear magnetic resonance calculations [31].

Until recently, correlation-based rigid-body fitting [32–34] has been the most commonly used tool to achieve the goal of fitting high-resolution structures into reconstructions from electron microscopy. Because of the increased availability of subnanometer resolution reconstructions where secondary structural elements are often visible as rods (α -helices) and sheets (β -sheets), significant efforts have been directed towards developing 'flexible' fitting methods that allow the high-resolution structures to be distorted in some way, subject to different types of constraints, in order to improve the fit with the density [35–49]. However, these flexible fitting methods are not necessarily useful for resolutions above the subnanometer range where secondary structural elements are not discernible. Recent test

calculations indicate that the conceptually simpler modular rigid-body fitting of domain structures often surpasses the achievable accuracy of the models obtained by flexible fitting [50**].

The need for validation tools

At intermediate resolution, depending on the shape of the structure, the number of parameters that can be determined can be severely limited. Care must be taken that the number of the degrees of freedom used during the fitting procedure does not exceed the number of independent observations. Otherwise, overfitting will inevitably ensue. Even the fitting of a rigid body using only the six rotational and translational degrees of freedom can lead to ambiguities in the resulting models [51]. Recently developed methods for incorporating data from other data sources such as Förster resonance energy transfer [52*], proteomics [53*], or sparse distance restraints [54*] into the fitting process are helping to resolve some of these ambiguities.

However, it is clear that rigorous and objective statistics-based evaluation criteria are needed to corroborate conclusions drawn from these models. The inherent uncertainties could be expressed by considering the various possible conformational changes and orientations that fit the observed data equally well. A promising step into this direction is the use of statistical tools to obtain confidence intervals for the orientation parameters in modular fitting of rigid body domains, which allows determining ensembles of structures that fit the data equally well [50**]. These ensembles can then be used to estimate the uncertainties in the positioning of the atoms or in interaction surfaces.

Unfortunately, statistical procedures are sometimes applied in a very casual manner to density fitting procedures so that the conclusions deduced are not always reliable. A recent example is the comparison of different scoring functions for the quality of fit [55]. The authors calculate and compare confidence intervals by assuming that all scoring functions follow Gaussian, normal distributions. However, it is well known that, for example, the correlation coefficient, one of the scoring functions analyzed, does not follow a Gaussian distribution at all and needs to be subjected to a variance-stabilizing variable transformation [56] before reliable confidence intervals can be calculated. Since no normality tests were performed for the other scoring functions and a visual inspection of the distributions does not convey a particularly Gaussian shape for any of those, the confidence intervals calculated under the normality assumption can not be considered to be supported by the data.

Cellular electron tomography

Electron tomography is the most widely applicable method for obtaining three-dimensional information of large assemblies. In fact, it is the only method suitable for investigating unique structures such as organelles, cells, and tissues at a relatively high resolution of 4–8 nm. The reasons for the limited resolution as compared to other electron microscopy techniques are manifold. Primary bottlenecks include extremely low signal-to-noise ratios and low contrast in the tomograms. Technical developments are under way to improve upon both of these issues. Phase plates that are mounted inside the microscope can modify the microscope characteristics so that the contrast in tomograms is significantly improved [57] [58]. Direct electron detection devices promise to improve the signal-to-noise ratio of the cameras as well as optimize the efficiency of signal detection [59].

Structure determination by electron microscopy and image reconstruction requires the sample to be thin enough to allow transmission of the image forming electrons. This limits the sample thickness to about 500 nm, which is sufficient for small bacteria [60] and viruses

[61] but can be problematic for eukaryotic cells. Cryo-sectioning is one method that can produce thin sections [62–65] but tends to produce cutting artifacts. Focused ion beam milling can also produce thin enough sections [9*,66,67] but the technique is still in its infancy. Alternatively, electron tomography can be restricted to thin regions of the cell. Electron cryotomography has first been used to investigate the actin cytoskeleton at the cell edges of Dictyostelium cells [68*,69]. More recently, cell edges of eukaryotic cells have also been investigated [70–72]. The computational tools for interpreting these dense, crowded tomograms are still in their infancy and, for the most part, interpretation is carried out manually. The inherent subjectivity of the process has led to a major controversy in the field [73,74], corroborating the need for objective computational tools in this area.

Extracting structural information from electron tomograms

The primary tool for extracting molecular level information from electron tomograms is currently based on template matching [75–78]. It has, so far, mostly been applied to the detection of isolated macromolecular assemblies such as ribosomes [79,80], but has also been used for detecting filaments [81] and membranes [82,83]. The major challenge in this framework is to distinguish true positive from false positive detections. The detection performance depends on tomogram-specific parameters such as sample thickness, data acquisition settings, and the degree of molecular crowding. It also depends on target-specific parameters, such as abundance in the cell, molecular weights, and cellular abundance of assemblies with similar structural signature competing for detections.

In a recent study, proteomics experiments for detecting the identity and concentrations of cellular proteins of the pathogen *Leptospira interrogans* were performed and combined with electron tomography-based template matching to detect spatial localizations [78,84]. This experiment allows estimating the detection performance of the template matching approach in light of the proteomics data. The study showed that ribosomes can be discovered at an estimated true positive rate of better than 90% but discovery rates higher than 50% are difficult to achieve for targets of smaller molecular weights, indicating that there is room for improvements. The detection of low abundance target assemblies did not work out at all. A recently introduced alternative approach for detecting macromolecules in cellular tomograms is based on an initial template-free classification using rotation-invariant features of the tomogram, which is then refined using a Gaussian Hidden Markov Random Field [85]. The advantage of this approach is that it does not depend on templates. However, the current performance on simulated data is relatively poor and indicates that further development is necessary before this approach will be a viable alternative to template-based methods.

Correlating high-resolution information with electron tomograms

The quality and resolution of the raw densities of macromolecular assemblies extracted as subvolumes from electron tomograms are generally not good enough for direct structural interpretation or meaningful fitting of atomic models. In order to boost the signal to make this feasible, the subvolumes must be aligned, classified, and averaged. Several approaches have been developed recently to address these issues [86–92]. A recent study uses kernel density estimator self-organizing maps for classification of the extracted subvolumes [93**], which shows very encouraging results not only for the classification step itself but also for cross-validation of template-matching algorithms applied to electron tomograms. Once classification, alignment and averaging is achieved, the quality of the density maps is greatly improved and fitting of high-resolution atomic models can be pursued in an analogous fashion to that used for other electron microscopy reconstructions. Because the resolution tends to be lower than that of single-particle reconstructions (current limit about 2.5 nm), it

is of major importance to minimize the degrees of freedom exploited in the fitting process and to employ validation procedures to detect ambiguities.

Concluding Remarks

Electron microscopy and electron tomography, in conjunction with computational tools for integrating atomic-resolution information, are already making it possible to provide a bridge between cell biological function and molecular mechanism. With further improvements in experimental methods and hardware, in conjunction with emerging technologies such as correlative light and electron microscopy [8,9] and iPALM [94,95], these approaches will not only allow high-resolution mapping and interpretation of macromolecular assemblies and cytoskeleton elements in eukaryotic cells but will also allow direct correlation with dynamics information from life-cell imaging. Current bottlenecks include the relatively low signal-to-noise ratio and high noise level of electron tomograms as well as the lack of validation tools for incorporating atomic level information. However, in both areas promising progress has been made in the last few years. In summary, electron microscopy is likely to provide major contributions for defining the detailed spatiotemporal framework that is necessary for pushing the understanding of cell structure and dynamics to the next level. Further technical progress combined with systematic integration of atomic-resolution and dynamics information should allow electron microscopy to be a major player in the future of structural cell biology.

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Highlights

- Electron microscopy provides nm-range information on large biological assemblies
- Cellular context can be provided by electron tomography
- Atomic-resolution information can be combined with both types of data
- These approaches provide an essential link between structure and cell function

Figure 1. Schematic workflow for correlating atomic-level information with large, dynamic cellular structures. As an example, we place actin atoms into a cellular, actin-rich protrusion of a mouse embryonic fibroblast [71]

A. Structure of actin obtained by X-ray crystallography [96] (pdb accession code: 1atn).

B. Modularization of the structure into the four sub-domains.

C. Density of actin filament at 0.7-nm resolution [15**] (emdb accession code: emd_5168).

D. Single actin monomer density segmented from the filament density using the model-free, three-dimensional watershed procedure [97].

E. Iterative modular fitting [50**] of the actin subdomain structures into the monomer density segmented from the electron microscopy reconstruction of filamentous actin.

F. Slice through a tomogram of an actin-rich protrusion of a mouse embryonic fibroblast [71].

G. Template-based automatic segmentation of tomogram shown in F.

H. Watershed-based, template-free segmentation of filament extracted from tomogram shown in F using a mask derived from G. Note that single actin monomers can be segmented from the density.

I. Result of fitting the actin filament atomic model derived in E into the filament density extracted from the cellular tomogram in F-H. The left hand side shows the fit into the unprocessed extracted density. The right hand side shows the fit after the actin symmetry was applied to the extracted density. Comparison of the symmetrized density with the atomic model indicates a resolution of about 0.4 nm.