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Single-particle reconstruction from EM images of helical filaments

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Summary

Helical filaments were the first structures to be reconstructed in three-dimensions from electron microscopic images, and continue to be extensively studied due to the large number of such helical polymers found in biology. In principle, a single image of a helical polymer provides all of the different projections needed to reconstruct the three-dimensional structure. Unfortunately, many helical filaments have been refractory to the application of traditional (Fourier-Bessel) methods due to variability, heterogeneity and weak scattering. Over the past several years many of these problems have been surmounted using single-particle type approaches that can do substantially better than Fourier-Bessel approaches. Applications of these new methods to viruses, actin filaments, pili and many other polymers show the great advantages of the new methods.

Introduction

A significant fraction of the proteins found in prokaryotic, eukaryotic and archaeal cells exist in the form of helical polymers. Many viruses and bacteriophages either have a helical coat protein or helical components, such as nucleocapsids or tails. Pathologies, such as sickle-cell hemoglobin or amyloid formation, occur when proteins that do not normally polymerize undergo an aberrant helical polymerization. Despite the central importance of all of these polymers to many aspects of biology, in only a very few cases do we have atomic or near-atomic structures. This is because x-ray crystallography requires (by definition) a crystal, and the only helices that can pack with crystalline symmetry are those that have exactly two, three, four or six subunits per turn. The implication of this restriction is that the vast majority of these polymers will never crystallize in their native polymeric state. Other techniques, such as x-ray fiber diffraction, can be very useful, but typically require a model-building approach, and there is frequently a concern about whether the model built to match an observed fiber diffraction pattern is unique.

Electron microscopy (EM), on the other hand, has played a central role in the elucidation of the structure of helical polymers. EM images are two-dimensional, while the structures of all macromolecules and macromolecular complexes are three-dimensional. A great leap in structural biology occurred when DeRosier and Klug [1] showed how three-dimensional structure could be recovered from two-dimensional EM images of a helical bacteriophage tail. Helical symmetry (objects related by a coupled rotation about an axis with a translation parallel to the axis) generates the many different views of a single subunit that would otherwise be obtained by tomographic tilting of objects [2], or by finding particles that are randomly oriented on the EM grid [3] which provide the different views needed. Since the Fourier transform of

a helical structure can be simply decomposed into Bessel functions [4], DeRosier and Klug took advantage of this relationship to use Fourier transforms of EM images and Fourier-Bessel inversion of these transforms to generate the first three-dimensional EM reconstruction [1].

For more than 30 years, the Fourier-Bessel method was almost the only method to be used for reconstruction of helical objects from EM images. It was widely applied to such diverse specimens as acto-myosin filaments [5], bacterial flagellar filaments [6] and viruses [7]. For some highly ordered helical structures, such as for a flagellar filament [8] and lipid tubes containing the acetylcholine receptor [9], an unprecedented resolution of $\sim 4 \text{ \AA}$ has been achieved in the EM. At this resolution, the polypeptide chain can be traced. Although these two studies used substantial refinements of the classical Fourier-Bessel approach, needed to correct for the deviations of real structures from an ideal helical symmetry, they were able to achieve this very high resolution due to the high degree of order in both of these specimens. For instances where the intrinsic order of a specimen is even higher, such as in two-dimensional crystals of the aquaporin channel, a resolution of $1.9 \text{ \AA}$ has now been obtained in the EM [10].

A New Approach

For many real helical polymers, however, Fourier-Bessel methods are greatly limited or cannot be used at all, due to weak scattering, disorder and Bessel overlap [11]. That is why single-particle type approaches can be very useful, since they do not require long filaments with uniform order whose images diffract strongly enough to yield visible layer lines. A specific algorithm for single-particle type reconstruction of helical filaments was published in 2000 [12], and applications of this method have now been published in more than 35 papers. While many of these papers come from my own laboratory, an increasing number of applications are coming from other groups [13-21]. The algorithm, called the Iterative Helical Real Space Reconstruction (IHRSR) method, takes advantage of the helical symmetry present in images of helical filaments to impose helical symmetry on asymmetric reconstructions. By an iterative process an initial model, which can be a featureless solid cylinder, is “refined” each cycle and used as a new reference for a subsequent round of alignment and reconstruction (Figure 1). The helical symmetry is measured experimentally each cycle before being imposed, and can thus deviate from an initial guess at the correct symmetry. Figure 2 shows a typical application of this method to F-actin, where the starting model is a solid cylinder. Because of the helical symmetry present in such filaments, one is able to iteratively surmount one of the fundamental problems in single-particle reconstruction: how can one assign Euler angles to individual projection images without having a reference structure?

For highly ordered helical filaments with a uniform symmetry the IHRSR method may not offer any advantage. In fact, when one cuts such long filaments into short segments the ability to align these segments is related to the length of the segment, so the errors in alignment will become greater as the segment length becomes shorter. In such cases, one may be able to do better using the classical approach [19]. The exception is that even if a filament is highly ordered, but has a symmetry (such as a small integer number of units per helical turn) that results in a limited number of different views of a subunit from the image of any single filament, the Fourier-Bessel approach can be extremely problematic. It has been shown with the myosin thick filament that the IHRSR approach is actually transparent to such problems [22]. However, highly ordered helical polymers may be the exception, and not the rule. One particularly highly ordered helical polymer, Tobacco Mosaic Virus (TMV), has recently been reconstructed using a single particle approach (Figure 3) at $\sim 4.7 \text{ \AA}$ resolution [23]. This contrasts with a resolution of $\sim 10 \text{ \AA}$ that was previously achieved using conventional Fourier-Bessel methods with cryo-EM images of TMV [7]. Of course, these two results cannot be directly compared as there are undoubtedly improvements in microscopes and imaging, as well as the different reconstruction

methods. The high degree of order in TMV allowed relatively long segments (700 Å) to be used, in contrast to more variable filaments such as F-actin where we typically use segment lengths of ~ 480 Å. The greatly improved resolution of the TMV reconstruction has allowed for significant differences to be observed in certain regions when compared with a previous atomic model of TMV generated from x-ray fiber diffraction [24]. An interesting question for future studies is whether within such highly ordered polymers as TMV and the bacterial flagellar filament structural variability and polymorphisms will be discernable using single-particle type approaches.

Helical order may be a necessary, but not sufficient, condition for achieving high resolution when using Fourier-Bessel methods. There are some rather well-ordered helical polymers, such as a Type IV bacterial pilus, where the diffraction from individual unstained filaments in ice is so weak that no structural information is apparent [25]. This problem can be quite easily overcome with the IHRSR method, as one does not need to search for the rare filaments that might give rise to some visible diffraction. Instead, a power spectrum can be generated by adding together the diffraction intensities from thousands of short segments, and this power spectrum may unambiguously show the helical symmetry. This symmetry may then be used for the initial guess needed in the IHRSR procedure about the rotation and axial rise per subunit (i.e., the screw operator that relates one subunit to the next in the filament). For the Type IV pilus from *Neisseria gonorrhoeae* a resolution of ~ 12.5 Å was obtained from filaments that do not give rise to any visible diffraction [25]. Application of the IHRSR method to another bacterial pilus, the P-pilus from a pathogenic form of *E. coli* [18], has yielded a resolution of ~ 10 Å.

Structural Heterogeneity

Many helical filaments are much more structurally heterogeneous and disordered than such highly ordered polymers as TMV and the bacterial flagellar filament. In some cases, this heterogeneity and variability can be easily seen by eye (aided by an electron microscope, of course). In other cases, the heterogeneity can be much less obvious. We have shown that the EspA filament, an extension of the needle apparatus within the bacterial Type III Secretion System, has a rather fixed twist, but the axial rise per subunit within the filament can vary by more than 50% [26]. This contrasts with F-actin, where subunits have a fixed axial rise, but a variable twist [27]. The variability in EspA filaments is not readily apparent in images of either negatively stained or frozen-hydrated specimens, but the variability can explain why diffraction from such filaments is so poor. The variability does place restrictions on the resolution that can be achieved, as truly homogeneous subsets of filaments (all having nearly identical parameters) are never going to be obtained. As one sorts segments from such filaments into more and more homogeneous subsets the subsets become smaller and smaller. The task, therefore, is to balance the signal-to-noise ratio (which will depend upon the number of segments being used in a three-dimensional reconstruction) with the homogeneity.

A surprising degree of structural heterogeneity was found in a filamentous bacteriophage, where it was shown by the IHRSR approach that it could exist in at least two different structural states [28]. Filamentous bacteriophage are examples of weakly diffracting polymers, due to the fact that they are thin (~ 70 Å in diameter) and relatively featureless. Bacteriophage fd (which differs from the widely used M13 by only a single amino acid) has a very small capsid protein containing only 50 residues. The main techniques that have been used previously to generate models for the subunit structure and packing have been x-ray fiber diffraction [29] and solid-state NMR [30]. However, these models have differed considerably, and now we have two different EM reconstructions [28] that cannot be reconciled with either the x-ray or the NMR models. It was recently shown [29] that the same NMR constraints used to generate the NMR model could also be satisfied by the x-ray fiber diffraction model, suggesting that

the NMR constraints were not sufficient to generate a unique model. On the other hand, it has been known that due to the cylindrical averaging and overlap of different Bessel functions in x-ray fiber diffraction, models derived from this technique may not be unique, either. For example, a fiber diffraction model for F-actin [31] provided “an almost perfect fit” to the observed x-ray diffraction pattern, but has subsequently been shown to have little experimental support [32]. Hopefully, higher resolution EM reconstructions of such filamentous phages as fd will be able to resolve this controversy generated by the different models from NMR, x-ray fiber diffraction and EM.

Future Directions

While Fourier-Bessel approaches to EM reconstructions of helical polymers [1] have been responsible for the birth of the entire field of three-dimensional EM, it is now clear that many helical filaments deviate from the ideal helical symmetry that is needed for the best applications of the classical methods. In addition, many polymers are too weakly scattering for the traditional approach to be used at all, or have a symmetry that leads to an overlap of layer lines that is not easily solved with Fourier-Bessel methods. That is why single particle approaches will likely emerge in the very near future as the dominant method for reconstructing EM images of helical filaments. With the exception of polymers that maintain near-crystallographic order over long distances, and these are likely the exception and not the rule, it has been shown that reconstructions can be done better using the single particle approach than with the classical method. We should expect increased resolution in many single-particle helical reconstructions in the future, as well as an understanding of the structural variability that occurs within many polymers. Rather than such variability being seen only as an impediment to structural studies, it is likely that in many cases this variability reflects functional properties of these polymers. Thus, we will be able to pose and answer the question “what are the structures of a helical polymer?” rather than “what is the structure of a helical polymer?”

Acknowledgements

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Figure 1.

A schematic diagram showing the IHRSR algorithm. One cycle of IHRSR is depicted, with a reference volume at the top. This reference volume can start as a featureless solid cylinder, and has always been found to converge to the correct structure when the approximate helical symmetry can be initially determined. As one moves in a clockwise manner around the cycle: 1) projections of the reference volume are generated, which typically involve only azimuthal rotations about the filament axis (out-of-plane tilt can also be incorporated, but will be ignored here). These reference projections are used for multi-reference alignment against the images (one finds the single reference projection that gives the highest cross-correlation with an image, and this determines the azimuthal orientation of the segment being examined). The number of reference projections needed depends upon both the resolution and the diameter of the filament. 2) the multi-reference alignment determines the x-shift, y-shift and in-plane rotation needed to bring any image into register with a reference projection, and these parameters are applied to the images. 3) A back-projection is now generated using the aligned image segments. The resulting volume is clearly helical, but helical symmetry has not been imposed. A search is made within this volume for the screw operator (rotation and axial translation) that minimizes the deviation of density from points related by the screw symmetry. 4) The helical symmetry that was found for this cycle is now imposed, and the resulting helically symmetric volume (top) is used as the new reference for the next cycle. The entire procedure is run within the SPIDER software system [33], with calls to external programs that determine and impose the helical symmetry each cycle.

Figure 2.

The application of IHRSR to a subset of F-actin segments that have been classified as having a common twist. A solid cylinder (left) is used as an initial reference volume for two different runs of the IHRSR procedure, starting with twist values of either -160.0° or -168.0° . Both starting values converge to a solution of $\sim -166^\circ$. The reconstructed volume on the right will be almost indistinguishable for a large range of different initial reference volumes and starting symmetries, which is why the algorithm is called “robust”.

Figure 3.

A remarkably high resolution of 4-5 Å has been achieved using a single-particle approach to the helical reconstruction of Tobacco Mosaic Virus [23]. A portion of the density map is shown, along with an atomic model that has been built into it. Image courtesy of Niko Grigorieff.