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An approach to improve the resolution of helical filaments with a large axial rise and flexible subunits

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ABSTRACT

Single particle analysis is widely used for three-dimensional reconstruction of helical filaments. Near-atomic resolution has been obtained for several well-ordered filaments. However, it is still a challenge to achieve high resolution for filaments with flexible subunits and a large axial rise per subunit relative to pixel size. Here, we describe an approach that improves the resolution in such cases. In filaments with a large axial rise, many segments must be shifted a long distance along the filament axis to match with a reference projection, potentially causing loss of alignment accuracy and hence resolution. In our study of myosin filaments, we overcame this problem by pre-determining the axial positions of myosin head crowns within segments to decrease the alignment error. In addition, homogeneous, well-ordered segments were selected from the raw data set by checking the assigned azimuthal rotation angle of segments in each filament against those expected for perfect helical symmetry. These procedures improved the resolution of the filament reconstruction from 30 Å to 13 Å. This approach could be useful in other helical filaments with a large axial rise and/or flexible subunits.

1. Introduction

Helical filaments occur widely throughout nature in the form of bacterial, viral, cytoskeletal, and nucleoprotein complexes. Many of these filaments lack long-range helical order (Egelman, 2007), due to the flexibility required to carry out their versatile functions in different systems. Three-dimensional (3D) reconstructions of helical filaments can be calculated from EM images by extracting information from their Fourier transforms and processing through Fourier–Bessel helical methods (Egelman, 2015). The first 3D reconstruction of a helical structure (the tail of bacteriophage T4) was determined in this way (De Rosier and Klug, 1968). However, generating near-atomic structures by this approach is difficult due to the lack of long-range helical order and to Bessel function overlap. To take advantage of the local helical order along filaments, a single particle, real space approach was developed: Iterative Helical Real Space Reconstruction, or IHRSR (Egelman, 2000, 2010). This method surmounts limitations of Fourier–Bessel helical reconstruction and has been widely applied for reconstructing many different helical filaments (Egelman, 2015). Combined with advances in electron detectors (Ruskin et al., 2013) and data analysis methods (Li et al., 2013), this method has reached near-atomic resolution in several cases, including 3.35 Å with TMV (Fromm et al., 2015), 3.5 Å with type VI secretion system contractile tube (Ge et al., 2015; Kudryashev et al., 2015) and 3.7 Å with the F-actin-tropomyosin complex (von der Ecken et al., 2015).

To implement IHRSR, short segments are computationally cut from filaments by sequentially shifting a short distance along the filament axis (Egelman, 2000, 2010). The translation and orientation of segments are determined through projection matching, making it possible for most segments to match with projections of a reference model if the axial rise per subunit is not very large relative to the pixel size. However, in filaments with a large axial rise, many segments must be translated a long distance (i.e. a large number of pixels when the pixel size is small) along the filament axis to match with reference projections. This can create difficulty in obtaining correct alignment because the low signal-to-noise ratio and non-uniform background in the cryo-EM images can result in multiple cross-correlation maxima (Frank, 2006; Sigworth, 2010). When large image shifts are needed to bring segments into alignment, a correspondingly large search

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range is required to find the associated alignment peak. However, this can result in the choice of a spurious correlation peak that is not correct for the best alignment. This will result in degradation in quality of the resulting reconstruction.

Another factor limiting the resolution of a reconstruction is subunit flexibility, which causes departure of subunits from their strict helical positions dictated by the symmetry of the filament. Two parameters, axial rise (Δz) and azimuthal rotation angle between axially adjacent subunits ($\Delta\varphi$) can be used to describe the symmetry of helical filaments (Egelman, 2007). Subunit flexibility can cause either or both parameters to be distributed over a range of values. However, segments can be sorted into homogeneous subsets based on these helical parameters (Yang et al., 2001; Galkin et al., 2001). Initial helical parameters can be obtained by analyzing the diffraction pattern (Owen et al., 1996; Carragher et al., 1996), refined using IHRSR, then imposed on the reconstruction of each subset cycle by cycle (Egelman, 2000, 2010). Segments in a perfectly ordered filament should obey the helical symmetry if they are cut using the axial rise as the step size, resulting in the difference in the rotational angles assigned to two adjacent segments being approximately equal to the difference expected from helical symmetry. If the segments are well-ordered, then this symmetric relationship between segments should be reflected in the alignment parameters obtained from projection matching. This symmetry restriction can therefore be used to select the best-ordered segments and filaments.

The thick (myosin-containing) filaments of muscle are a system in which a large axial rise per subunit (145 Å) can limit the resolution of the reconstruction. Thick filaments of tarantula have been used as a model system to investigate the structural basis of the relaxed state of muscle. In relaxed thick filaments, pairs of myosin heads are helically organized on the filament backbone, forming asymmetric "J motifs" in which the two heads of each myosin molecule interact, inhibiting each other's activity. This motif is conserved across multiple vertebrate and invertebrate species (Woodhead et al., 2005; Zhao et al., 2009; Pinto et al., 2012; Woodhead et al., 2013; Zoghbi et al., 2008). In the tarantula, these motifs are axially separated by 145 Å (i.e. Δz = 145 Å) and lie on the filament surface along four equally spaced helical strands, resulting in fourfold rotational symmetry (cf. Fig. 5a). Successive motifs along each strand are related by an azimuthal rotation of 30° ($\Delta\varphi$ = 30°) giving a helical repeat of 435 Å (3 × 145 Å). This arrangement results in successive "crowns" of subunits 145 Å apart axially, with each crown containing four subunits separated azimuthally by 90° at every level.

Myosin filaments also display substantial subunit flexibility: myosin heads are of necessity flexibly connected to the filament so that they can cyclically attach to and detach from thin (actin-containing) filaments to generate force and cause sarcomere shortening (Craig and Padrón, 2004). This flexibility causes variations in helical order which severely limits the resolution of myosin filament reconstructions.

Here we have devised a new approach for processing EM images of helical filaments with a large axial rise and flexible subunits in order to improve the reconstruction resolution. The axial positions of crowns of myosin heads in tarantula thick filaments are determined first to make certain that segments approximately correspond with those of an identical reference particle. This precentering leads to a small search range being required in the alignment procedure and therefore to better alignment precision (Liu et al., 2007). We address subunit flexibility by selecting well-ordered segments based on their adherence to helical symmetry and removing whole filaments or parts of filaments with poorly ordered segments. The resolution is dramatically improved by combining these two procedures.

2. Materials and methods

2.1. Sample preparation and cryo-EM

Tarantula thick filaments were prepared as previously described (Woodhead et al., 2005). Blebbistatin was added to the sample to stabilize the myosin heads and improve their helical order (Zhao et al., 2008). 2 μl of sample was applied to a Quantifoil holey grid, and the grid rinsed with relaxing buffer and plunged into liquid ethane after manual blotting for 2 s. Cryo-EM images were recorded with a Gatan US4000 CCD camera on a Titan Krios transmission electron microscope (FEI, Hillsboro OR) at Florida State University, operated at 120 kV. Images were automatically collected in three positions per hole using LEGINON (Suloway et al., 2005). The magnification was calibrated to be 109,000 × based on the 72.5 Å layer line of tarantula thick filaments, corresponding to a pixel size of 1.37 Å in the specimen.

2.2. Image processing

690 EM images were selected following visual screening to remove those with bad ice. Defocus was determined to be within the range 1.0–5.0 μm using CTFFIND3 (Mindell and Grigorieff, 2003). 1430 filaments of width 480 pixels were boxed using helixboxer in EMAN2 (Tang et al., 2007). 320 filaments were selected for further image processing based on the appearance of more than three symmetric layer lines (coming from the 435 Å repeat) in the power spectrum computed from each filament image. These filaments were then band-pass filtered to remove excessive noise (see Section 3.2). The locations of subunit peaks were still not discernible after filtering; therefore the positions of the myosin head crowns in each filament were estimated as follows. Consecutive 480 × 480 pixel segments along the filament were cut by shifting ~145 Å from the previous one to produce 5107 segments. CTF correction was applied to these segments by multiplying the Fourier transform of each segment by the CTF (Sachse et al., 2007). Each segment comprised 4.5 crowns. A 1D projection of the averaged density of the segments from each filament (perpendicular to the filament axis) was used to determine the filament crown positions (see Section 3.2). Based on this, a second set of segments was cut with the crowns now centered in each box. A total of 4886 segments were cut in this way from the 320 filaments. Segments were then decimated to 240 × 240 pixels to improve the signal-to-noise ratio in subsequent projection matching, resulting in a pixel size of 2.74 Å. To better understand the degree of helical order of the myosin heads, class averages of these segments were computed using correspondence analysis and hierarchical clustering, after first performing reference-free alignment in SPIDER (Frank et al., 1996).

2.3. 3D reconstructions

An initial reconstruction was calculated using the data set (5107 segments) in which crowns were not positioned specifically in the box center. A cylinder of constant density was used as the initial model for multi-reference alignment in SPIDER (Frank et al., 1996) to compute the 3D reconstruction using IHRSR (Egelman, 2000, 2010). The polarity of each filament was determined after the reconstruction converged. A second reconstruction was computed from the data set (4886 segments) cut with the crown position in the box center. The differences in the azimuthal angles ($\Delta\varphi$) between successive segments in each filament assigned during the IHRSR procedure for this reconstruction were determined in order to obtain a measure of adherence to perfect helical symmetry ($\Delta\varphi$ should be 30° for the tarantula filament). Segments that did not closely obey helical symmetry (to within 5°) were removed, as were