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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.

Keywords

single particle analysis; Cryo-EM; thick filament; 3D reconstruction; muscle contraction

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

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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; Egelman, 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; Egelman, 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 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 (z) and azimuthal rotation angle between axially adjacent subunits (ϕ) 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; Egelman, 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. $z = 145 \text{ Å}$) and lie on the filament surface along four equally spaced helical strands, resulting in four-fold rotational symmetry (cf. Fig. 5a). Successive motifs along each strand are related by an azimuthal rotation of 30° ($\phi = 30^\circ$) giving a helical repeat of 435 Å ($3 \times 145 \text{ Å}$). 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 pre-centering 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 seconds. 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 LEGION (Suloway et al., 2005). The magnification was calibrated to be $109,000\times$ 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 heliboxer 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 $\text{\AA}$ 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 $\sim 145 \text{\AA}$ 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 $\text{\AA}$. 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; Egelman, 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 (ϕ) 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 (ϕ should be 30° for the tarantula filament). Segments that did not closely obey helical symmetry (to within 5°) were removed, as were any filaments that contained more than 30% of such segments. The remaining 3174 segments were used in a third reconstruction. Out-of-plane tilt was taken into consideration in all these reconstructions. Wiener filtering was applied in the last iteration of each reconstruction (Sachse et al., 2007). Fourier shell correlation (FSC) was used to estimate the resolution (Supplementary Fig. 1) and each reconstruction was Fermi low-pass filtered to the corresponding 0.5 FSC resolution (Fig. 5a,b,c). For simplicity, the reconstructions were not corrected for overfitting (Scheres and Chen, 2012; Henderson, 2013) as our goal was simply to demonstrate the importance of particle centering and quality (Liu et al., 2007) for optimizing resolution with helical filaments having a large axial rise. The observed trend in resolution would be expected to be similar using gold standard

procedures (Scheres and Chen, 2012), which we will use for final reconstructions in future work.

The three reconstructions have been deposited in the Electron Microscopy Data Bank (EMDB) with accessions codes EMD-6512, EMD-6513 and EMD-6514.

3. Results

3.1. Helical order of filaments

Myosin heads are flexibly attached to the thick filament backbone to enable their interaction with actin during muscle contraction. This flexibility causes imperfect head ordering, which places a key limitation on the resolution that can be achieved in a filament reconstruction. Fig. 1 shows that thick filaments in the relaxed state can be well ordered, partially ordered or completely disordered. In Fig. 1a, the arrow shapes of density coming from superposition of far and near myosin heads are similar to each other, indicating good helical order in this filament, confirmed by a symmetric series of layer lines in its power spectrum (Fig. 1d). In partially ordered filaments some arrows disappear or have different angles (Fig. 1b), and the number of layer lines in the power spectrum is less (Fig. 1e). Completely disordered filaments show only the backbone; the myosin heads are randomly oriented and not readily seen (Fig. 1c) and the layer lines are weak or absent (Fig. 1f). Because the main helical features of the thick filaments result from the myosin heads and not the backbone (Fig. 1d-f), the alignment precision of segments and thus the global resolution of the reconstruction depends on the degree of head ordering.

The varying degrees of intrinsic head disorder in thick filaments are also demonstrated by computing class averages of all segments using correspondence analysis and hierarchical clustering, after first performing reference-free alignment (Frank et al., 1996). The segments used for classification were cut using the approach in section 3.2. The class averages can be divided into two groups as indicated by the black and white ovals in Fig. 2b. The densities in one group clearly show the arrow shape resulting from helical ordering of the heads, consistent with the reference projection in Fig. 2a. In the other group, the main feature is four lines parallel to the filament axis (white arrows in Fig. 2b), coming from superposition of myosin tail subfilaments on the far and near sides of the filament backbone (Woodhead et al., 2005); the two fuzzy borders (red arrows in Fig. 2b) result from disordered myosin heads.

Inclusion of disordered segments in the reconstruction increases noise and reduces the resolution. Disordered filaments or segments were therefore removed in two steps (see Methods): first by rejecting filaments that did not show at least three layer lines in the power spectrum, and second by removing filaments that had >30% segments that did not follow the known helical symmetry of the filaments (see 2.3, 3.5). These steps substantially improved the resolution of the reconstruction.

3.2. Estimating the crown positions in each filament

To implement IHRSR (Egelman, 2000; Egelman, 2010), segments are successively cut along a filament, starting from the beginning and shifting a certain distance, and these are

treated as single particles. This method works successfully for many different samples (Egelman, 2015). However, segments cut using the routine IHRSR procedure are not identical particles and must be axially translated to match with equivalent reference projections (Fig. 3). This requires an axial search to find the best alignment. It is preferable that the translation required is minimized so that only a small search range is needed. Non-uniform ice thickness, random high densities in the background (e.g. due to impurities), and low signal-to-noise ratio can produce multiple cross-correlation maxima. Only one of these peaks will give the best alignment, and it may not have the highest value (Frank, 2006; Sigworth, 2010). With a large search range there are more peaks, increasing the likelihood that a spurious peak is chosen, therefore leading to the wrong alignment. Another problem introduced by a large axial translation is the image wrap-around that occurs during alignment (Fig. 3i,j). This causes a mismatch at the end of the image, resulting in reduction in resolution at the ends of the reconstruction.

An effective search range (defined as the distance from the search origin in a given direction) is dependent on both axial rise and pixel size. If the crown positions have random axial distances from the segment center, the percentage of segments that can be properly aligned and make a useful contribution to the 3D reconstruction for a given search range is given by:

$$[\text{Translational search range} \times \text{pixel size} / (\text{axial rise} / 2)] \times 100$$

where the translational search range is in pixels and the pixel size and axial rise are in Å. Only half the axial rise is used because the search is performed from the origin in both axial directions so that the total distance searched is twice the range (Egelman, 2000; Egelman, 2010). If the axial rise per subunit is not large in relation to the pixel size, segments only need to be translated a short distance along the filament axis for correct alignment with a reference projection, so a small search range is effective (Fig. 3h,i). However, if the axial rise is very large relative to the pixel size, as exhibited by tarantula thick filaments, only a small percentage of segments makes a useful contribution to the reconstruction if a small search range is used. Tarantula filaments have an axial rise of 145 Å; therefore, for a pixel size of 1.37 Å and a search range of 5 pixels, only about 9% of the segments will be aligned correctly during the multi-reference alignment procedure. The other 91% exceed this range and cannot be aligned correctly. These misaligned segments will add noise to the 3D reconstruction and decrease the resolution. If the pixel size is increased to 2.74 Å and the translational search range remains unchanged, 19 % of segments can be aligned properly. However, using a large pixel size is not a good way to increase the number of usable segments. In the above case, although this number doubles with twice the pixel size, the percentage of segments still remains low. Moreover, the maximum resolution that could theoretically be obtained drops in inverse proportion to the pixel size. This problem can be solved if the axial positions of the segments are pre-aligned as closely as possible to those of the reference projection so that they can be refined effectively in the alignment step using a minimal search range (Liu et al., 2007).

We achieved this using a different method to cut the segments. The 1D density projection of a filament perpendicular to the filament axis is a periodic function with the period equal to the axial rise. This fact can be used to cut segments with the maximum density of a crown at

the box center. Fig. 4b shows such a 1D projection for a single filament. However, no obvious periodic features can be observed due to the low signal-to-noise ratio (Fig. 4a). Therefore, both low- and high-pass filters were applied to filaments to remove high frequency ($> 1/10 \text{ \AA}^{-1}$) and low frequency ($< 1/400 \text{ \AA}^{-1}$) noise. To further reduce the noise, segments cut using the IHRSR procedure and successively shifted by the axial rise ($145 \text{ \AA}$) were averaged together, producing a 1D projection with greatly improved periodic features (Fig. 4d cf. Fig. 4b). The segment polarity is evident in the asymmetrical nature of the density peaks. The position required to cut the first segment of the filament with a crown in its center could now be readily calculated, and successive shifts of $145 \text{ \AA}$ along the filament axis were then used to cut the other segments. Note that in this procedure, two sets of segments were cut. One set comprised temporary images used to estimate the crown position. In this set, from all filaments combined, the crown positions randomly distribute within segments and so the computed global average does not show the crown positions (Fig. 4e). This data set was used in an initial 3D reconstruction (section 3.3). In the second set of segments from all the filaments, a crown has been positioned close to the box center. The global average of this data set shows five crowns, with one at the box center (Fig. 4f). The polarity seen in Fig. 2a is not observed here because the segment polarity was not corrected in this step and was averaged out. The absence of polarity is indicated by the symmetrical peaks in the 1D projection (Fig. 4g). This second data set was used in the image classification described in section 3.1 and the second and third 3D reconstructions described in sections 3.4 and 3.5.

3.3. Initial 3D reconstruction

An initial reconstruction was calculated using the first data set of 5107 segments (see 2.2, 2.3), in which the axial positions of the crowns were randomly distributed with respect to the box center; this is the normal IHRSR procedure (Egelman, 2000; Egelman, 2010). The first segment was cut at the beginning of each filament and subsequent segments were then cut by successively shifting the box in $145 \text{ \AA}$ increments. The box and pixel sizes were 240 pixels and $2.74 \text{ \AA/pixel}$ for this data set, producing a reconstruction (Fig. 5a,b) with a resolution of $30 \text{ \AA}$ (according to the 0.5 Fourier shell correlation criterion; Supplementary Fig. 1). The reconstruction exhibited a clear J motif similar to that in previous reconstructions (Woodhead et al., 2005; Zhao et al., 2009; Pinto et al., 2012; Woodhead et al., 2013). The resolution was lower than the $25 \text{ \AA}$ resolution of the original 3D reconstruction (Woodhead et al., 2005). As discussed in Section 3.2, ~ 970 segments (19%) would be correctly aligned for this data set. In the original reconstruction (Woodhead et al., 2005), ~ 5000 segments with pixel size $5.3 \text{ \AA}$ were cut for 3D reconstruction and the translational search range was chosen to be 10 pixels. A maximum of 3655 segments ($\sim [10 \times 5.3/72.5] \times 100 = 73\%$) would have been correctly aligned for 3D reconstruction. The number of segments for the 3D reconstruction would thus be almost three times more than in our first data set, explaining the poorer resolution of the latter. We tried further decimating the data set (to $5.48 \text{ \AA/pixel}$) to obtain a preliminary alignment (Kudryashev et al., 2015). This resulted in a greater number of correctly aligned segments using the same translational search range (see 3.2). However, using the original data set together with the alignment parameters from the decimated data set still produced a reconstruction with a limited resolution ($\sim 22 \text{ \AA}$) (not shown).

3.4. Improved 3D reconstruction with centered crowns

A second reconstruction was computed from the data set composed of the 4886 segments cut as in Section 3.2 where a crown was now positioned in the box center for every segment. Therefore, the initial center of each single particle is close to the correct value. A small translational search range could then be used to match a segment with a reference projection, improving the alignment precision for this data set. In this way, all segments had the possibility to be correctly aligned instead of only 970 segments in the first data set. The possibility of a large shift for segments after alignment and the smearing of density at the ends of the 3D reconstruction is avoided. The 3D reconstruction (Fig. 5d,e) had a resolution of 17 Å (Supplementary Fig. 1) and showed more detailed features in the J motif.

3.5 Optimized 3D reconstruction using only well-ordered segments

To further optimize the structure, we computed a third reconstruction in which disordered segments were rejected. This was accomplished by comparing the observed positions of myosin heads in each segment with those expected for perfect helical symmetry. The relationship among subunits in a helical filament is described by the helical parameters z and ϕ . If the segments are cut using the method in section 3.2 (i.e. the shift of the box is the same as the axial rise (z)), the difference between the azimuthal rotation angles assigned to adjacent segments in the multi-reference alignment step of IHRSR should have the same value as ϕ (for a filament with perfect helical order). A decrease in helical order will be reflected in a deviation in ϕ from the expected value. We used this symmetry restriction to choose segments or filaments with the best helical order.

Cryo-EM images of tarantula thick filaments were analyzed using the above method by studying the azimuthal rotation angles assigned to adjacent segments during the 3D reconstruction procedure in section 3.4. The angular difference (ϕ) between successive segments should be 30° for perfect helical symmetry in a tarantula thick filament when the box shift is 145 Å (see Introduction for description of the filament symmetry). The assigned rotational angles between successive segments were used to calculate ϕ and compared to the expected value of 30° . The values determined for ϕ along example filaments 1, 2 and 4 are shown in Table 1. ϕ is quite close to 30° for filaments 1 and 2, showing that the myosin heads in these filaments have good helical order. However, ϕ for filament 4 varies over a large range, especially for segments 23 to 30, suggesting that the myosin heads in this region have bad helical order. On the other hand, ϕ for segments 31 to 37 is close to 30° . Therefore, filament 4 is clearly divided into two regions: ordered and disordered, consistent with the raw image. We set the error threshold to be 5° to select each segment: segments with $\phi > 5^\circ$ were discarded. Segments such as those in filaments 1 and 2 were chosen for 3D reconstruction.

In the complete filament there are intermolecular interactions between J motifs along its length (Woodhead et al., 2005). Any structural change in one J motif could be transmitted to another. Thus the helical order of some potentially good segments could be affected by the bad segments in a filament. The percentage of segments with $\phi > 5^\circ$ was calculated for each filament. If this number was greater than 30%, the whole filament was treated as a bad filament and discarded for image processing. Both the criteria described above to select

segments and filaments were chosen empirically so as to include as many good segments as possible. ~ 4300 segments had $\phi = 5^\circ$. The remaining 580 segments mainly had disordered myosin heads. 3174 segments were finally selected after also excluding the segments in the bad filaments.

A third reconstruction was computed using only these 3174 segments that were homogeneous and exhibited good helical order. The resolution of the reconstruction improved to 13 Å (Fig. 5g,h; Supplementary Fig. 1). This reconstruction showed better defined density for the coiled-coil α -helical tail of the myosin molecule (myosin subfragment 2) where it leaves the heads, compared with the other reconstructions (Fig. 5h cf. 5 b,e; Woodhead et al., 2005). The paramyosin core (Zhao et al., 2009) also had more detail (not shown). The current resolution does not allow secondary structure to be visualized. However, atomic fitting using molecular dynamics flexible fitting (Trabuco et al., 2008) indicates that several loops in the crystal structure have large conformational changes when the J motifs are assembled into filaments. A detailed discussion will appear in another paper.

4. Discussion

4.1. Intrinsic flexibility of myosin heads limits the resolution of the reconstruction

Flexibility exists in many multi-component complexes and plays an essential role in their functions, such as molecular recognition (Janin and Sternberg, 2013). In the case of myosin filaments, this flexibility is required for the cyclic attachment of myosin heads to actin followed by a large conformational change which drives thick and thin filaments past each other, producing muscle contraction. This flexibility places a critical limitation on the resolution that can be achieved in a filament reconstruction. The key site of flexibility is the hinge between each head and the coiled-coil tail, which allows almost complete freedom of head rotation with respect to the tail (Elliott and Offer, 1978). This flexibility is moderated by multiple but weak intramolecular interactions between the two heads within the repeating J-motif, together with intermolecular interactions between J-motifs and between the J-motif and the filament backbone (Woodhead et al., 2005). Myosin head order can be improved during specimen preparation by stabilizing the myosin heads with blebbistatin (Zhao et al., 2008) (see 2.1). Phosphorylation of Ser-35 in the regulatory light chain of one of the heads in the J-motif is thought to promote head flexibility (Sulbaran et al., 2013). This may lead to some of the disorder that we observe, and we have not found a way to fully inhibit this phosphorylation.

Because of disorder, data sets are a mixture of good and bad particles, and even good particles may have a heterogeneous structure. Many methods have been used to tackle this type of situation in other systems. A multipath simulated annealing optimization algorithm was developed to correctly align particles and to remove bad particles (Liu et al., 2007). Protein secondary structure was resolved by selecting several hundred icosahedral particles using this method. In RELION (Scheres, 2012), good particles are selected using 2D and 3D classification, or a heterogeneous data set is sorted into several homogeneous data sets based on 3D classification. Several near-atomic structures have recently been determined in this way. Our method is designed for helical filaments with a large axial rise per subunit where

the subunits exhibit significant flexibility. In this case, the helical symmetry was taken into account in the alignment step so that only good particles or segments (i.e. well-ordered and obeying the correct helical symmetry) were used in the reconstruction. Our results (section 3.5) showed that good segment quality was essential for obtaining the best resolution.

4.2. Importance of determining crown position

Single particle reconstruction usually involves discrete particles with defined boundaries and centers. Once the center has been determined, the problem essentially becomes one of orientation determination. However, errors in locating the true center can cause degradation of accuracy in the orientation steps; it is therefore important that the center is located as precisely as possible by pre-centering the particles (Liu et al., 2007). Only a small search range is then required for refinement of the particle centers.

In contrast, the segments used in IHRSR are not true single particles as they have been cut from a parent helical filament starting at an arbitrary location. Despite this, if the axial rise per subunit is small, then the search origin will always be located within a few pixels of one of the successive subunits, so that axial refinement will require only a small search range (just as for “true” single particles). However, in the case of a large axial rise, the axial location of the centers of segments cut using the normal IHRSR protocol will vary considerably (Fig. 4e), requiring a large search range to find the centers that correspond to the reference segments. In this situation, the low signal to noise ratio in cryo-EM images can create a significant problem when performing reference-based image matching procedures. This is due to background noise variation (e.g. Fig. 4b) that can result in multiple cross-correlation maxima. A large search range can produce multiple peaks, of variable magnitude, only one of which will give the best alignment (Frank, 2006; Sigworth, 2010). The misalignments resulting from the use of wrong peak positions add noise to the reconstruction and decrease resolution. Decreasing the search range to deal with this problem lowers the number of segments that can be accurately matched, which also decreases the resolution of the reconstruction. Increasing the pixel size to reduce the number of pixels searched likewise decreases the potential resolution.

These issues are addressed by the procedure described in section 3.2, where the segments are cut from the filament in such a way that they all have similar axial alignment to the reference segments, thus requiring only a minimal translational search range; this is thus similar to the case for centered “true” single particles, or segments from a helical structure with a low axial rise. A similar method was used to determine troponin positions in thin filaments (Yang et al., 2014). The difference here is that the period of the 1D projection along the filament axis was taken into consideration for the thick filaments. Each segment cut using this method had its central crown close to the box center. The average image density is highest at this point, and it is therefore likely that the cross-correlation peak closest to this location will also give the best alignment (and that any other peaks will be excluded by the small search range). Due to pre-centering, these segments are now projections from nearly identical particles, but with different azimuthal rotations. Therefore, most segments should align properly, and the problem of incorporating an insufficient number of segments into the reconstruction is avoided even though the translational search

range used in the alignment is small. This method is straightforward and readily accomplished using a SPIDER script (Frank et al., 1996). However, caution must be used if the axial rise is variable. In this case, segments should be sorted based on the axial rise before implementing this method.

An alternative method has been suggested to address the crown position in thick filament segments (Pinto et al., 2012). Reference projections were shifted different distances along the filament axis so as to match with the segments that have crowns in different positions. This approach becomes inefficient and computationally intensive for helical filaments with a large axial rise but small pixel size. If each reference projection was shifted 7 times, the total number of reference projections would increase 7 times, leading to a dramatic increase in computing time in the alignment step. Moreover, the large shift in a reference projection causes a corresponding shift for a segment that matches this reference projection. One end of such a segment is filled with density cut from the other end owing to the wrap around effect that occurs when the image is translated within a box (Fig. 3i,j). This causes a large proportion of densities near the ends of the 3D reconstruction to become smeared because of the shifted segments, which reduces the number of subunits that can be used to impose the helical symmetry in IHRSR (Egelman, 2000; Egelman, 2010). The long wrap-around in some of the reference images would further induce alignment errors.

4.3. Comparison of data sets

Three different sets of segments were generated from the cryo-EM images of the tarantula thick filaments. The first set (5107 segments) was produced using the standard IHRSR approach (Egelman, 2000; Egelman, 2010) where no attempt was made to perform axial pre-alignment of segments before 3D reconstruction (see 3.3). In the ensuing reconstruction, only ~970 segments would be correctly aligned when using a 5 pixel search range, resulting in a resolution of ~ 30 Å (Fig. 5a,b). The second set (4886 segments) had the crowns centered in the box so that all segments could be correctly aligned in the reconstruction (see 3.4). In this case the resolution improved to ~ 17 Å (Fig. 5d,e). The final set (3174 segments) included only those segments from the second set that exhibited good helical symmetry (3.5), resulting in improvement of the reconstruction resolution to ~ 13 Å (Fig. 5g,h).

It is clear that increasing the number of particles included in a reconstruction does not necessarily result in better resolution. Particles that have been pre-aligned, even if fewer in number, give significantly better results. The best results are obtained when the data set is further refined to include only well-ordered particles.

5. Conclusion

We have devised a method to improve the resolution of helical filaments with large axial rise (in relation to pixel size) and flexible subunits. The problem of alignment precision was surmounted by estimating the positions of the crowns in the segments, thus maximizing segment utilization, and the helical symmetry between segments along the filament was checked to select segments or filaments with good helical order. These steps greatly improved the resolution (from 30 Å to 13 Å). This method should be useful for other helical

filaments with large axial rise relative to pixel size. This applies to myosin filaments from all muscles, which have a common axial rise of 145 Å. Their helical symmetries vary substantially, however, and comparison of their diverse structures has led to key insights into the principles of their assembly and regulation (Wray et al., 1975; Wray, 1979; Woodhead et al., 2005; Woodhead et al., 2013). While other helical structures have smaller axial rises, using the principles described here may also be of value, as researchers use smaller pixel sizes in the quest for higher resolution reconstructions. For helical filaments with a very small axial rise, the method may not be necessary or may need to be modified.

Although the resolution of the thick filament reconstruction in this study is not high enough to visualize secondary structure, the method is clearly a powerful way to improve the resolution of helical filaments with large axial rise. Using a direct electron detector, optimizing the thick filament preparation, and increasing the number of images should improve the resolution further, providing more detailed insight into the structure and function of thick filaments in the relaxed state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

6. Acknowledgments

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Fig. 1.

Cryo-EM images show that myosin heads have intrinsic subunit flexibility (protein is black). Dashed lines placed next to the main densities in the images highlight helically ordered regions. (a) The periodic pattern of lines (forming arrows pointing to the right) indicates that this filament has good helical order. (b) The angle of the lines varies in different positions, and one disappears in the middle. Myosin heads in this filament partially deviate from helical symmetry. (c) Four parallel lines of density (arrowed) are seen in this filament instead of arrow-shaped densities. In this case, myosin heads randomly project from the filament surface making the underlying backbone more visible. The heads in this filament are completely disordered. (d) The power spectrum of (a) has four meridional layer lines at orders of 145 Å, indicated by arrows. (e) Only two meridional layer lines can be identified in the power spectrum of (b). (f) No meridional layer line can be clearly identified in the power spectrum of (c), and the other layer lines are weak. The whole raw filaments were used to compute the power spectra, but the corresponding images shown in (a) - (c) were cropped to the same length. Scale bar is 500 Å.

Fig. 2.

Two types of class average are observed following the classification of segments, corresponding to ordered and disordered filaments (protein is white). (a) A reference projection from a 3D reconstruction shows arrow-shaped densities along the filament axis similar to those in Fig. 1a. (b) In one type of class average arrow-shaped densities are seen (e.g. white oval), consistent with the reference projection (a). This class corresponds to filaments with good helical order. In other class averages, no arrow-shaped densities are observed (e.g. black oval); instead, four lines (white arrows) in the center reflect the thick filament backbone, while disordered heads give rise to fuzzy material at the filament edges (red arrows). Scale bar is 500 Å.

Fig. 3.

Illustration of the alignment problem for helical filaments with large axial rise. (a) Two segments were boxed from a model of the tarantula thick filament. The first segment was cut with the cyan box as shown in (b). The second segment was cut with the tan box by shifting 72.5 Å from the first one as shown in (c). These two segments are different particles and cannot be aligned through azimuthal rotation. Segment (c) matches (b) by translating 72.5 Å along the filament axis. (f) and (g) show this translation. The tan box in (g) now matches the cyan box in (f) but there is a 72.5 Å mismatch at each end. This illustrates how it is impossible to completely match a segment projection from a cryo-EM image with a model reference projection. (d) and (e) are the projections of (b) and (c), respectively. (h) and (i) are the projections of (f) and (g), showing the 72.5 Å translation between (h) and (i) needed to match them. The 72.5 Å-long tan rectangle at the top of (i) makes a poor contribution to the 3D reconstruction as the IHRSR procedure moves it to the bottom of the segment as in (j). This shows how a large translational search results in poor image matching at the projection ends owing to the large wrap-around effect that is introduced. However, this can be essentially eliminated by accurate pre-alignment of segments before performing 3D reconstruction.

Fig. 4.

Determination of crown positions by 1D projection. (a) Cryo-EM image of tarantula thick filament; scale bar is 200 Å. (b) 1D projection computed directly from (a). No obvious periodic pattern was discerned because of the low signal to noise ratio. (c) Average image for the segments in filament (a); 480×480 pixel segments were cut by sequentially shifting 145 Å along the filament axis. (d) 1D projection perpendicular to the filament axis of the average in (c). The five peaks (arrowed) correspond to five crowns in the segment average from the single filament. The peaks are asymmetric because all crowns have the same polarity. (e) Global average of segments cut from all filaments without allowing for initial crown position. Crown positions are not apparent, indicating that they are randomly distributed within the segments and thus become smeared out in the average. (f) Global average of segments cut from all filaments where a crown has been set in the box center. The five horizontal stripes correspond to the five crowns, which are no longer smeared because the middle crown in every segment has been set at the box center; scale bar is 200 Å. (g) 1D projection perpendicular to the filament axis of the average in (f). The five peaks, corresponding to the five crowns, are symmetric because similar numbers of segments in (f) point up and down.

Fig. 5.

Comparison of 3D reconstructions using three different data sets. (a) Reconstruction using the segments that were cut using the standard IHRSR procedure. (d) Using the segments that have a crown located in the box center. (g) Using the segments that were selected from the data set in (d) by checking the helical symmetry of segments in a filament. The resolutions were determined to be 30 Å, 17 Å and 13 Å for (a), (d), and (g), respectively (Supplementary Fig. 1), and the reconstructions were Fermi-filtered to their respective resolutions. (b), (e) and (h) Single J motifs from the reconstructions in (a), (d) and (g), respectively. (c), (f) and (i) Atomic models of the motif (obtained by molecular dynamics flexible fitting of the volume in (h); see 3.5) filtered to resolutions of 30 Å, 17 Å and 13 Å, respectively, for comparison with the motifs derived from the three reconstructions at the same resolutions. The red arrows in (b), (e) and (h) show clear improvement in the definition of the weak, coiled-coil (myosin subfragment 2) portion of the reconstruction as the resolution improves. Some features (a groove in the SH3 domain (yellow arrow) and density at the N-terminal of the regulatory light chain (green arrow)) are seen only in the 13 Å reconstruction (compare (e) and (h)). The presence of these features in the 13 Å but not the 17 Å atomic model (compare (i) and (f)) supports the validity of 13 Å as the resolution for (h). This is further supported by the presence of a 13 Å layer line in the power spectrum generated from the projection of this reconstruction (arrow in Supplementary Fig. 2).

Table 1

The azimuthal rotation angle error of a segment computed from the difference between the assigned rotation angle (ϕ) and the theoretical estimated value.

Filament	Segment	ϕ^a (°)	ϕ^b (°)	error (°)
Filament 1	1	1		
	2	32	31	1
	3	64	32	2
	4	4	30	0
	5	36	32	2
	6	65	29	1
	7	4	29	1
Filament 2	8	76		
	9	46	−30	0
	10	20	−26	4
	11	81	−29	1
	12	52	−29	1
	13	25	−27	3
	14	84	−31	1
Filament 4	23	74		
	24	80	6	24
	25	38	−42	12
	26	24	−14	16
	27	85	29	1
	28	43	44	14
	29	78	−55	25
	30	25	−53	23
	31	85	−30	0
	32	57	−28	2
	33	28	−29	1
	34	89	−29	1
	35	62	−27	3
	36	28	−34	4
	37	1	−27	3

^cAngle error between the observed ϕ and the theoretical value (30° for tarantula thick filaments; see Introduction).

^aAzimuthal rotation angle (ϕ) assigned to a segment in the alignment step;

^bRotational angle difference ϕ computed between two adjacent segments in a filament. The different polarities of filaments 1 and 2 lead to the different ϕ sign. To compute ϕ , the four-fold symmetry around the filament axis must be taken into consideration due to the repeat of the symmetry every 90° of azimuthal rotation.