

## CryoEM Structure of *Drosophila* Flight Muscle Thick Filaments at 7 Å Resolution

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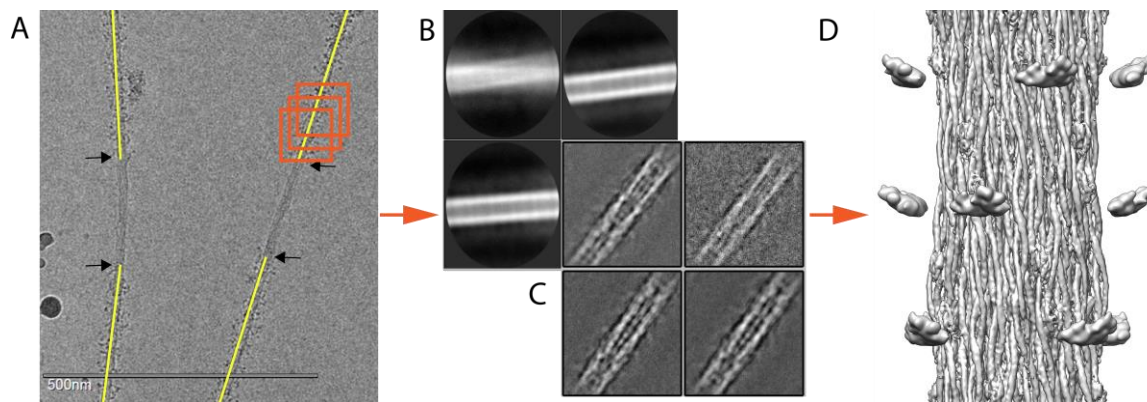
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Myosin II, composed of two heavy chains and two light chains, is the main component of bipolar thick filaments of striated muscle. Compared to thin filaments, sarcomeric thick filaments are poorly understood, especially in the backbone. The *Drosophila* flight muscle myosin rod sequence compared to human cardiac  $\beta$ -myosin (MYH7) is 56% identical, 74% similar with no insertions or deletions between sequences of the two species. Here we report a  $\sim 7$  Å resolution structure of isolated thick filaments from asynchronous flight muscle with disordered heads. *Drosophila* thick filaments are 3.2  $\mu\text{m}$  in length and 250 Å in diameter and have a helical structure with C4 rotational symmetry and a helical rise of 145 Å and a helical angle of  $3.86^\circ - 33.92^\circ$ . The 145 Å repeat is generally referred to as a “crown”. Relaxed myosin filaments from multiple species are observed to fold in an asymmetric conformation called Interacting Heads Motif (IHM). IHM shows two myosin heads interacting asymmetrically with each other which inhibits their interaction with actin filaments. Our reconstruction surprisingly failed to show densities resembling the IHM of relaxed myosin. Thick filaments of *Lethocerus indicus*, the only other thick filament structure reported at comparable resolution had well resolved myosin heads in a modified IHM positioned very differently from all previously-reported structures. Instead of being positioned against the proximal S2 via an interaction with the “so-called” blocked head, the free head was positioned against the thick filament backbone and the “blocked” head appearing to pin the free head against the backbone. In *Drosophila* densities were found in the expected axial position of myosin heads but unconnected to the filament surface.

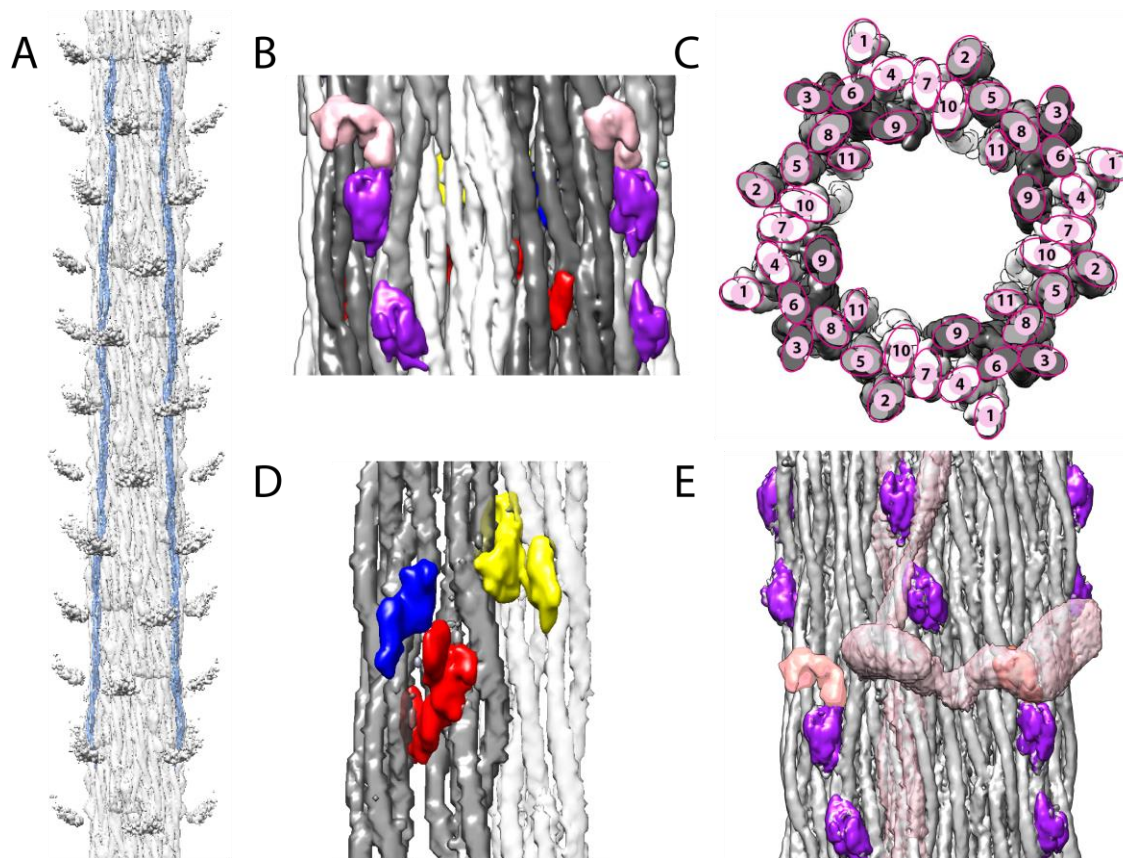
Thick filaments were isolated from flight muscles of two *Drosophila* strains, a wild type, W1118, and a strain designated Dmlc2<sup>[D2-46; S66A,S67A]</sup> with regulatory light chain mutations. Regulatory light chain phosphorylation is known to disorder the heads, but would be impossible in the mutant. Both samples were imaged on a FEI Titan Krios transmission electron microscope operated at 300kV. Images were recorded on a Direct Electron DE-64 camera operated in integrating mode. Data for the mutant was collected using the Volta phase plate.

Filaments were manually picked and helically extracted using Relion. In addition to a generally low signal to noise ratio, the myosin heads in our thick filaments are disordered, which could throw off the alignment significantly, unless other features within the backbone can define the axial repeat period. Initial 2D classification in Relion was inconclusive, since the class averages were featureless and showed the program’s inability to align particles and classify them effectively. To obtain better definition among the classes, we tested ROME 1.1 for 2D classification. ROME 1.1 uses statistical manifold learning (SML) which turned out to be a powerful classification tool for this data. We then imported the best classes obtained from *rome\_sml* and reprocessed them using cisTEM 1.0. An individual myosin molecule is 11 crowns or 1600 Å in length (11 x 145 Å). To obtain a density map of a single myosin rod structure as well as determine the rod arrangement within ribbons, required extending the reconstruction to 11-crowns in length, which was done utilizing the helical symmetry determined by Relion.

We identified several densities from non-myosin proteins. The major reported non-myosin proteins in *Drosophila* flight muscle thick filaments are paramyosin and miniparamyosin, myofilin, flightin, kettin, projectin, stretchin and obscurin. The reconstruction shows four densities that cannot be assigned to myosin at this time. Two of those densities are similar enough to non-myosin densities in *Lethocerus* thick filaments, that we believe them to be the thought to be flightin and myofilin in *Lethocerus* thick filaments. Flightin penetrates a curved layer, as does the similar density in *Lethocerus*. Myofilin, is contained completely within the myosin rod annulus and penetrates between two curved layers. The best candidate for the surface decorating densities seems to be a kettin-like protein, referred to as stretchin-klp, the form detected in our proteomics. We were able to fit the PDB of I-set domains (2YXM) from human myosin binding protein C to these densities. Although the resolution for these densities is not very high, combined with mass spectroscopy data, we believe the most reasonable hypothesis is that the density is stretchin-klp. Extensive averaging of multiple filaments and multiple repeating segments of each filament occurs in the reconstruction process. Consequently, structural elements that do not follow the myosin symmetry or that follow it but are present in less than equimolar amounts will not show up in the reconstruction. Those elements that appear in the reconstruction at the same contour threshold as the myosin tails are present at close to equimolar amounts with respect to myosin.



**Figure 1.** (A) Myosin filaments are seen in micrographs and manually picked from the M-band and extracted in overlapping boxes. (B) 2D classification using Relion resulted in blurry class-averages. (C) ROME Statistical Manifold Learning produced higher class averages and better classification. (D) Using best classes from ROME a 3D reconstruction was produced in cisTEM while other software packages failed to achieve comparable resolutions.



**Figure 2.** (A) *relion\_helix\_toolbox* was used to expand the map and resolve one complete myosin tail. Colored in light blue are individual myosin molecules highlighted in the backbone. Myofilin is colored yellow, flightin red, purple and pink densities decorating the surface we believe to be stretchin-klp and the blue density is unidentified (B) The map showed ribbons and densities on the surface. Different ribbons are colored in white, light grey and dark grey. (C) Ribbons are made of myosin tail with an offset of 3 repeats. (D) Non-myosin proteins are observed among myosin tails. (E) Fitting *Lethocerus* myosin in our map shows our non-myosin densities in the surface coincide with the location where the myosin free head is contacting the backbone; a possible explanation for why the interacting heads motif is not observed.

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