Structure and function of myosin filaments
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Myosin filaments interact with actin to generate muscle contraction and many forms of cell motility. X-ray and electron microscopy (EM) studies have revealed the general organization of myosin molecules in relaxed filaments, but technical difficulties have prevented a detailed description. Recent studies using improved ultrastructural and image analysis techniques are overcoming these problems. Three-dimensional reconstructions using single-particle methods have provided many new insights into the organization of the myosin heads and tails. Docking of atomic structures into cryo-EM density maps suggests how regulated myosin filaments are ‘switched off’, bringing about muscle relaxation. Additionally, sequence analysis suggests probable interactions between myosin tails in the backbone, whereas crystallographic and EM studies are starting to reveal tail interactions directly in three dimensions.

Introduction
Myosin filaments (also called thick filaments) are key components of muscle and non-muscle cells. In striated muscle, they overlap with thin (actin-containing) filaments in an orderly array, making a repeating pattern of sarcomeres, the basic units of contraction [1] (Figure 1a). In smooth and non-muscle cells, myosin filaments play two key roles in muscle contraction and many forms of cell motility. The myosin heads (or crossbridges), which lie on the surface of the filaments, bring about contraction by cyclic interaction with actin subunits in the thin filaments. This ‘crossbridge cycle’ causes the thick and thin filaments to slide past each other, producing movement [2,3]. Thick filaments in many types of muscle and non-muscle cells also participate in regulating or modulating contractile activity. Understanding how thick filaments are assembled and carry out these functions requires a detailed knowledge of their structure.

The structure of thick filaments has been elucidated over the past 40 years by electron microscopy (EM), X-ray diffraction and other techniques. Although this has provided many insights into the principles of myosin assembly, critical molecular details have been missing and the relationship of filament structure to function has been unclear. Here, we review the key advances that have led to our current understanding of thick filament structure and its relation to function, with an emphasis on recent progress. We focus on myosin organization in striated muscle filaments in the relaxed state, in which the filament structure is stable and unperturbed by interaction with actin. We also discuss myosin filaments in smooth and non-muscle cells, and the role of myosin-binding proteins in thick filament assembly and function.

Myosin molecules and thick filaments
The major component of thick filaments is myosin II, a member of the myosin superfamily of motor proteins that produce motility by moving actin [4]. Myosin II is an elongated, two-headed molecule consisting of two identical heavy chains and two pairs of light chains [5] (Figure 1d). The C-terminal half of each heavy chain is an essential light chain, which contains a regulatory light chain and an essential light chain, and functions as a lever responsible for filament sliding in contraction [3]. In many muscles, the light chain domain regulates (or modulates) contractile activity, either by phosphorylation of the regulatory light chains or by binding Ca2+ [6]. The α helices coil around each other, forming a long coiled-coil tail that is insoluble at physiological ionic strength.

Thick filaments are formed by the staggered association of myosin tails running approximately parallel to the filament axis. The distal (C-terminal) two-thirds of the tail (light meromyosin [LMM]; Figure 1d) is responsible for self-association and is tightly packed in the filament backbone. The more soluble, proximal one-third of the tail (subfragment 2 or S2) acts as a flexible link between LMM and the heads, which lie on the filament surface, where they can interact with actin. Non-myosin proteins, usually in small amounts, may be incorporated into the filament core, or serve structural or regulatory roles on the filament surface.
Striated muscle thick filaments

In striated muscle, thick filaments are bipolar structures, formed by antiparallel tail interactions at the filament center (creating a ‘bare zone’ free of myosin heads) and parallel interactions at either end (the head or ‘cross-bridge’ region) [7] (Figure 1b,c). In most muscles, thick filaments are connected to each other at their bare zones via cytoskeletal bridges constituting the M-line (Figure 1a) [8]. These connections help organize the filament lattice and maintain filament register.

Head organization

In the relaxed state, the myosin heads are helically or quasi-helically ordered (reflecting the underlying organization of the tails), with an axial spacing of ~14.5 nm between adjacent levels (Figure 1b) [9,10]. This helical
organization, first revealed by X-ray diffraction, was for many years not observed by EM. The disordering of the heads that occurred during filament preparation for negative staining was first overcome using invertebrate thick filaments, which are relatively stable (Figure 2a) [11]. Fourier analysis and 3D helical reconstructions of negatively stained invertebrate [12–15], and subsequently vertebrate [16], filaments revealed information about the number of helical tracks, as well as the conformation and arrangement of the heads at moderate resolution (Figure 2b).

Invertebrate thick filaments have become model systems for study owing to their marked stability, which may result from their ability to ‘switch off’ ATPase and actin-binding activity (in contrast to vertebrates, in which myosin ATPase is unregulated and the thin filaments regulate actin-binding activity [6]). The number of parallel helical tracks of heads varies from four to seven in invertebrates and their helical repeat varies from 30 to 48 nm, depending on both muscle type and species [10,13,14,17]. Various interpretations of arthropod reconstructions [13,14], in some cases based on docking with the atomic structure of S1 [18], all suggested that the myosin heads were splayed axially, with interactions occurring between heads from different 14.5 nm levels. However, the resolution was limited to ~5 nm (Figure 2b) and the accuracy of the atomic fitting was therefore uncertain.

A quite different organization is suggested by cryo-EM of purified [19], frozen-hydrated tarantula filaments analyzed by single-particle reconstruction methods [20**]. Cryo-EM preserves native structure without staining artifacts, whereas single-particle reconstruction avoids the problem of information overlap that can occur with helical reconstruction methods [13]. With a resolution of 2.5 nm, the reconstruction clearly reveals the two heads of a single myosin as a ‘tilted-J’ motif (Figure 2c). This motif enables unambiguous fitting of the atomic model of an ‘off-state’ (dephosphorylated) myosin molecule from vertebrate smooth muscle [21–23] (Figure 2d). The fit shows that the heads of a single myosin are not splayed but lie close together, interacting with each other and both pointing towards the bare zone. These intramolecular interactions between the heads of a single myosin, as well as intermolecular interactions between the heads of axially adjacent molecules, appear to block key functional sites in the motor domain (Figure 2d), suggesting a simple structural mechanism for switching off the filament in the relaxed state [20**,21]. The reconstruction also reveals how S2 connects the heads to the filament backbone and shows possible interactions between S2 and the heads [20**,23] (Figure 2d). The fit of a vertebrate smooth muscle myosin molecule into an invertebrate striated muscle filament is extraordinary, and suggests that the intramolecular interacting-head structure is highly conserved and likely to represent the structural basis of the off-state in most regulated myosin filaments. An
exception may be the highly specialized indirect flight muscle of insects, for which modeling of the relaxed X-ray diffraction pattern (to 6.5 nm resolution) suggests that one myosin head projects out from the filament backbone, while the other wraps around it [24]. It will be important to test this model against high-resolution cryo-EM reconstructions of filaments.

In contrast to invertebrates, all vertebrate thick filaments have a similar structure, with the heads arranged in three tracks and with a repeat of 43 nm. This was first established in skeletal filaments [16] and has now also been demonstrated in cardiac filaments [25]. Suggestions that vertebrate filaments are two stranded [26] are incorrect [25]. Unlike the helical arrays in invertebrates, the heads in vertebrate muscle have only a quasi-helical arrangement [9,16], their positions and possibly their conformations being perturbed, probably by the binding of myosin to non-myosin proteins (see below). Using helical reconstruction methods [27], it has been suggested that the myosin heads are close to each other and project to high radius, tilting away from the bare zone. The difference between this proposed head orientation and that in tarantula muscle [20**] may result from the limited (~6 nm) resolution of the reconstruction or from the absence of an off-state in vertebrate filaments. Although helical reconstruction provides only an average of the different head positions and conformations, the perturbation is preserved in reconstructions using tilt views. In frog filaments, it appears to involve differences in radial, azimuthal and axial positions of the heads compared with those of a strictly helical filament [16], but individual heads are not resolved. Single-particle methods [28] should distinguish different head conformations at different levels and improve the resolution, but may require frozen-hydrated filaments for definitive conclusions to be reached [20**].

The ordering of the heads that occurs in relaxing conditions depends on the ‘closing’ of the switch 2 element [3**] of the myosin active site, located in the motor domain [29,30]. This traps inorganic phosphate, inhibiting its release from the head and minimizing ATP turnover — a key characteristic of relaxation. Relaxed ordering of heads in regulated filaments produces head–head interactions [20**] that contribute to the switched-off state and may also underlie the efficient switching on of contraction [30].

**Backbone structure**

The backbone of the myosin filament consists of staggered, closely packed myosin tails running approximately parallel to the filament axis. The detailed packing of the tails has not been determined because of their length (~160 nm), narrow diameter (~2 nm) and tight packing. Theoretical models have been proposed using equivalent or quasi-equivalent interactions between myosin tails that are similar between species, with only small changes leading to the diverse filament symmetries found in the animal kingdom [31]. In one class of model, the tails assemble individually to form the backbone (molecular crystal model; Figure 3a), whereas in another class they form small groups (subfilaments) that make up the backbone (subfilament model [31]; Figure 3b). X-ray diffraction supports a 4 nm diameter subfilament model (Figure 3b) as a common structural theme in crustacea, with variations in subfilament number and tilt accounting for filaments with different symmetries [17]. The reconstruction of tarantula filaments provides direct support for this model [20**] (Figures 2c and 3c), suggesting that it might also be common throughout the arthropods. The backbone organization in other invertebrates is not known and remains speculative for vertebrate filaments. The subfilament model could be modified slightly to account for the quasi-helical symmetry in vertebrates.

![Figure 3](image_url)

**Figure 3**

Backbone structure of striated muscle thick filaments (transverse views, shown for a filament with fourfold rotational symmetry). (a) Molecular crystal model. Each circle represents one myosin tail (based on [64]). (b) Subfilament model. Each subfilament is 4 nm in diameter and contains three myosin tails. Based on [17]. (c) Projected density of a 43.5 nm repeat of tarantula thick filament reconstruction (protein white) [20**]. Twelve subunits, 4 nm in diameter and of high density (red circles), representing the thick filament backbone, run parallel to the filament axis — as predicted by the subfilament model [17] for a filament with the tarantula symmetry. Note that (c) is shown at a smaller scale than (a,b); lower density material at higher radius represents heads.
[17] and EM shows evidence of a substructure approximately parallel to the filament axis that is consistent with subfilaments [32]. However, sequence considerations are suggested to favor the molecular crystal model [33]. Any model of vertebrate filaments must account for their fraying into three large subunits at low ionic strength [34].

Tail interactions

Insights into likely tail interactions in the filament backbone have come from sequence analysis of Caenorhabditis elegans myosin [35]. The tail shows a repeating structural unit with a length of 28 residues, in which positively and negatively charged regions are separated by 14 residues. Strong charge attraction is predicted between tails staggered by odd multiples of 14 residues. Attraction is especially strong with stagger of 98 or 294 residues, corresponding to displacements of 14.4 and 43.2 nm between adjacent tails — two of the fundamental periodicities seen in myosin filaments. Analysis of the sequences of vertebrate skeletal, smooth and non-muscle myosin tails reveals the presence of long-range oscillations of charge and the importance of skip residues (single-residue insertions in the α-helical sequence that cause local instability of the coiled-coil) in determining the dominant stagger interactions [33]. Longer odd-number multiples of 14.4 nm staggers are considered to stabilize, and even-number multiples to destabilize, thick filament structure.

Non-myosin components

Striated muscle thick filaments contain non-myosin components that play structural and regulatory roles. Most invertebrate filaments contain a core of paramyosin (a coiled-coil protein about 124 nm in length, similar to the myosin tail) in molar ratios up to 50% of myosin [36]. This may serve to strengthen these filaments against the high loads they must bear due to their greater length (and their larger number of actin filament interactions) compared with vertebrate muscle. In Drosophila flight muscle, paramyosin must be phosphorylated for optimal power output [37]. Most invertebrates also contain small versions of titin-like proteins (see below), which contribute to the mechanical properties of these muscles [38,39]. Substantial molar amounts of additional proteins may also be present, particularly in insect muscles (myofilin [40], flightin [40,41] and myosin rod protein [42]), where they play a role in filament assembly and stability, and in modulation of contraction. The 3D structural organization is not known for any of these non-myosin components.

Thick filaments from vertebrate skeletal and cardiac muscle are associated both with the giant elastic protein titin and with myosin-binding proteins. Titin (also called connectin) is a giant polypeptide (~3 MDa) that extends from the center of the thick filament through the I-band (where the thick filaments are absent) to the Z-line [39,43] (Figure 4). It functions as a template for the assembly of the sarcomere during development and as a molecular spring responsible for key aspects of the mechanical behavior of muscle. In the region associated with the thick filament, titin consists of immunoglobulin-like (Ig) and fibronectin-like (Fn) domains, arranged in super-repeat patterns with a periodicity of ~43 nm. These repeats seem to dictate the positioning of the myosin-binding proteins (Figure 4) and the organization of myosin. There appear to be six molecules of titin associated with each half of a thick filament, probably running parallel to the filament axis on the filament surface [39], but their 3D organization remains uncertain [32,44].

The myosin-binding proteins of vertebrate thick filaments (primarily myosin-binding protein C [MyBP-C]) bind at 11 sites, 43 nm apart (every third level of heads), in each half of the filament [43,45]. They are an important target of mutations in familial hypertrophic cardiomyopathy [45]. Like titin, these myosin-binding proteins (probably three molecules at each axial level) are made largely from Ig and Fn modules. MyBP-C presumably contributes to thick filament images and reconstructions, but distinguishing it from the dominant organization of myosin molecules remains difficult [27] and its 3D organization therefore remains unknown. The C-terminal end
of MyBP-C may wrap around the filament backbone, forming a collar [45\*, or run axially along the filament surface [46], while its N-terminal end may project from the filament, interacting with S2 [45\*,47] and possibly extending as far as the neighboring actin filament [46]. MyBP-C may play a role in sarcomere assembly [45\*] and, in cardiac muscle, it appears to modulate contractility by interacting with S2 in a phosphorylation-dependent way [45\*,47].

Smooth muscle and non-muscle thick filaments

Myosin filaments are also present in smooth muscle and non-muscle cells, where they pull on actin to produce filament sliding, as in striated muscle. In vertebrates, smooth muscle and non-muscle filaments are more labile than those of striated muscle. Under *in vitro* relaxing conditions, dephosphorylated filaments disassemble into monomers with a folded structure and low ATPase activity. Upon phosphorylation, the molecules unfold and assemble into filaments that have high ATPase activity and are capable of interacting with actin [48\*]. Phosphorylation-dependent regulation of ATPase activity and filament assembly provides a simple device for the storage of inactive molecules in non-muscle cells, and for their transient assembly into filaments when and where they are needed for motility. In the case of smooth muscle, the lability of dephosphorylated filaments caused uncertainty as to their very existence in the relaxed state [48\*]. It now appears that, although filaments are present in relaxed smooth muscle, their number or length may, in some cases, increase upon activation by assembly from a pool of the folded monomers [48\*,49\*].

Vertebrate smooth muscle filaments have a different arrangement of myosin molecules from that of striated filaments. In this non-helical, ‘side-polar’ structure, the orientation of the myosin molecules reverses on opposite sides of the filament, rather than at opposite ends [50,51] (Figure 5). The tail interactions responsible for this different mode of assembly may be explained by subtle differences in the tail sequence compared with striated muscle myosin [33\*]. The side-polar structure would still pull thin filaments of opposite polarity towards each other, and could help to account for the extreme shortening of which smooth muscle is capable [50]. *In vivo*, smooth muscle filaments appear to have four molecules at each 14.4 nm level [52], but the lability and non-helical structure of the filaments has so far prevented 3D reconstruction. Two-dimensional crystals of vertebrate smooth muscle myosin show that the heads of individual molecules interact with each other in the dephosphorylated state, switching each other off [21,22]. This is the same interaction that has now been demonstrated in off-state striated muscle filaments from tarantula [20\*] (Figure 2d). The presence of this interhead interaction across phyla and muscle types implies that it will also be present in native smooth muscle myosin filaments.

Smooth muscle filaments contain, in addition to myosin, smitin (a titin analog, thought to play a role in smooth muscle mechanics) and two other proteins (telokin and a 38 kDa protein), which may be involved in stabilizing filaments in relaxed muscle [48\*]. The 3D organization of these components is unknown.

Invertebrate smooth muscles, such as the catch muscles of molluscs, contain large bipolar thick filaments with a large core of paramyosin. In these filaments, paramyosin has a paracrystalline organization, with the myosin forming a thin surface layer of loosely attached molecules, possibly organized in a helical fashion [53]. The thick paramyosin core may strengthen these large filaments against the high tensions that they must bear. These filaments also contain small amounts of additional proteins, including catchin and twitchin, which may play a role in the catch mechanism of invertebrate smooth muscle [53].

Assembly of thick filaments

The correct assembly of myosin molecules into thick filaments in muscle and non-muscle cells has several key requirements, which have been well demonstrated by genetic and other analyses [54]. These requirements

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**Figure 5**

Schematic diagram of a side-polar thick filament. In the side-polar mode of assembly, found in smooth muscle thick filaments, antiparallel tail interactions occur along the entire length of the filament (c.f. bipolar filaments), so that opposite sides, rather than opposite ends, of the filament have opposite polarity. Functionally, the filament is still bipolar and able to pull actin filaments in opposite directions from opposite ends. The double arrows indicate how bipolar myosin dimers could easily associate with and dissociate from the filament ends, consistent with assembly-disassembly processes thought to occur in some smooth muscles in different physiological states.
Myosin tail domains involved in thick filament assembly. Schematic representation of the myosin tail sequence showing the locations of various regions thought to be involved in the correct assembly of thick filaments (composite diagram based on different myosins; not all sites are involved in all myosins). These include a negative charge cluster, N1 (red), and two positive charge clusters, P1 (blue) and P2 (purple) [58**]; an assembly competence domain or ACD (yellow) [55,58**]; the non-helical tailpiece or NHT (green) [56,57,58**,60,61*]; and four skip residues (dark blue) [33]. Smooth muscle and non-muscle myosins lack the indicated skip residue.

Acanthamoeba myosin assembles into octameric filaments in vitro and in vivo. Truncation studies have defined key regions of the tail essential for the sequential steps of assembly. Crystallographic studies of such defined tail fragments may provide the first insights at high resolution into the 3D interactions involved in the assembly process [62**]. Acanthamoeba may be a model for the early stages of assembly of other species of filament.

**Conclusions**

Myosin filaments comprise a diverse array of macromolecular assemblies. Although their basic structure has been understood for more than 40 years, progress towards an atomic-level description has been slow. Recent innovative studies have finally revealed details of the organization of the heads and tails in tarantula filaments at near-atomic level [20**], suggesting a structural model for myosin filament regulation. Sequence analysis and crystallographic studies of other systems are providing new insights into the structural basis of thick filament assembly. Important unresolved issues remain. Cryo-EM and single-particle reconstruction of additional thick filament species should reveal whether subfilaments and an interacting head structure, both seen in tarantula, are common themes of thick filament structure. Improvements in resolution may directly resolve individual myosin tails and non-myosin proteins, and also provide more detail on head interactions. Interpretation of structure should be aided by further sequencing of the relevant proteins from these species. Understanding vertebrate smooth muscle thick filament structure will require tomographic or single-particle reconstruction, as these filaments lack helical symmetry. Further progress in understanding assembly can be expected from higher resolution EM and X-ray studies.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


This detailed review of the atomic basis of the crossbridge mechanism of muscle contraction relates crystallographic structures of S1 to biochemical and kinetic data.


The authors summarize current knowledge of myosin superfamily structure and function, placing myosin II in context.


A review of the structural components and function of the striated muscle M-line.


The first reconstruction of a thick filament to unambiguously reveal the organization of myosin heads and the probable arrangement of the tails in subfilaments. The head organization directly suggests how these filaments are switched off in the relaxed state.


An elegant study of rabbit cardiac thick filaments using negative staining and Fourier analysis.


This useful review outlines the use of single-particle methods to reconstruct filamentous particles, including those lacking strict helical symmetry, such as vertebrate thick filaments.


A negative-stain EM study of tarantula thick filaments in the presence of nucleotide analogs simulating different states of ATP. Quantification of head ordering by Fourier transformation of images shows that only analogs that produce the switch 2 closed state cause the heads to be ordered.


This detailed analysis of the sequence and charge patterns of the tails of human striated, smooth and non-muscle myosin molecules suggests likely tail interactions in thick filaments.


A review of the structure and function of MyBP-C, comparing different models of its arrangement on the thick filament, and discussing its role in physiology and disease.


A concise review of vertebrate smooth muscle thick filament composition, structure, assembly and stability.


Birefringence studies of tracheal smooth muscle are consistent with an increase in thick filament number or length upon activation. This in vivo study supports and discusses earlier EM and birefringence evidence in favor of changes in filament density in different physiological and mechanical states of smooth muscle.


By studying the solubility of truncated rod fragments of non-muscle myosin, two domains in the C-terminal region of the coiled-coil are identified as critical for thick filament assembly. Interactions between positive and negative charge clusters in these regions are proposed to initiate filament assembly.


Constructs of *Acanthamoeba* myosin II tail are used to define the tail domains required for assembly into the octameric minifilaments that may occur in vivo. This model system may provide clues to the initial stages of the assembly of thick filaments in other systems.


An elegant 3D EM and X-ray crystallographic study of the interactions in the antiparallel dimer of coiled-coil tails that guide the first step of myosin assembly in *Acanthamoeba* thick filaments.
