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Isolation of Striated Muscle Thick Filaments for Cryo-EM

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The detailed structure of filaments formed from myosin II is poorly understood. In so far as it is known, vertebrate thick filaments follow a single structural model; invertebrate thick filaments show high variability between species and between muscles of the same species. To date, high resolution structures have been reported only for myosin filaments isolated from invertebrates and no structures have been reported for myosin filaments in which one or more thick filament proteins have been mutated. A structure of thick filaments from vertebrates would be the most useful for understanding the effects of mutations on muscle function in humans. Despite numerous cryo-EM advances, structures of vertebrate thick filaments are limited to low resolution from negatively stained specimens. The main problem seems to arise from difficulties in preserving the apparently more fragile vertebrate thick filaments using techniques that work well for invertebrates. Here, we report progress in the cryo-EM sample preparation of thick filaments which preserves the structure of both backbone and myosin heads.

Long strips of the glycerinated rabbit psoas muscle were excised and placed in a relaxing solution containing 80 mM KCl, 20 M MOPS, 5 mM EGTA, 2 mM dithiothreitol, 5 mM $MgCl_2$, and 2.5 mM ATP (pH 7) at 4°C. After washing off the glycerol, each muscle strip was gently teased into very thin bundles (0.5 mm - 1 mm). The muscle bundles were then transferred into fresh relaxing solution plus 1% Triton x-100 and left overnight at 4°C while shaking gently on a rocker. The following day, the muscle was minced with a razor blade and homogenized on ice with two 10 s bursts at setting 5 of a Sorvall Omni- Mixer. The homogenate was diluted with additional relaxing solution plus 20 mg of a His-tagged, Ca^{2+}-insensitive gelsolin fragment (residues 25 to 406 of plasma gelsolin), which we expressed in *Escherichia coli* and purified by standard techniques [1], to remove as much of the sarcomeric actin as possible. All subsequent steps incorporate calcium-insensitive gelsolin in the buffers. Myofibrils can be stored at this step in 80% glycerol at -80°C for further experiments. We diluted 0.1 ml of myofibrils with 0.3 ml of the relaxing buffer, centrifuged for 2 min at 6000g to pellet the myofibrils, resuspended in 0.25 ml of relaxing buffer with 5 mM $CaCl_2$ added, and incubated for 60 min at room temp with 2 mg of m-calpain [2]. The m-calpain was necessary to obtain sufficient yield of intact filaments. Calpain-digested myofibrils were pelleted as before and resuspended in 0.1 ml of fresh relaxing buffer and sheared by pulling the suspension through a 26-gauge needle with a syringe 5 times to release filaments. The preparation was centrifuged for 2 min at 3500g to pellet unsheared fibrils, leaving a mixture of thick and thin filaments in the supernatant. To remove the remaining actin filaments, the supernatant was incubated for 20 min on ice with more gelsolin (1:1). After gelsolin treatment, the filament suspension was checked by conventional negative-stain EM for quality and yield and then used directly for cryo-EM specimens. R1.2/1.3 Quantifoil grids were cleaned using a Gatan Solarus 950.M plasma cleaner in argon gas for 22 s. Samples were vitrified on a Vitrobot Mark IV (FEI) with the environmental chamber set to 90% relative humidity and 27°C. Thick filaments were applied to a grid directly after cleaning, incubated for 60 s, and washed with 0.5% glutaraldehyde in 0.1M Cacodylate buffer to preserve ordered heads which otherwise disorder rapidly in vertebrate thick

filament cryo-EM samples. Grids were plunged into liquid ethane to vitrify and examined on the Titan Krios operated at 300keV.

The aim of this experiment was to find a simple and solid method for purifying and freezing native thick filaments from a vertebrate model striated muscle in near-physiological ionic conditions for structural studies. Here, we propose a consistent method by which one can improve a heterogenous broken specimen to a more homogenous full length one in which you can clearly locate the bare zone required for 3D reconstruction analysis (Fig. 1a-1c). This same method without the glutaraldehyde works well with invertebrate thick filaments. Negative staining electron microscopy of vertebrates' myosin filaments showed ordered heads arrangement (Fig. 1d-1g). However, disordered heads and disassembled backbone is seen in Cryo-electron microscopy images (Fig 2a). We realized that even increasing the temperature of the Vitrobot chamber to 27°C during the plunge freezing will not prevent the nature of the disordered state populated by lowering temperature at rest [3]. After several unsuccessful attempts for preserving myosin heads in frozen samples, we tried to fix the specimen using 0.5% glutaraldehyde. The primary results showing heads lying on the surface of the filament (Fig 2b). However, since the axial repeat does not seem be clearly seen in the power spectrum, we assume that heads are not perfectly ordered (Fig 2c-d). Single particle image processing is in progress. Simultaneously, we are still trying to optimize the Cryo-EM sample preparation condition for a more reliable data. Supported by NIH/NIAMSD.

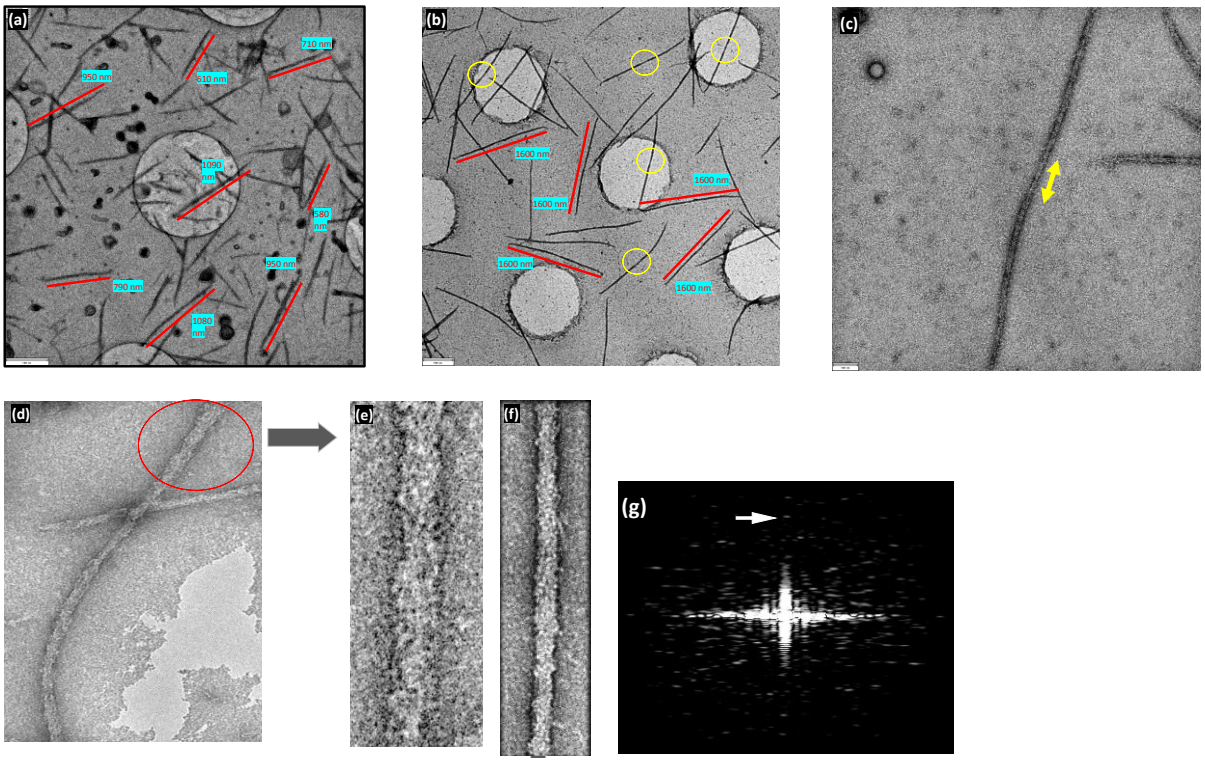

Figure 1. Negative staining electron microscopy and image analysis of rabbit psoas muscle thick filament purification **(a)** Filament suspension preparation without an incubation in Gelsolin resulting in an heterogeneity in filaments' length. The average length of filaments is less than 1000 nm **(b)** Filament suspension preparation using calpain and gelsolin treatment resulting in filaments with homogenous lengths. Most of the filaments show the full-length of 1600 nm. Bare zones are marked with yellow

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circles. **(c)** Higher mag image of an intact thick filament with its bare zone **(d,e)** Higher mag image of a filament over a hole showing ordered heads. **(f,g)** A selected thick filament and its power spectrum. The arrow points to the visible layer linear on the power spectrum indicating the thick filament axial repeat of ~14.5 nm.

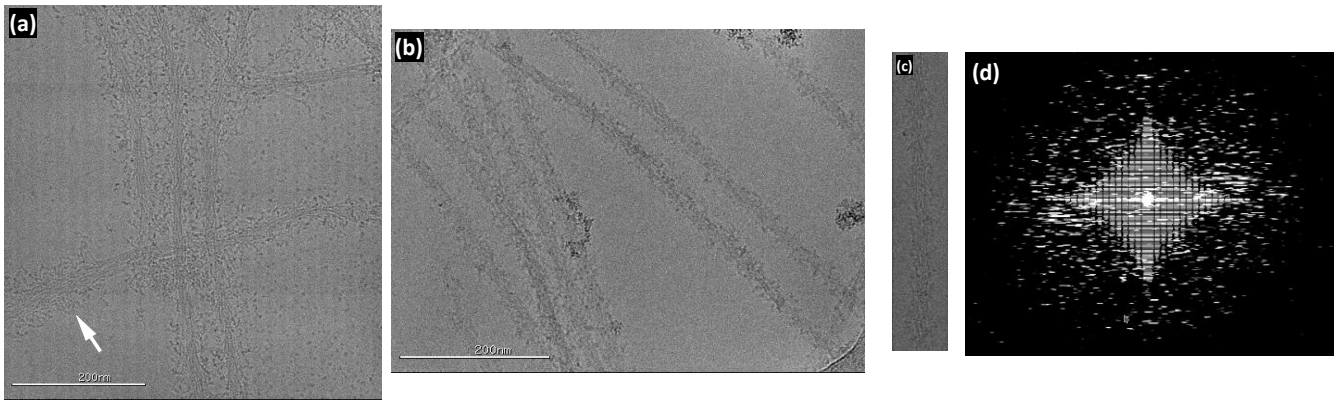

Figure 2. Cryo-electron microscopy and image analysis of rabbit psoas muscle thick filaments **(a)** Filament suspension vitrified on a Vitrobot with the environmental chamber set to 90% humidity and 27°C. Heads look disordered and filament backbone marked with an arrow does not look normal. **(b)** Thick filament suspension fixed on the grid with 0.5% glutaraldehyde in 0.1M cacodylate buffer. Filaments are more homogenous, and heads look at least compact against the filament backbone. **(c,d)** Power spectrum of a selected filament. Unlike the spectrum seen in Fig. 1g, the axial repeat does not seem be clearly seen in the power spectrum while other layer lines can be seen.

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