Zebrafish—Topical, Transparent, and Tractable for Ultrastructural Studies

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The zebrafish (*Danio rerio*) has become a model animal of great value in the study of disease and development because of its fecundity, its similarities in structure and physiology to mammalian tissues, and the availability of immensely powerful genetic tools (Briggs, 2001; Wienholds et al., 2003; Zon and Peterson, 2005; for a complete story see http://zfin.org). Even adult fish that have been made transparent for ease of observing their internal organs have become available (White et al., 2008). This allows, for example, individual cancer cells to be seen directly and followed in real time as they spread. Here, prompted by the pioneering paper by Dou et al. in this issue (see p. 445), we consider just one aspect of zebrafish, namely the ultrastructure and physiology of their muscles. For many years the study of muscle structure at the molecular level has concentrated on only a small number of species—frog, rabbit, and chicken because of their availability, their ease of dissection, and their relatively well-aligned muscle fibers. The fibers of frog sartorius and semitendinosus muscles were used for studies of muscle mechanics from the early 1900s (see Wilkie, 1976; Squire, 1981). Starting in the 1950s, Hugh Huxley and others used these preparations for x-ray diffraction and electron microscope studies and an enormous wealth of information was obtained about the molecular arrangements within the muscle sarcomeres (e.g., Huxley and Brown, 1967). Shortly after this the asynchronous flight muscles of insects, particularly of the giant water bug *Lethocerus maximus*, became of great interest because the normal active state of the muscles was oscillatory and displayed the property of stretch activation (Pringle, 1967). Subsequent studies found that these insect flight muscles were also by far the most highly ordered of all known invertebrate muscles (Reedy, 1968); they gave really beautiful and well sampled low-angle x-ray diffraction patterns and they gave electron micrograph images which, because of their regularity, could be subjected to detailed image processing and 3D reconstruction (Taylor et al., 2007). What about the vertebrates, then? Are the fibers of frogs, chickens, and rabbits the most highly regular of all the vertebrate muscles? Surprisingly it turns out that they are not. In recent years it has been found that the muscles of bony fish, the teleosts, are intrinsically much better ordered than those of any of the higher vertebrates, including humans (Luther et al., 1996). There are, therefore, great advantages in studying the ultrastructures and physiological properties of bony fish muscles simply because of the intrinsically high order in their sarcomeres. Among the bony fish, the zebrafish become a logical choice of species, even though the usefulness of zebrafish for studies of disease and development was pursued and established without any thought for their ultrastructure. In particular, zebrafish muscles have not previously been used for studies of the molecular events that take place during muscle contraction. Now, in their new paper in this issue, Dou et al. (2008) have used whole zebrafish early larvae, ~1.5 mm long, both for direct studies of their muscle mechanics and for low-angle x-ray diffraction from the whole animal, which can show evidence of molecular movements within the body muscles while force is being produced. Zebrafish, therefore, not only provide a wonderful genetic tool, but they also have the kind of vertebrate muscle that, of all the vertebrate muscles, is the most amenable to ultrastructural studies. The two approaches combined promise to open up a plethora of new research opportunities.

Superlattices and Simple Lattices in Vertebrate Muscles

To understand why bony fish, including zebrafish, offer an inherent advantage for muscle ultrastructural studies it is necessary to look closely at the vertebrate muscle sarcomere (Squire et al., 2005). Fig. 1 shows the well-known breakdown of the sarcomere into the A-band and I-band. These bands are defined by the protein filaments that produce them. Myosin filaments are confined to the A-band, and they have a cross-linking structure called the M-band at their centers. Actin filaments originate at the Z-band, cross the I-band, and partly overlap the myosin filaments in the A-band. The myosin filaments are formed mainly from myosin molecules, along with the giant protein titin, which also extends through the I-band to the Z-band, and C-protein

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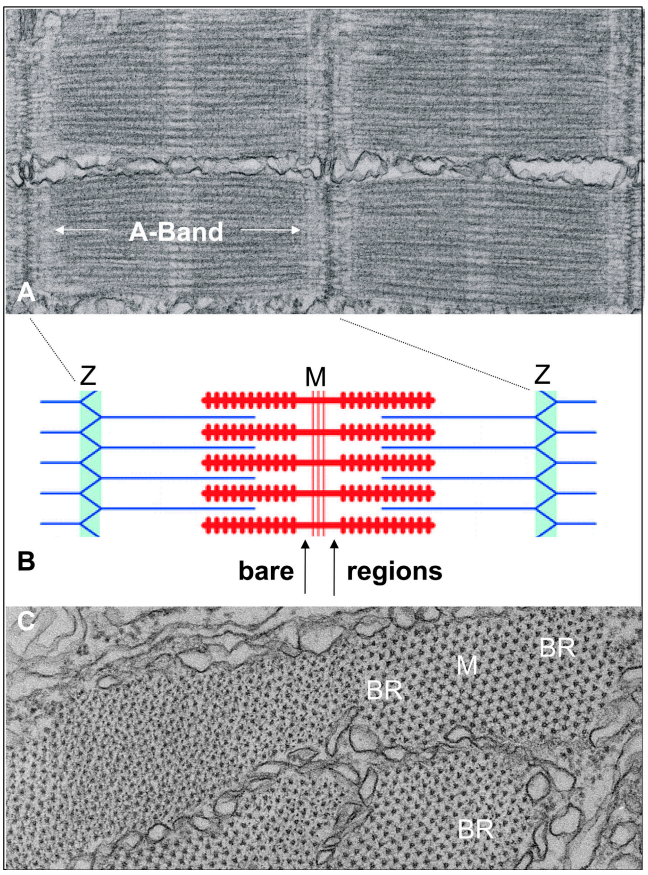

Figure 1. (A) Electron micrograph of a longitudinal section through zebrafish myotomal muscle showing the typical sarcomere striations of vertebrate striated muscle. The sarcomere (B), which extends between Z-bands (Z) and is ~2.2 μm long, consists of the centrally placed A-bands and the less densely packed I-bands, which extend between successive A-bands. The A-band is formed by an array of myosin filaments carrying myosin head projections and cross-linked halfway along their length at the M-band (M). Each side of the M-band are the bare regions where the myosin filament backbones appear triangular. (C) Electron micrograph of zebrafish myotomal muscle in cross section showing myosin filament profiles near to the M-band (M) and in the adjacent bare regions (BR). The triangular profiles in one bare region all point in the same direction indicating the presence of a simple lattice arrangement.

(MyBP-C), which occurs in the central third of each half of the myosin filaments. Myosin molecules have a two-chain α-helical coiled-coil rod region with two globular myosin heads on the end. The rods pack together to form the filament backbone and the heads, which are ATPases, are on the filament surface where they can interact with the neighboring actin filaments (Fig. 1 B). The myosin rods in the two halves of the myosin filament on each side of the M-band have opposite polarities, which means that the central part of the myosin filament has overlapping antiparallel myosin rods and no heads. This is the so-called bare zone. The myosin filaments have threefold rotational symmetry, which means that the heads of three myosin molecules occur

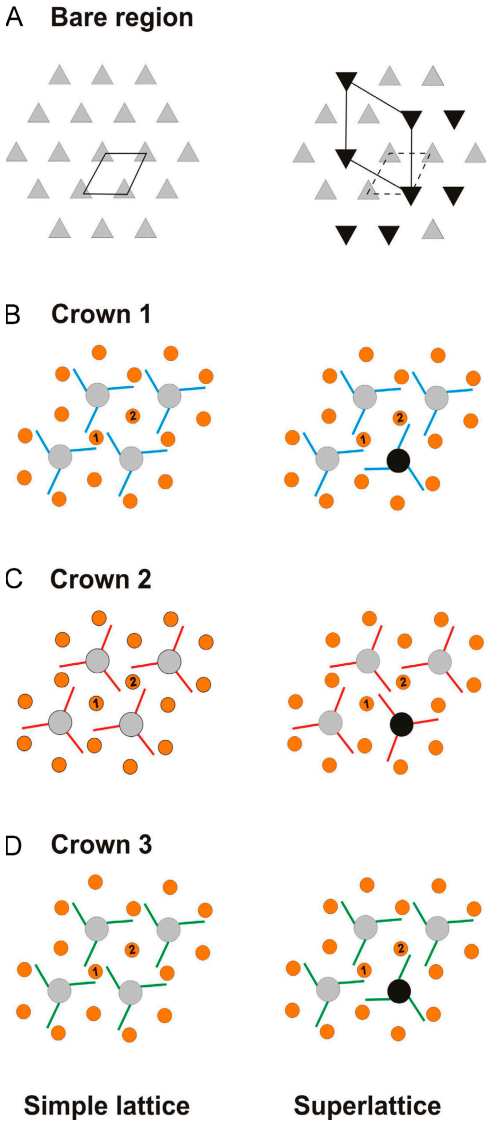

Figure 2. (A) Illustrations of the bare region arrangements of myosin filament profiles in a simple lattice (left) and a superlattice (right). The simple lattice has identically oriented triangular profiles throughout. The superlattice has two filament orientations with an irregular, statistical arrangement. (B–D) The different effects of the simple lattices and superlattices on the myosin head arrangements on the three 14.3-nm spaced crowns of myosin heads within the 42.9-nm repeat that occurs along vertebrate muscle myosin filaments. Each radiating line from the myosin filament backbones (blue) represents a pair of myosin heads. On crown 1 the simple lattice has three head pairs approaching one of the actin filaments (brown) and no heads approaching the other actin filament in the unit cell. On the other hand the superlattice spreads the myosin heads more evenly along the actin filaments so that on crown 1 there are two head pairs for one actin filament and one head pair for the second filament. Similar effects occur on crowns 2 and 3.

at 120° intervals around the filament surface at a particular position along the myosin filament (Fig. 2 B). One such set of three head pairs is called a crown and successive crowns along the filament are separated axially by ~14.3 nm on average.

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TABLE I

Forces Generated by Different Muscle Types

Animal	Temp °C	Speed	Force/ Unit Area (Nm^{-2})	Lattice Type	Reference
Frog	3	Fast	270	Super	Gordon et al. (1966)
Rat	12	Fast (Ave)	360	Super	Bottinelli et al. (1991)
Rat	12	Slow	211	Super	Bottinelli et al. (1991)
Dogfish	12	Fast	289	Super	Lou et al. (2002)
Dogfish	12	Slow	142	Simple	Lou et al. (2002)
Sculpin	3	Fast	281	Simple	Altringham and Johnston (1988)
Carp	15	Fast	230	Simple	Wakeling and Johnston (1999)
Carp	8	Slow	202	Simple	Langfeld et al. (1991)

The threefold symmetry of the myosin filaments means that in parts of the bare zone, namely in the "bare regions" on each side of the M-band (Fig. 1, B and C), the myosin filament cross sections appear triangular. It was studies on the relative orientations of these triangular profiles in different muscles that led to the realization that the A-bands of bony fish are characteristically different from other vertebrate muscles (Luther and Squire, 1980). In electron micrographs of thin cross sections through the bare regions of frog and other higher vertebrate muscles it was found that the triangular profiles pointed in two different directions, but that the arrangement of these two orientations was not regular. Although the organization followed specific rules, these produced a rather complicated statistical "superlattice" arrangement (Fig. 2 A, right). The consequence of this is that there is no long range rotational myosin filament order in the A-bands of higher vertebrate muscles. The difference found in the A-bands of bony fish was that all the triangular myosin filament profiles pointed in exactly the same direction (see Fig. 1 C for zebrafish). In other words, in fish muscle, all the myosin filaments have identical rotations around their long axes. In this case the structure is simple and regular, the myosin filaments are arranged in a "simple lattice" (Fig. 2 A, left) and there is good long range order.

This difference in A-band lattice may seem a subtle thing, but for those carrying out ultrastructural studies it makes a huge difference. For example, electron microscopy these days is rarely enough on its own. It is usually followed up by image processing and analysis, which usually involves the averaging together of images of regularly arranged adjacent objects. This can be done in the case of fish muscle where adjacent myosin filaments are identically oriented, but not for higher vertebrate muscles where the A-band array is irregular. Structural techniques like x-ray diffraction are also rendered much simpler if the specimen is quasi-crystalline, as in fish muscle. The diffraction patterns become well sampled, which makes them easier to analyze (Harford and Squire, 1986). For the invertebrates, insect flight muscle has the same advantage in that the myosin filaments there, albeit having fourfold symmetry rather than the vertebrate threefold, also have identical myosin filament orientations through the A-band. Because of this regularity they give beautifully sampled x-ray diffraction patterns that are amenable to rigorous analysis (AL-Khayat et al., 2003). So, for the invertebrates, insect flight muscle is the muscle of choice for ultrastructural studies and, for the vertebrates, bony fish muscle is the muscle of choice.

Evolutionary Advantages of the Simple Lattice

A question that immediately comes to mind on finding out that vertebrate muscles come in two varieties, simple lattice and superlattice, is what evolutionary difference there might be in having one structure rather than the other. In an attempt to answer this and to map the evolutionary history of lattice development, Luther et al. (1996) found, perhaps surprisingly, that the early craniates like lamprey and hagfish have superlattice muscles. Teleosts and Bowfin have simple lattice muscles; sharks, rays, and other cartilaginous fish have some of each, the fast muscles tending to be superlattice and the slow muscles simple lattice; and tetrapods and Dipnoi (all relatively recent vertebrates) have the superlattice. The teleosts have been an incredibly successful group so it would appear that they adopted the simple lattice arrangement because it was in some way to their advantage. We have puzzled about what this advantage might be. An immediate effect of the different lattices is that an actin filament in the muscle A-band will "see" different arrangements of myosin heads around them (Fig. 2, B–D). In fact, the superlattice arrangement spreads the myosin heads more evenly along the actin filaments, so with a superlattice there is presumably a better chance for the heads to attach to actin in active muscle. It has been found that fish muscles generally produce a smaller force per unit cross-sectional area than higher vertebrate muscles. We have done a quick trawl across many fish and higher vertebrate muscle papers quoting forces per unit area and will present the results elsewhere, but Table I lists a few representative examples that illustrate the trend.

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In summary, the strongest superlattice muscles can produce over 350 Nm^{-2}, whereas, in our trawl, the strongest simple lattice muscles produced forces in the range 200–280 Nm^{-2}. Remembering the different ways that these measurements were made, the variations in temperature that have a big effect on isometric force, the presence of different protein isoforms, particularly between slow and fast muscles, and the usual mix of fiber types in different muscles, this nevertheless seems to show that there may be a trend where simple lattice muscles produce less force per unit area than superlattice muscles. And this could simply be because heads in simple lattice muscles have to compete for actin binding sites more than in superlattice muscles.

Why then might fish want their muscles to be weaker? In land animals it is clear that muscles with high force and low mass will be advantageous since the animals have to carry the weight of their muscles around with them. Fish on the other hand use their myotomal muscles not only to produce movement but also to bulk out their volume to generate a streamline shape. In addition their muscle mass is partially offset by the buoyancy provided by their aqueous environment. A little extra volume for a given muscle force may not therefore be a disadvantage and may allow economies in ATP usage. What about the cartilaginous fish? They have some superlattice muscles, albeit giving higher force per unit area as expected, but they are also fish. Why do they not have simple lattice muscles too? Here it gets harder, but one thought that still requires further analysis is that it may be to do with the very different swimming, lifestyles, and feeding habits of sharks compared with most teleosts.

The Recent Study

Studies of muscle in zebrafish really started with the major ultrastructural survey by Waterman (1969) and, later, effects on myofibril formation were reported by Felsenfeld et al. (1990). Since then it has been found that good models of various diseases can be developed, including studies of dystrophin (Bassett et al., 2003), dystroglycan (Parsons et al., 2002), and cardiomyopathy induced by modified titin (Xu et al., 2002). However, little work has been done so far on the contractile properties of zebrafish muscles. The new work of Dou et al. (2008) combining muscle mechanics and low angle x-ray diffraction, which can give the value of the A-band lattice spacing and report molecular movements, has now changed all that. Results from 5–7-d larvae showed muscle fibers more or less axially aligned, whereas at a later stage (2 mo) they were angled at 25°. x-ray diffraction from activated muscles showed changes characteristic of myosin head movement to actin to produce contraction (see Squire and Knupp, 2005). Although more detailed diffraction data will be needed to take this kind of analysis the next level, already Dou et al. have shown that the zebrafish is not just a good model organism for studies of development and genetic manipulation. Of all the teleosts, with their beautifully ordered simple lattice A-bands, the zebrafish may well be an appropriate fish to spend more time with for ultrastructural studies, preferably also combined with targeted genetic manipulations. It is evident that the use of the zebrafish system for studies of integrative biology has enormous potential.

Some of the data in Table 1 were compiled by Felicity Eakins.

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