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From Cell Biology to Tissue Engineering

History and development of staining methods for skeletal muscle fiber types

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Summary. The contractile and metabolic properties of skeletal muscles depend on the composition of muscle fibers. There are two major fiber types: type 1 and type 2. Type 2 fibers are further subdivided into type 2A, 2X, and 2B fibers. Muscle fiber type composition is an important property that affects sports performance and metabolic ability in humans, and meat quality in domestic animals. In this review, we summarize the history of muscle fiber type classification based on various staining methods for skeletal muscle sections. The history illustrates the development of an experimental method to detect myosin heavy chain (MyHC) proteins, which are the most common marker molecules for muscle fiber type. Metabolic enzymes, such as nicotinamide adenine dinucleotide-tetrazolium reductase and succinate dehydrogenase are also described for histochemical staining combined with myosin ATPase staining. We found an improvement in the quality of antibodies used for immunostaining of MyHC, from polyclonal antibodies to monoclonal antibodies (mAbs) and then to mAbs produced by synthetic peptides as antigens. We believe that the information presented herein will assist researchers in selecting optimal staining methods, dependent on the experimental conditions and purposes.

Key words: Skeletal muscle, Fiber type, Myosin ATPase, NADH-TR, Immunostaining, Myosin heavy chain (MyHC)

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Skeletal muscle fiber type and myosin

Skeletal muscle tissue is composed of thousands of muscle fibers, and the contractile and metabolic properties of skeletal muscle tissues depend on their fiber-type composition. There are two major fiber types: type 1 (slow-oxidative, red muscle) and type 2 (fast-glycolytic, white muscle), which are further subdivided into type 2A, 2X (also referred to as 2D), and 2B fibers. Although Roman numerals, such as type I and type II, are often used, this review uses Arabic numerals.

Type 1 fibers are rich in mitochondria, possess a high oxidative capacity, and are resistant to fatigue. Conversely, type 2 (2A, 2X, and 2B) muscle fibers exhibit high rates of glycolytic metabolism and fatigue. Consequently, muscles enriched in type 1 fibers, such as the soleus, typically perform sustained and tonic contractile activities, such as postural tension, whereas muscles enriched in type 2 fibers, such as the extensor digitorum longus, are typically involved in intense and rapid activities of short duration. Research on the human vastus lateralis muscles suggests that there is a large variation in the composition of muscle fiber types between individuals (Simoneau and Bouchard, 1995). In all mammalian species, the contractile speed is lower in type 1 than in type 2 fibers, whereas the speed increases in the order 2A<2X<2B in fast fibers (Barany, 1967). Similarly, oxidative metabolic properties and fatigue resistance are higher in type 1 than in type 2 fibers, and decrease in the order 2A>2X>2B in type 2 fibers (Rivero et al., 1999). Type 2B fibers appear to be specific to small mammals and marsupials and are not detected in most human (Smerdu et al., 1994) and bovine (Tanabe et al., 1998; Toniolo et al., 2005) muscles.

Fiber type composition affects exercise performance, fatigue resistance, and metabolic capacity

in animals (Zierath and Hawley, 2004). Furthermore, animal model studies have demonstrated a strong relationship between muscle fiber type and the development of diabetes and obesity (Wang et al., 2004; Schuler et al., 2006). Meanwhile, certain diseases can interfere with the composition or distribution of muscle fiber types, which can subsequently result in clinical manifestations (D'Amico and Bertini, 2008). The associations between muscle fiber type and meat quality, such as pH, color, tenderness, and nutritional values, are well examined (Picard and Gagaoua, 2020). Thus, elucidating the mechanism of muscle fiber type regulation would likely enhance our understanding of human metabolic disorders, exercise performance, skeletal muscle diseases, and various meat properties.

Myosin is a myofibrillar protein found in skeletal muscles and accounts for about 50-60% of muscle proteins, together with actin. Myosin is a hexameric protein consisting of two myosin heavy chains (MyHC) and four myosin light chain subunits. Since the ATPase activity and actin-binding domain are located in MyHC (Rayment, 1996), MyHC is regarded as the core of the myosin protein complex. There are reportedly multiple isoforms of MyHC, each with different ATPase activity (i.e., molecular motor properties). In adult mammalian skeletal muscles, four MyHC isoforms, namely MyHC1 (also called MyHC-β), MyHC2A, MyHC2X (also called MyHC2D), and MyHC2B, are encoded by independent *MYH7, MYH2, MYH1,* and *MYH4* genes, respectively (Schiaffino and Reggiani, 2011). It is important to note that the gene numbers and fiber types (MyHC isoform numbers) differ completely. The number of MyHC isoform proteins corresponds to the muscle fiber types (Table 1); hence, muscle fiber type determination is currently synonymous with identification of the present MyHC isoforms. As fiber type composition affects various muscle properties, the composition of MyHC isoforms largely determines whole muscle contractile (Harridge et al., 1996) and metabolic properties (Talmadge et al., 1996). MyHC

isoforms in birds are quite different from those in mammals. At present, five types of fast MyHC and four types of slow MyHC are known to be expressed in birds (Bandman and Rosser, 2000), and the classification of fast and slow muscles is similar to that of mammals. However, the correspondence between the mammalian MyHC isoforms and these nine avian MyHC isoforms is still unclear.

In most cases, a single muscle fiber (muscle cell) expresses only one MyHC isoform, and is called a "pure fiber." Conversely, some minor muscle fibers express two or more MyHC isoforms in one cell body and are called "hybrid fibers". Analysis of a rat hindlimb muscle revealed that more than 90% of the isolated myofibers were pure fibers (Staron and Pette, 1993), and in the human vastus lateralis muscle, approximately 90% of the myofibers were reported to be pure fibers (Galpin et al., 2012), whereas another study reported about 70% of fibers were pure fibers (Paoli et al., 2013). The observation of hybrid fibers in various animal species suggests that skeletal muscles of many organisms allow for multiple myofibrillar protein isoforms to be expressed in a single fiber (Lutz et al., 2001; Stephenson, 2001; Medler et al., 2004; Perry et al., 2009). Hybrid fibers are essentially hybrids of two neighboring isoforms when the types are arranged in the order 1, 2A, 2X, and 2B. However, there are some exceptions to this rule, such as hybrids that deviate from the order. This is especially true in skeletal muscle, where the fiber type is in transition. For example, the most common combination of type 1 and 2X hybrids has been reported in the soleus muscle of adult rats 60 days after spinal cord transection (Grossman et al., 1998; Stephenson, 2001). However, it is almost certain that most myofibers are composed of pure fibers, indicating the muscle fiber type is induced to be uniform in a single fiber once determined. Using any method, identification of the type of MyHC isoform in skeletal muscle, either based on enzyme activities or protein detection, is regarded as the identification of the fiber type.

Table 1. Conventional muscle fiber types (classified by ATPase staining) and staining pattern related to their corresponding myosin heavy chain (MyHC) isoforms in mammals.

	type 1	type 2A	type 2X[a]	type 2B
Biochemical	slow-twitch oxidative (SO)	fast-twitch oxidative (FOG)	fast-twitch glycolytic (FG)	
MyHC isoform (protein)	MyHC1 (MyHCβ)	MyHC2A	MyHC2X(2D)	MyHC2B[b]
MyHC gene	*MYH7*	*MYH2*	*MYH1*	*MYH4*
ATPase staining pH 4.3	++	-	-	-
ATPase staining pH 4.6	++	-	+	+
ATPase staining pH 10.3	-	++	++	+
NADH-TR[c]	+	+ (++)	- ~ ++	-
SDH[c]	+	+ (++)	- ~ ++	-

[a]Note that type 2X is a novel fiber type after the identification of MyHC2X by Bär and Pette (1988). [b]MyHC2X is expressed in human fibers and is classified as type 2B using the ATPase reaction. MyHC2B is not expressed in most skeletal muscle tissues of large mammals, such as humans. [c]The intensity of staining depends on species. Please refer to the main text.

Myosin ATPase staining

In the 1960s and 1970s, many papers were published regarding the classification of muscle fiber types. Initially, three fiber types were defined based on color tone: red, white, and intermediate (Padykula and Gauthier, 1967). These criteria were morphologically based on mitochondrial content as seen by electron microscopy; Dubowitz classified muscle fibers into type 1 and type 2 based on the histochemical properties of enzymes (Dubowitz and Pearse, 1960). Type 1 fibers display greater levels of oxidative enzymes and lower levels of phosphorylase and ATPase, whereas type 2 fibers display the opposite. Engel developed this histochemical method and enabled the characterization of the fiber type using ATPase reactions alone (Engel, 1962). The staining method, currently known as myosin ATPase staining organized by Brooke and Kaiser (Brooke and Kaiser, 1970b), has become a common and customary procedure, which has been widely adopted since the mid-twentieth century as a standard method to classify fiber types in skeletal muscles, especially in clinicopathological examinations (Engel, 2015). Brooke and Kaiser used human, rat, and rabbit muscle samples, and the results were almost identical in these three animal species. The staining method was able to be applied to muscle sections of bovine (Gotoh, 2003), porcine (Lefaucheur, 2010), equine (Gunn, 1972), wild boar (Rehfeldt et al., 2008), and even chickens (Barnard et al., 1982; Sosnicki and Cassens, 1988), suggesting this tissue staining method is very versatile.

The principle of the myosin ATPase staining method is to inactivate a specific type of ATPase by treating muscle sections at different pH conditions and observing differences in the amount of inorganic phosphate released from ATP hydrolysis. The phosphate is then reacted with calcium chloride to produce calcium phosphate and then with cobalt to produce cobalt phosphate. This is visualized by reacting the product with ammonium sulfide to produce cobalt sulfide, which is black in color. During alkaline (pH 10.3) pretreatment, myosin ATPase activity of type 1 fibers is lost, and only type 2A and 2B fibers are stained. In contrast, acidic (pH 4.3) pretreatment stains only type 1 fibers, and type 2A and 2B fibers are inactivated. Further, only type 2B fibers appear stained in intermediate colors at pH 4.6 when the pH is gradually increased from 4.3, and type 2A and 2B fibers can be distinguished. This staining pattern is summarized in Table 1. Some fibers react in both acid and alkaline pretreatments and are called type 2C fibers. These fibers are now thought to be hybrid fibers (i.e., hybrids of type 1 and type 2A (or other type 2) fibers). In fact, very few type 2C fibers are observed in normal muscles. The low number of hybrid fibers is consistent with the very low number of type 2C fibers (Suzuki and Tamate, 1988; Suzuki and Hayama, 1994).

It is still unclear how ATPase enzyme activity is affected by specific pH levels. It is possible that the ATP binding site in a myosin molecule becomes denatured by pH alterations. Two regions of amino acids in the myosin sub-fragment 1 (S1) head are thought to play a particularly important role. These regions are called surface loops, as they are considered flexible and do not crystallize in the crystal structure of S1, as shown by Rayment (Rayment, 1996). These regions may contribute to the regulation of myosin ATPase activity either by affecting the dissociation rate of ADP (surface loop 1) (Murphy and Spudich, 1998) or by modulating the interaction between myosin and actin (surface loop 2) (Uyeda et al., 1994). Brooke and Kaiser speculated the effects of the sulfhydryl group of cysteine and the carboxyl group of glutamic acid in MyHC for ATPase inactivation via pH alteration (Brooke and Kaiser, 1970a).

When the oxygen supply decreases during strenuous exercise, lactic acid accumulates in the muscle fibers and the pH of the muscle fibers declines. Nonetheless, at low pH (acidic) conditions, the activity of myosin ATPase enzyme in type 1 fibers is still high, suggesting that the muscle may continue contracting even if lactic acid accumulates during exercise. Consequently, type 1 fibers appeared to have fewer fatigable properties.

This myosin ATPase staining method cannot distinguish between the type 2X and 2B fibers, as these two myosin isoforms have the same pH lability (the proteins also show high amino acid sequence homology). However, in human muscles, this is not a serious problem because MyHC2B is rarely expressed. It is assumed that MyHC2X is expressed in type 2B fibers. However, since both MyHC2X and MyHC2B are expressed in significant amounts in laboratory mouse and rat samples, which are common animal models, it is necessary to know that myosin ATPase staining cannot be used to classify type 2X and type 2B in mice and rats.

One disadvantage of myosin ATPase staining is that this method requires the preparation of multiple serial sections for comparison (usually at least three sections are required for pre-incubation at pH 4.3, 4.6, and 10.3, respectively); therefore, a high level of cryostat-based sectioning technique is required. Furthermore, it is very time-consuming to manually observe the tissue sections under a microscope after staining and match the identical region in a section.

To overcome the disadvantages of multiple sections, an improved method of myosin ATPase staining was developed and named metachromatic ATP staining (Doriguzzi et al., 1983). In this method, Azure A or Toluidine Blue dyes produce metachromatic or orthochromatic staining at different phosphate contents after incubation in the reaction medium, thus allowing fiber type distinction. In metachromatic ATP staining, pre-incubation and incubation temperatures have been found to affect the conditions under which metachromasia occurs. According to Olgivie and Feeback, in human muscle sections, pre-incubation at pH 4.5 in the temperature range of 22-24°C, staining with toluidine blue for 10 s, followed by a quick wash with distilled water, strongly stained type 1 fibers blue-

green, type 2B fibers dark red, and type 2C fibers blue (Ogilvie and Feeback, 1990). After dehydration with increasing ethanol concentrations, type 1 fibers remained blue-green, type 2B fibers appeared purple, and type 2A fibers appeared light purple. According to the authors, this method can clearly distinguish between different muscle fiber types. Although not as prevalent as the conventional myosin ATPase staining, this method contributed to the discovery of the surprising effects of PGC1α (Lin et al., 2002) and PPARδ (Wang et al., 2004) on the regulation of muscle fiber type in the early 2000s.

NADH-TR and SDH staining

A staining method based on the enzymatic activity of myosin ATPase is often used in conjunction with nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) or succinate dehydrogenase (SDH) staining, which is sometimes used instead of NADH-TR staining. Both the NADH-TR and SDH staining methods showed similar results. Histochemical studies have shown that type 1 and 2A fibers contain more mitochondria and have better oxidative metabolism than type 2B (MyHC2X-positive) fibers in human muscles (Yang et al., 1990). The enzyme commission numbers (EC number) of NADH-TR and SDH are EC 7.1.1.2 and EC 1.3.5.1, respectively. NADH-TR can transfer electrons from the reduced NADH to various electron acceptors. Electron transport systems working in the mitochondria are involved in this electron transfer. In the NADH-TR staining method, the target chemical reaction is a multicomponent enzyme reaction that includes complex I of the electron transport chain, NADH dehydrogenase, and portions of the microsomal (sarcoplasmic reticulum) electron transport system, including cytochrome b5 reductase (Charles-Schoeman and Verity, 2012). SDH, an enzyme complex found in the inner membrane of mitochondria, catalyzes the oxidation of succinate in the tricarboxylic acid cycle and transfers electrons to intramembrane quinones during oxidative phosphorylation. It forms part of the aerobic respiratory chain (complex II), which transfers electrons to quinones during oxidative phosphorylation (Rutter et al., 2010). Since these two staining methods mainly visualize mitochondrial enzymatic activity, this staining technique is used not only for muscle fiber type classification, but also for the diagnosis of mitochondria-related muscle diseases. NADH-TR or SDH staining is utilized in the biochemical classification of muscle fiber types. Slow-oxidative (SO) fibers are positive for acidic treatment (pH 4.3 or 4.6) of myosin ATPase staining and NADH-TR or SDH staining. Muscle fibers that are negative for both are referred to as fast glycolytic (FG) fibers. In contrast, myofibers that are negative for acidic treatment of myosin ATPase staining (pH 4.3 or 4.6) and positive for NADH-TR or SDH staining are classified as fast-oxidative-glycolytic (FOG) fibers because of their intermediate traits (Table 1).

The staining principle relies on the transfer of electrons from NADH to a colorless soluble tetrazolium salt such as nitro blue tetrazolium (NBT), which is converted to an insoluble blue formazan compound. Thereafter, stained and unstained fibers can be distinguished. The staining procedure is also noteworthy in that type 2A fibers showed very high NADH-TR oxidase activity in rat skeletal muscle (Yang et al., 1990). Presumably, this is due to mitochondrial content, as a hierarchy of 2A>2X>1>2B was observed in mice, 2A>1>2X>2B in rats, and 1>2A>2B (2X) in human skeletal muscle (Gouspillou et al., 2014). The principle of SDH staining was similar to that of NADH-TR staining. SDH staining utilizes the reduced blue formazan compound produced from tetrazolium salt by the oxidation of succinate to fumarate as an indicator.

As noted by Bottinelli and Reggiani, the classification of muscle fiber types based on metabolic properties, such as NADH-TR, does not necessarily coincide with the classification of muscle fiber types based on myosin ATPase (Bottinelli and Reggiani, 2000). Indeed, type 1 fibers are generally more oxidative than type 2 fibers. However, within each type of fiber based on ATPase, there is a wide range of metabolic enzyme activities (Hintz et al., 1984), and there is a large overlap between different fiber types (Reichmann and Pette, 1982). For this reason, attempts to combine the classification of these metabolic enzymes with myosin ATPase have generally been unsuccessful. For example, single-fiber analyses of enzyme activities show a continuous distribution of aerobic-oxidative (Bottinelli and Reggiani, 2000) and anaerobic (e.g., lactate dehydrogenase) enzyme activities (Pette et al., 1980; Nemeth et al., 1981; Pette and Spamer, 1986), independent of the type defined by myofibrillar ATPase activity. This means that classification by metabolic enzyme activity may not be complete.

Immunostaining method for MyHC

In the myosin ATPase staining method, myosin ATPase is detected by visualizing enzyme activity. Based on this principle, the detection of myosin ATPase protein instead of enzyme activity would provide the same result. The experimental method used to detect the distribution and localization of specific proteins within tissues is defined as immunostaining. This method utilizes an antibody targeting a specific molecule, referred to as the primary antibody, to detect its presence. A secondary antibody conjugated with either a fluorophore or enzyme (horseradish peroxidase is most common) is used to detect the primary antibody, and the presence of the target protein can be visualized and/or quantified using a fluorescence microscope or by the addition of a colored substrate. Immunostaining with specific anti-MyHC antibodies was used to identify MyHC isoforms. Currently, this technique is commonly used for muscle fiber type classification. Many antibodies that specifically recognize MyHC isoforms are already readily available through reagent companies

or the Developmental Studies Hybridoma Bank (Table 2). Both myosin ATPase staining and anti-MyHC antibody immunostaining eventually produce similar staining patterns, although the experimental procedures differ. The marked difference from myosin ATPase staining is that type 2X and type 2B fibers can be distinguished. This is because immunostaining is highly specific and can more precisely distinguish between fiber types.

Anti-MyHC antibodies have been developed over the past four decades. In 1981, Pierobon-Bormioli et al. first identified two fast-twitch fiber types, types 2A and 2B, through immunohistochemistry using two rabbit polyclonal antibodies (Pierobon-Bormioli et al., 1981). However, polyclonal antibodies are not well suited for continuous mass production of antibodies of the same quality.

The antibody binding site of an antigen is called an epitope. In many cases, animals produce many antibodies that recognize independent epitopes on the antigen. Individual antibodies recognizing a particular epitope are produced by different plasma clone cells, each of which secretes antibodies that bind to only one epitope. In 1975, Köhler and Milstein published the first method of producing monoclonal antibodies (mAbs) (Köhler and Milstein, 1975). They isolated the precursors of plasma cells and fused them with a mouse myeloma cell line capable of producing immunoglobulins. The fused hybridoma cells were single-cell clones that could be grown as individual clones secreting only one type of antibody. These antibodies are known as mAbs, all of which have the same epitope. Since mAbs have very high specificity, they are effective against MyHC, which has relatively high homology amongst isoforms. Recently, mAbs have been the most common choice for immunostaining targeting MyHC proteins. Table 2 shows a list of the prevalent anti-MyHC primary mAbs.

In 1989, Schiaffino et al. reported about mouse mAbs that could distinguish between the four MyHC isoforms (Schiaffino et al., 1989). In the antibodies developed in this study, the mAbs BA-D5, SC-71, and BF-F3 were introduced as specific antibodies against MyHC1, 2A, and 2B, respectively. However, antibodies against MyHC2X were not available. Alternatively, the BF-35 antibody reacts with three MyHCs, except 2X, which can be applied to identify 2X fibers.

During the development of these mAbs, the MyHC2X isoform was discovered; MyHC2X was first identified independently as a distinct band in gel electrophoresis by Bär and Pette (Bär and Pette, 1988). Analysis using *in situ* hybridization provided definitive evidence for the presence of this isoform (DeNardi et al., 1993). In human skeletal muscles, immunohistochemical and *in situ* hybridization studies clearly showed that fibers previously classified as type 2B by myosin ATPase staining were actually type 2X (i.e., MyHC2X expressing fibers). MyHC2B is rarely expressed in human muscle tissues.

The use of BF-35, which reacts with three types of MyHCs except for MyHC2X, for the detection of type 2X fibers is not straightforward. Furthermore, hybrid fibers of 2X and 2A or 2X and 2B could not be identified. To overcome these issues, Lucas et al. challenged to produce a new anti-MyHC antibody in 2000 (Lucas et al., 2000). In this study, they finally succeeded in producing a specific antibody for MyHC2X, and clone 6H1 was the hybridoma that produced this antibody.

Generally, in immunostaining protocols using secondary antibodies, simultaneous multiplex staining of a single section is possible if the antibody-producing animal species differ (e.g., mouse and rabbit). Currently, anti-MyHC mAbs are derived from mouse or rat, thus

Table 2. List of monoclonal antibodies used for MyHC immunostaining.

MyHC specificity	Antibody name (clone)	Antigen (Immunogen)	Host animal	Isotype	Reference	Current supplier
MyHC1	NOQ7.5.4D	Purified native human myosin	mouse	IgG	Draeger et al., 1987	several companies
MyHC1	BA-D5	Native bovine protein	mouse	IgG2b	Schiaffino et al., 1989	DSHB etc.
MyHC1	A4.840	Purified native human myosin	mouse	IgM	Hughes and Blau, 1992	DSHB etc.
MyHC1	4B51E8	Synthetic peptide	rat	IgG2aκ	Sawano et al., 2016	Merck KGaA
MyHC2A[1]	SC-71	Native bovine protein	mouse	IgG1	Schiaffino et al., 1989	DSHB etc.
MyHC2A	A4.74	Purified native human myosin	mouse	IgG1	Hughes and Blau, 1992	DSHB etc.
MyHC2A	2F7	Feline denatured myosin	mouse	IgG1κ	Lucas et al., 2000	DSHB etc.
MyHC2A	8F72C8	Synthetic peptide	rat	IgG2aκ	Sawano et al., 2016	Merck KGaA
MyHC2X	6H1	Native rabbit muscle extract	mouse	IgMκ	Lucas et al., 2000	DSHB etc.
MyHC2X	6F12H3	Synthetic peptide	rat	IgG1κ	Sawano et al., 2016	Merck KGaA
MyHC2B	BF-F3	Purified fetal bovine myosin	mouse	IgM	Schiaffino et al., 1989	DSHB etc.
MyHC2B	10F5	Native rabbit muscle extract	mouse	IgMκ	Lucas et al., 2000	DSHB etc.
MyHC2B	2G72F10	Synthetic peptide	rat	IgG1κ	Sawano et al., 2016	Merck KGaA
MyHC2A, 2X, and 2B	MY-32	Rabbit muscle myosin	mouse	IgG1	Cohen-Haguenauer et al., 1988	several companies
All sarcomeric MyHC	MF20	Purified native chicken myosin	mouse	IgG2b	Bader et al., 1982	DSHB etc.

[1]Moderately reactive with human MyHC2X.

labeled secondary antibody staining is not feasible for multiplex staining. To overcome this problem, the use of mouse IgG isotype-specific secondary antibodies (Bloemberg and Quadrilatero, 2012; Ribaric and Cebasek, 2013) or fluorophore-conjugated primary antibodies (Gregorevic et al., 2008) have been reported.
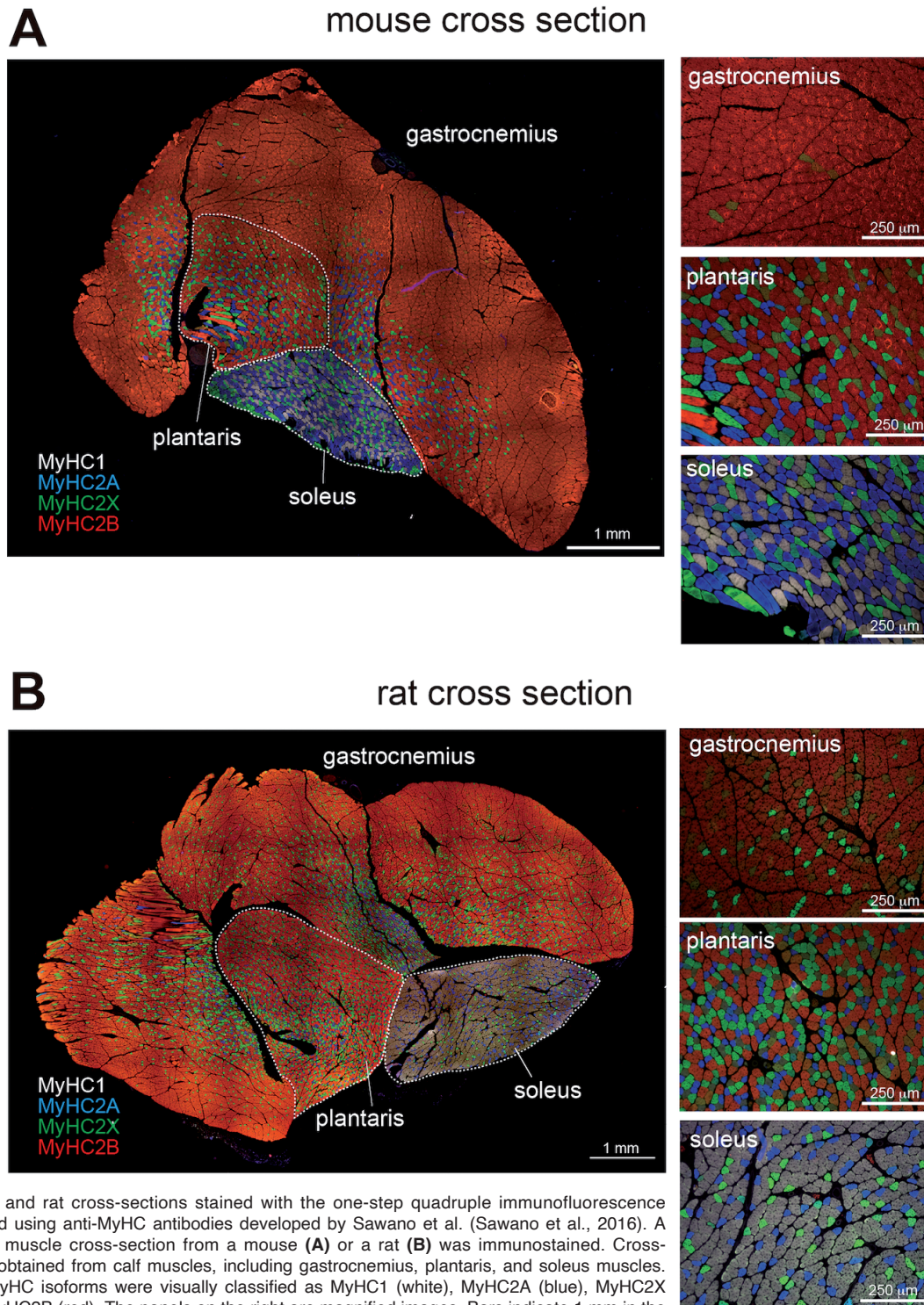
Fig. 1. Mouse and rat cross-sections stained with the one-step quadruple immunofluorescence staining method using anti-MyHC antibodies developed by Sawano et al. (Sawano et al., 2016). A single skeletal muscle cross-section from a mouse **(A)** or a rat **(B)** was immunostained. Cross-sections were obtained from calf muscles, including gastrocnemius, plantaris, and soleus muscles. The specific MyHC isoforms were visually classified as MyHC1 (white), MyHC2A (blue), MyHC2X (green), and MyHC2B (red). The panels on the right are magnified images. Bars indicate 1 mm in the whole muscle image, and 250 μm in the magnified images. Figure reproduced from the previous article (Sawano et al., 2016).

However, in the direct labeling of primary antibodies, the stability of the antibody to withstand the labeling procedures is important. Since all commercially available anti-MyHC2B (BF-F3 and 10F5) and anti-MyHC2X antibodies (6H1) are IgM mAbs, maintaining antibody activity is difficult. Freeze-thaw cycles can be particularly damaging to IgM mAbs (Goding, 1986). In addition, IgM mAbs are prone to aggregation when stored at 4°C for long periods. Furthermore, if ascites are not defatted, insoluble lipoprotein precipitates form in samples stored for long periods at 4°C (Draber et al., 1995). Since IgM is less durable than IgG, many IgM mAbs are irreversibly denatured by freeze-drying. Therefore, labeling IgM mAbs is difficult due to this instability. There is an advantage in producing new anti-MyHC2X and -MyHC2B antibodies with stable IgG as a subclass.

In 2016, Sawano et al. developed anti-MyHC2B (2G72F10) and -MyHC2X antibodies (6F12H3), which are IgG (Sawano et al., 2016). Since these antibodies are stable IgGs, it is possible to label primary antibodies. Together with these two antibodies, Sawano labeled the other anti-MyHC2A (8F72C8) and -MyHC1 (4B51E8) antibodies with four different fluorescent dyes, enabling one-step quadruple immunostaining (Fig. 1). This means that the four anti-MyHC antibodies cover all four adult MyHC isoforms that are expressed in the skeletal muscle tissues of mammals. This method can be applied not only to muscle sections, but also to cell cultures, making it possible to detect hybrid fibers in cultured isolated muscle fibers. This was not possible with conventional primary antibodies.

Cross-reactivity of anti-MyHC antibodies among animal species

One of the disadvantages of immunostaining is the strict specificity of antibodies, but this can also be an advantage in terms of assay purpose. Sometimes, the specificity is not conserved across species due to slight differences in the amino acid sequence and may be restricted to a small number of species. For example, the anti-MyHC2A mAb (SC-71) generated by Schiaffino et al. (1989) cross-reacted with MyHC2A and 2X in rabbits (Lucas and Hoh, 1997). The mAbs against MyHC2A (SC-71) and MyHC2B (BF-F3) did not react with guinea pig tissues (Rivero et al., 1998). Furthermore, they were reportedly unable to detect the MyHC2A, 2X, and 2B isoforms expressed in cats (Talmadge et al., 1996), horses (Rivero et al., 1996), and cattle (Gagnière et al., 1999) using mAbs available at that time. The anti-MyHC mAbs produced by Lucas et al. were reported to cross-react with mice, guinea pigs, rabbits, cats, and baboons, allowing evaluation of muscle fiber types in a wider range of animal species (Lucas et al., 2000).

Sawano et al. changed the antigen to a synthetic peptide to create a mAb (Sawano et al., 2016). This change is a major difference compared to previous

MyHC1 (*MYH7*)

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Mouse (Mus musculus)         ELESSQKEARSLSTELFKLKNAYEESLEH
Rat (Rattus norvegicus)      ELESSQKEARSLSTELFKLKNAYEESLEH
Bovine (Bos taurus)          ELESSQKEARSLSTELFKLKNAYEESLEH
Monkey (Macaca mulatta)      ELESSQKEARSLSTELFKLKNAYEESLEH
Human (Homo sapiens)         ELESSQKEARSLSTELFKLKNAYEESLEH
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MyHC2A (*MYH2*)

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Mouse (Mus musculus)         ELEASQKEARSLGTELFKMKNAYEESLDQ
Rat (Rattus norvegicus)      ELEASQKEARSLGTELFKMKNAYEESLDQ
Bovine (Bos taurus)          ELEAAQKEARSLGTELFKMKNAYEESLDQ
Monkey (Macaca mulatta)      ELEASQKEARSLGTELFKMKNAYEESLDQ
Human (Homo sapiens)         ELEASQKEARSLGTELFKIKNAYEESLDQ
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MyHC2X (*MYH1*)

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Mouse (Mus musculus)         ELEASQKESRSLSTELFKIKNAYEESLDH
Rat (Rattus norvegicus)      ELEASQKESRSLSTELFKIKNAYEESLDQ
Bovine (Bos taurus)          ELEASQKESRSLSTELFKIKNAYEESLDQ
Monkey (Macaca mulatta)      ELEASQKESRSLSTELFKIKNAYEESLDQ
Human (Homo sapiens)         ELEASQKESRSLSTELFKIKNAYEESLDQ
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MyHC2B (*MYH4*)

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Mouse (Mus musculus)         ELEASQKESRSLSTELFKVKNAYEESLDQ
Rat (Rattus norvegicus)      ELEASQKESRSLSTELFKVKNAYEESLDQ
Bovine (Bos taurus)          DLEASQKESRSLSTELFKVKNAYEESLDQ
Monkey (Macaca mulatta)      ELEASQKESRSLSTELFKVKNAYEESLDH
Human (Homo sapiens)         ELEASQKESRSLSTELFKVKNAYEESLDH
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Fig. 2. Murine amino acid sequences of synthetic peptides used for the production of anti-MyHC antibodies (Sawano et al., 2016), and inter-species comparison of those amino acid sequences. The key amino acid residues to decide possible epitope site are highlighted in green. The amino acid sequence used as antigen peptides of murine MyHC2A differs from human MyHC2A (methionine into isoleucine) highlighted in red, which is similar to MyHC2X predicted epitope sites. Since little information is available on public databases regarding the Japanese monkey (*Macaca fuscata*), the amino acid sequence of the rhesus macaque (*Macaca mulatta*) is shown instead.

mAbs. Peptide antibodies were first described in the 1980s, shortly after Köhler and Milstein discovered hybridoma technology (Köhler and Milstein, 1975). The purpose of using synthetic peptides is to reveal antigen-antibody recognition sites (epitopes) to specific regions of the native protein. The use of synthetic peptides to produce mAbs has an advantage in terms of a relatively simple and inexpensive peptide production process and in increasing the specificity of the mAb. By using peptides as immunogens, it became possible to determine which epitopes of the antigen would be recognized and targeted by the host's immune system during the antibody generation phase. Subsequently, the use of these peptides allowed us to design screening techniques for antibody libraries in a cost-effective manner.

Sawano et al. used synthetic peptides to produce anti-MyHC antibodies. They prepared a 29-amino acid sequence peptide, whose sequence is identical to the tail domain of the MyHC protein that exhibits high levels of amino acid sequence homology among the four adult MyHC isoforms, except for one amino acid (Fig. 2). Although it is generally preferable to select a sequence that has as many different amino acids as possible, they chose to utilize a sequence that was similar among the proteins to ensure that each antibody exhibited similar levels of accessibility to intracellular MyHC. This peptide is based on a mouse amino acid sequence, but we examined the sequences of other animal species (Fig. 2). In rats, the amino acid sequence of this region was identical to that of mice, resulting in the same staining pattern as that observed in mice. In cattle and monkeys (Japanese macaque), the amino acid sequence of the presumed epitope site was the same, and the staining results were acceptable. The results of multiple immunostaining with antibodies from Sawano et al. in transverse sections of a region called the "outside round" in bovine showed the presence of type 1 (white), type 2A (blue), and type 2X (green) fibers (Fig. 3A). Moreover, type 1 (white), type 2A (blue), and type 2X (green) fibers were present in the gastrocnemius muscle of a monkey (Fig. 3B). However, the amino acid sequence used as antigen peptides of murine MyHC2A differs from human MyHC2A (methionine into isoleucine), which is similar to a predicted epitope site of human MyHC2X. In other words, the anti-MyHC2A antibody (8F72C8) produced by Sawano et al. was not expected to distinguish between human MyHC2A and MyHC2X. In fact, it was reported that MyHC2X and MyHC2A could not be distinguished by staining (Nakashima et al., 2020). In the current study, no MyHC2B-positive (red) fibers were detected in bovine, monkey, and human muscle sections with the anti-MyHC2B (2G72F10) antibody. This is consistent with reports that MyHC2B expression is not common in large mammals (Smerdu et al., 1994; Tanabe et al., 1998; Toniolo et al., 2005).
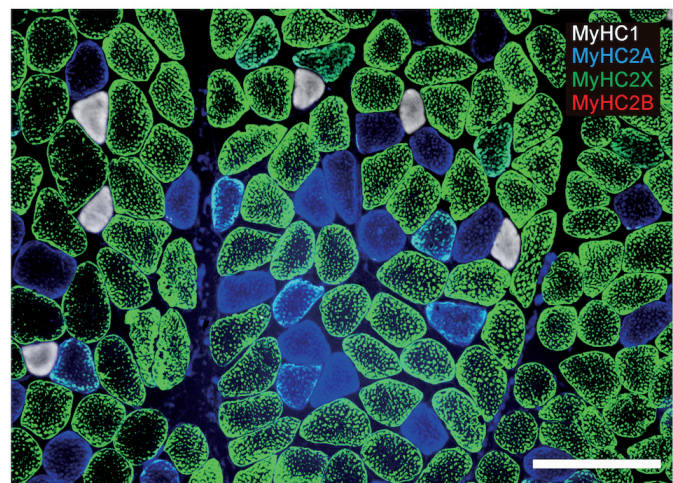
Concluding remarks

In summary, the history of muscle fiber type classification is also a history of muscle section staining.

a cattle leg muscle (outside round)
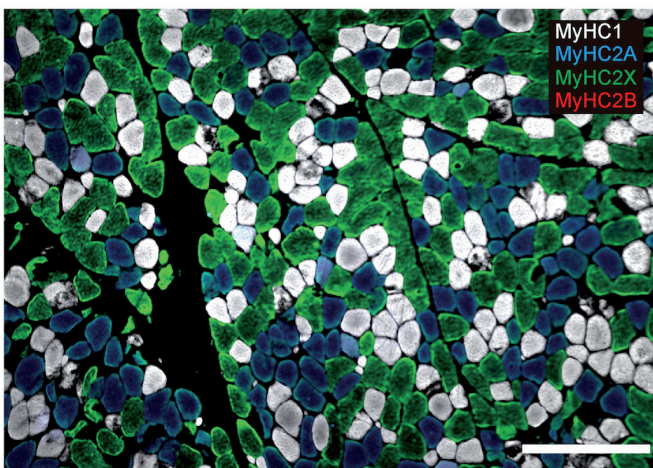
b monkey gastrocnemius muscle

Fig. 3. Cattle and monkey cross-sections stained using the one-step quadruple immunostaining method. **a.** A cross-section was obtained from a commercial "outside round" portion from bovine meat, and immunopositive myosin heavy chain (MyHC) isoforms were visually classified as MyHC1 (white), MyHC2A (blue), and MyHC2X (green). **b.** A cross-section was obtained from a monkey's (Macaca fuscata) gastrocnemius muscle, and immunopositive MyHC isoforms were visually classified as MyHC1 (white), MyHC2A (blue), and MyHC2X (green). Note that no MyHC2B-positive fibers were found in the shown cattle and monkey muscles. Scale bars: 250 μm.

The history of immunostaining for MyHC is also consistent with the history of antibody development, considering the shift from polyclonal to mAbs with high specificity and the shift from purified proteins to synthetic peptides as antigens. Tissue staining for myofiber type identification will continue to be used as an important tool for mammalian muscle fiber type identification, and it will also contribute greatly to the elucidation of molecular regulatory signaling pathways of muscle fiber types, muscle diseases, and meat quality improvement.

References

Bader D., Masaki T. and Fischman D.A. (1982) Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. J. Cell Biol. 95, 763-770.

Bandman E. and Rosser B.W. (2000). Evolutionary significance of myosin heavy chain heterogeneity in birds. Microsc. Res. Tech. 50, 473-491.

Bär A. and Pette D. (1988). Three fast myosin heavy chains in adult rat skeletal muscle. FEBS Lett. 235, 153-155.

Barany M. (1967). ATPase activity of myosin correlated with speed of muscle shortening. J. Gen. Physiol. 50, 197-218.

Barnard E.A., Lyles J.M. and Pizzey J.A. (1982). Fibre types in chicken skeletal muscles and their changes in muscular dystrophy. J. Physiol. 331, 333-354.

Bloemberg D. and Quadrilatero J. (2012). Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. PLoS One 7, e35273.

Bottinelli R. and Reggiani C. (2000). Human skeletal muscle fibres: molecular and functional diversity. Prog. Biophys. Mol. Biol. 73, 195-262.

Brooke M.H. and Kaiser K.K. (1970a). Three "myosin adenosine triphosphatase" systems: the nature of their ph lability and sulfhydryl dependence. J. Histochem. Cytochem. 18, 670-672.

Brooke M.H. and Kaiser K.K. (1970b). Muscle fiber types: How many and what kind? Arch. Neurol. 23, 369-379.

Charles-Schoeman C. and Verity M.A. (2012). Nicotinamide adenine dinucleotide tetrazolium reductase identifies microvasculature activation in muscle from adult patients with dermatomyositis. J. Rheumatol. 39, 94-99.

Cohen-Haguenauer O., Barton P.J., Nguyen V.C., Serero S., Gross M.S., Jegou-Foubert C., de Tand M.F., Robert B., Buckingham M. and Frézal J. (1988). Assignment of the human fast skeletal muscle myosin alkali light chains gene (MLC1F/MLC3F) to 2q 32.1-2qter. Hum. Genet. 78, 65-70.

D'Amico A. and Bertini E. (2008). Congenital myopathies. Curr. Neurol. Neurosci. Rep. 8, 73-79.

DeNardi C., Ausoni S., Moretti P., Gorza L., Velleca M., Buckingham M. and Schiaffino S. (1993). Type 2X-myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene. J. Cell Biol. 123, 823-835.

Doriguzzi C., Mongini T., Palmucci L. and Schiffer D. (1983). A new method for myofibrillar Ca++-ATPase reaction based on the use of metachromatic dyes: Its advantages in muscle fibre typing. Histochemistry 79, 289-294.

Draber P., Draberova E. and Novakova M. (1995). Stability of monoclonal igm antibodies freeze-dried in the presence of trehalose. J. Immunol. Methods 181, 37-43.

Draeger A., Weeds A.G. and Fitzsimons R.B. (1987). Primary, secondary and tertiary myotubes in developing skeletal muscle: a new approach to the analysis of human myogenesis. J. Neurol. Sci. 81, 19-43.

Dubowitz V. and Pearse A.E. (1960). A comparative histochemical study of oxidative enzyme and phosphorylase activity in skeletal muscle. Zeitschrift für Zellforschung und mikroskopische Anatomie Abt. Histochem. 2, 105-117.

Engel W.K. (1962). The essentiality of histo- and cytochemical studies of skeletal muscle in the investigation of neuromuscular disease. Neurology 12, 778-784.

Engel W.K. (2015). Diagnostic histochemistry and clinical-pathological testings as molecular pathways to pathogenesis and treatment of the ageing neuromuscular system: A personal view. Biochim. Biophys. Acta 1852, 563-584.

Gagnière H., Picard B. and Geay Y. (1999). Contractile differentiation of foetal cattle muscles: Intermuscular variability. Reprod. Nutr. Dev. 39, 637-655.

Galpin A.J., Raue U., Jemiolo B., Trappe T.A., Harber M.P., Minchev K. and Trappe S. (2012). Human skeletal muscle fiber type specific protein content. Anal. Biochem. 425, 175-182.

Goding J.W. (1986). Monoclonal antibodies: Principles and practice. Academic Press. London.

Gotoh T. (2003). Histochemical properties of skeletal muscles in japanese cattle and their meat production ability. Anim. Sci. J. 74, 339-354.

Gouspillou G., Sgarioto N., Norris B., Barbat-Artigas S., Aubertin-Leheudre M., Morais J.A., Burelle Y., Taivassalo T. and Hepple R.T. (2014). The relationship between muscle fiber type-specific PGC-1α content and mitochondrial content varies between rodent models and humans. PLoS One 9, e103044.

Gregorevic P., Meznarich N.A., Blankinship M.J., Crawford R.W. and Chamberlain J.S. (2008). Fluorophore-labeled myosin-specific antibodies simplify muscle-fiber phenotyping. Muscle Nerve 37, 104-106.

Grossman E.J., Roy R.R., Talmadge R.J., Zhong H. and Edgerton V.R. (1998). Effects of inactivity on myosin heavy chain composition and size of rat soleus fibers. Muscle Nerve 21, 375-389.

Gunn H.M. (1972). Histochemical observations on laryngeal skeletal muscle fibres in 'normal' horses. Equine Vet. J. 4, 144-148.

Harridge S.D.R., Bottinelli R., Canepari M., Pellegrino M.A., Reggiani C., Esbjörnsson M. and Saltin, B. (1996). Whole-muscle and single-fibre contractile properties and myosin heavy chain isoforms in humans. Pflügers Archiv. 432, 913-920.

Hintz C., Coyle E., Kaiser K., Chi M. and Lowry O. (1984). Comparison of muscle fiber typing by quantitative enzyme assays and by myosin ATPase staining. J. Histochem. Cytochem. 32, 655-660.

Hughes S.M. and Blau H.M. (1992). Muscle fiber pattern is independent

of cell lineage in postnatal rodent development. Cell 68, 659-671.

Köhler G. and Milstein C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-497.

Lefaucheur L. (2010). A second look into fibre typing--relation to meat quality. Meat Sci. 84, 257-270.

Lin J., Wu H., Tarr P.T., Zhang C.Y., Wu Z., Boss O., Michael L.F., Puigserver P., Isotani E., Olson E.N., Lowell B.B., Bassel-Duby R. and Spiegelman B.M. (2002). Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 418, 797-801.

Lucas C.A. and Hoh J. (1997). Extraocular fast myosin heavy chain expression in the levator palpebrae and retractor bulbi muscles. Invest. Ophthalmol. Vis. Sci. 38, 2817-2825.

Lucas C.A., Kang L.H. and Hoh J.F. (2000). Monospecific antibodies against the three mammalian fast limb myosin heavy chains. Biochem. Biophys. Res. Commun. 272, 303-308.

Lutz G.J., Bremner S.N., Bade M.J. and Lieber R.L. (2001). Identification of myosin light chains in *Rana pipiens* skeletal muscle and their expression patterns along single fibres. J. Exp. Biol. 204, 4237-4248.

Medler S., Lilley T. and Mykles D.L. (2004). Fiber polymorphism in skeletal muscles of the american lobster, *homarus americanus*: continuum between slow-twitch (S1) and slow-tonic (S2) fibers. J. Exp. Biol. 207, 2755-2767.

Murphy C.T. and Spudich J.A. (1998). Dictyostelium myosin 25-50k loop substitutions specifically affect adp release rates. Biochemistry (Mosc). 37, 6738-6744.

Nakashima A., Yamada T., Sugiyama G., Mizunoya W., Nakano H., Yasuda K., Takahashi I. and Mori Y. (2020). Masseter muscle properties differ between the left and right sides in mandibular class III patients with asymmetry. J. Hard Tissue Biol. 29, 25-30.

Nemeth P., Pette D. and Vrbova G. (1981). Comparison of enzyme activities among single muscle fibres within defined motor units. J. Physiol. 311, 489-495.

Ogilvie R.W. and Feeback D.L. (1990). A metachromatic dye-ATPase method for the simultaneous identification of skeletal muscle fiber types I, IIA, IIB and IIC. Stain Technol. 65, 231-241.

Padykula H.A. and Gauthier G.F. (1967). Morphological and cytochemical characteristics of fiber types in normal mammalian skeletal muscle. In: Exploratory concepts in muscular dystrophy and related disorders. Excerpta Medica Foundation Amsterdam. pp 117-128.

Paoli A., Pacelli Q.F., Cancellara P., Toniolo L., Moro T., Canato M., Miotti D. and Reggiani C. (2013). Myosin isoforms and contractile properties of single fibers of human latissimus dorsi muscle. Biomed. Res. Int. 2013, 249398.

Perry M.J., Tait J., Hu J., White S.C. and Medler S. (2009). Skeletal muscle fiber types in the ghost crab, ocypode quadrata: implications for running performance. J. Exp. Biol. 212, 673-683.

Pette D. and Spamer C. (1986). Metabolie properties of muscle fibers. Fed Proc. 45, 2910-2914.

Pette D., Wimmer M. and Nemeth P. (1980). Do enzyme activities vary along muscle fibres? Histochemistry 67, 225-231.

Picard B. and Gagaoua M. (2020). Muscle fiber properties in cattle and their relationships with meat qualities: an overview. J. Agric. Food Chem. 68, 6021-6039.

Pierobon-Bormioli S., Sartore S., Libera L.a., Vitadello M. and Schiaffino S. (1981). "Fast" isomyosins and fiber types in mammalian skeletal muscle. J. Histochem. Cytochem. 29, 1179-1188.

Rayment I. (1996). The structural basis of the myosin atpase activity. J.

Biol. Chem. 271, 15850-15853.

Rehfeldt C., Henning M. and Fiedler I. (2008). Consequences of pig domestication for skeletal muscle growth and cellularity. Livest. Sci. 116, 30-41.

Reichmann H. and Pette D. (1982). A comparative microphotometric study of succinate dehydrogenase activity levels in type I, IIA and IIB fibres of mammalian and human muscles. Histochemistry 74, 27-41.

Ribaric S. and Cebasek V. (2013). Simultaneous visualization of myosin heavy chain isoforms in single muscle sections. Cells Tissues Organs 197, 312-321.

Rivero J.L.L., Talmadge R.J. and Edgerton V.R. (1996). Myosin heavy chain isoforms in adult equine skeletal muscle: An immunohistochemical and electrophoretic study. Anat. Rec. 246, 185-194.

Rivero J.L., Talmadge R.J. and Edgerton V.R. (1998). Fibre size and metabolic properties of myosin heavy chain-based fibre types in rat skeletal muscle. J. Muscle Res. Cell Motil. 19, 733-742.

Rivero J.L., Talmadge R.J. and Edgerton V.R. (1999). Interrelationships of myofibrillar ATPase activity and metabolic properties of myosin heavy chain-based fibre types in rat skeletal muscle. Histochem. Cell Biol. 111, 277-287.

Rutter J., Winge D.R. and Schiffman J.D. (2010). Succinate dehydrogenase–assembly, regulation and role in human disease. Mitochondrion 10, 393-401.

Sawano S., Komiya Y., Ichitsubo R., Ohkawa Y., Nakamura M., Tatsumi R., Ikeuchi Y. and Mizunoya W. (2016). A one-step immunostaining method to visualize rodent muscle fiber type within a single specimen. PLoS One 11, e0166080.

Schiaffino S. and Reggiani C. (2011). Fiber types in mammalian skeletal muscles. Physiol. Rev. 91, 1447-1531.

Schiaffino S., Gorza L., Sartore S., Saggin L., Ausoni S., Vianello M., Gundersen K. and Lomo T. (1989). Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J. Muscle Res. Cell Motil. 10, 197-205.

Schuler M., Ali F., Chambon C., Duteil D., Bornert J.M., Tardivel A., Desvergne B., Wahli W., Chambon P. and Metzger D. (2006). PGC1alpha expression is controlled in skeletal muscles by PPARbeta, whose ablation results in fiber-type switching, obesity, and type 2 diabetes. Cell Metab. 4, 407-414.

Simoneau J.A. and Bouchard C. (1995). Genetic determinism of fiber type proportion in human skeletal muscle. FASEB J. 9, 1091-1095.

Smerdu V., Karsch-Mizrachi I., Campione M., Leinwand L. and Schiaffino S. (1994). Type IIX myosin heavy chain transcripts are expressed in type IIB fibers of human skeletal muscle. Am. J. Physiol. 267, C1723-1728.

Sosnicki A. and Cassens R.G. (1988). Determination of fiber types of chicken skeletal muscles based on reaction for actomyosin, calcium+2, magnesium+2-dependent adenosine triphosphatase. Poult. Sci. 67, 973-978.

Staron R.S. and Pette D. (1993). The continuum of pure and hybrid myosin heavy chain-based fibre types in rat skeletal muscle. Histochemistry 100, 149-153.

Stephenson G.M. (2001). Hybrid skeletal muscle fibres: A rare or common phenomenon? Clin. Exp. Pharmacol. Physiol. 28, 692-702.

Suzuki A. and Tamate H. (1988). Distribution of myofiber types in the hip and thigh musculature of sheep. Anat. Rec. 221, 494-502.

Suzuki A. and Hayama S. (1994). Individual variation in myofiber type composition in the triceps surae and flexor digitorum superficialis muscles of Japanese macaques. Anthropol. Sci. 102, 127-138.

Talmadge R.J., Grossman E.J. and Roy R.R. (1996). Myosin heavy chain composition of adult feline (*Felis catus*) limb and diaphragm muscles. J. Exp. Zool. 275, 413-420.

Tanabe R., Muroya S. and Chikuni K. (1998). Sequencing of the 2A, 2X, and slow isoforms of the bovine myosin heavy chain and the different expression among muscles. Mamm. Genome 9, 1056-1058.

Toniolo L., Maccatrozzo L., Patruno M., Caliaro F., Mascarello F. and Reggiani C. (2005). Expression of eight distinct MHC isoforms in bovine striated muscles: evidence for MHC-2B presence only in extraocular muscles. J. Exp. Biol. 208, 4243-4253.

Uyeda T.Q., Ruppel K.M. and Spudich J.A. (1994). Enzymatic activities correlate with chimaeric substitutions at the actin-binding face of myosin. Nature 368, 567-569.

Wang Y.X., Zhang C.L., Yu R.T., Cho H.K., Nelson M.C., Bayuga-Ocampo C.R., Ham J., Kang H. and Evans R.M. (2004). Regulation of muscle fiber type and running endurance by PPARdelta. PLoS Biol. 2, e294.

Yang S., Lu D., Li S. and Chen G. (1990). Histochemical identification of skeletal muscle fiber type-skeletal muscle in normal men and rats. Acta Histochem. Cytochem. 23, 825-830.

Zierath J.R. and Hawley J.A. (2004). Skeletal muscle fiber type: influence on contractile and metabolic properties. PLoS Biol. 2, e348.