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Abstract

We developed a staining protocol that enables simultaneous visualization of myosin heavy chain (MHC) pure and hybrid muscle fiber types in rat skeletal muscle. Up to eight different muscle fiber types can be visualized in a single section of the rat extensor digitorum longus muscle, which contains all four adult MHC isoforms and shows plasticity during the denervation-reinnervation process. Triple immunofluorescent staining of MHC-1, MHC-2a and MHC-2b with primary antibodies BA-D5 (isotype IgG2b), SC-71 (isotype IgG1) and BF-F3 (isotype IgM) and with three fluorophore-labeled isotype-specific secondary antibodies displays different muscle fiber types in a merged image of red, green and blue channel, each in its own color. Immunoperoxidase staining with primary antibody 6H1 directed against MHC-2x can be additionally applied on the same tissue section to facilitate the identification of muscle fibers containing MHC-2x. Triple staining can also be used in combination with other staining procedures to derive more information about the number of capillaries or the oxidative potential of muscle fiber types. Simultaneous visualization of multiple fiber types in a single merged image enables economical use of muscle samples and provides simple and rapid identification of all fiber types that are present in rat limb muscles.

Body**Introduction**

The rat extensor digitorum longus muscle (EDL) contains a mixture of all adult limb myosin heavy chain (MHC) types and is commonly used in physiological investigations of

fast-twitch muscle plasticity. Most studies have relied upon analysis of muscle fiber types, whose different contractile and metabolic features determine muscle work [Caiozzo, 2002; Caiozzo et al., 2003]. Morphological analyses of histochemically differently stained muscle fiber types are essential procedures for the estimation of skeletal muscle properties and for identification of early changes in muscles undergoing phenotype transformation.

Since the maximum shortening velocity of single muscle fibers is correlated with MHC isoform composition [Reiser et al., 1985; Bottinelli et al., 1994], immunohistochemical staining for MHCs has proven to be a reliable method for identifying slow and fast muscle fiber types [Schiaffino et al., 1989; Lucas et al., 2000].

The type and quantity of MHC isoforms expressed in muscle cells can be altered in response to various extrinsic or intrinsic perturbations [Roy et al., 1991; Pette and Vrbová, 1992; Härmäläinen and Pette, 2001; Pette, 2002; Asmussen et al., 2003]. Thus, MHC isoforms are used as markers for tracking phenotypic modulations of fiber types [Schiaffino and Reggiani, 1996; Schiaffino, 2010; Schiaffino and Reggiani, 2011].

All four MHC isoforms present in adult mammalian limb muscles are expressed in the EDL muscle: one slow MHC isoform type 1 (also known as β -cardiac) and three fast MHC isoforms, types 2a, 2x and 2b [Bär and Pette, 1988; Schiaffino et al., 1989; Termin et al., 1989; Schuenke et al., 2009] either in MHC pure or MHC hybrid muscle fiber types [Staron and Pette, 1993; Bortolotto et al., 2000; Caiozzo et al., 2003; Cebasek et al., 2007]. However, current morphometrical analyses of MHC hybrid muscle fiber types, i.e. measurement of muscle fiber type cross-sectional areas and assessment of areal and numerical percentage, are very demanding and time consuming. The same fiber must be recognized in multiple cross-sections, where binding of each of the specific primary antibody against the specific MHC isoform is visualized by the same (standard)

immunoenzyme technique using horseradish peroxidase (HRP) and 3,3-diaminobenzidine tetrachloride (DAB) chromogen [Gorza, 1990].

Multiple immunostaining techniques are well established in tissue research and are frequently used to visualize the spatial relationship of different cellular molecules or to study the organization of complex tissue structures [Nakane, 1968; Campbell and Bhatnagar, 1976; Brouns et al., 2002].

Various methods have been developed for simultaneous detection of multiple antigens in a single muscle or in a non-muscle tissue section, using either indirect [Pierobon-Bormioli et al., 1980; Wessel and McClay, 1986] or direct [Ferri et al., 1997; Tsurui et al., 2000] immune techniques.

Direct immunostaining methods, where different primary antibodies are directly labeled either by different enzymes or by different fluorescent dyes, are among the simplest ways to achieve multicolor staining, especially if primary antibodies are commercially available in conjugated form.

Primary antibodies directed against MHC isoforms are almost all raised in a mouse as host species, and therefore, almost all are mouse monoclonal antibodies. Usage of species-specific secondary antibodies is not possible, and the risk for cross-reactivity reactions with multiple staining is very high. Various techniques have been developed in the past to overcome this problem: (1) a high dilution of the first primary antibody in combination with tyramide signal amplification step; (2) heat or formaldehyde vapor treatments for selective destruction of the antigen-combining sites of the second layer of antibodies [Wang and Larsson, 1985; Wang et al., 1999]; (3) the use of fluorophore-conjugated secondary monovalent Fab antibody that prevents access of the third secondary antibody to the second primary antibody [Brouns et al., 2002], or (4) the use of different subclasses or isotype-specific secondary antibodies if different isotypes

or different subclasses of primary antibodies are available [Tidman et al., 1981]. We have tried several of the proposed methods, but the last method, where subclass or isotype-specific secondary antibodies were employed for visualization of different primary mouse monoclonal anti-MHC antibodies, proved to be the most simple, reliable and effective.

In this paper, we introduce a multicolor staining technique for the identification of different muscle fiber types from a single tissue cross-section. Staining and acquisition of images are described in detail. Identification of the muscle fiber types was verified on a series of successive tissue cross-sections conventionally stained with the immunoperoxidase staining technique. The proposed triple fluorescent staining clearly reveals all pure and hybrid fiber types of rat limb muscles and allows for additional staining combinations with enzyme, immunoenzyme or lectin histochemical techniques.

Materials and Methods

Muscle Samples

EDL muscles were dissected from three groups of Wistar rats (230 ± 20 g) after the animals were killed by cervical dislocation. Samples were collected from control animals (sham operated), denervated animals (the sciatic nerve was cut) and reinnervated animals (the sciatic nerve was crushed for 1 min with a serrated hemostat) on day 14 after the operation (control and denervated groups) and on day 28 after nerve lesion (reinnervated group). The denervation and crush experiments were performed under aseptic conditions. Details of the procedure are explained in our previous study [Cebasek et al., 2007]. Complete EDL muscles were frozen in liquid nitrogen at near physiological length and stored at -80°C for later analysis. Frozen muscles were cut with a razor blade through the middle of the muscle belly, embedded in Tissue-Tech Optimal

Cutting Temperature compound (Sakura Finetek Europe, Zoeterwonde, NL) and cut in a cryostat (Reichert-Jung Frigocut 2800) at -20°C into 5- or 10- μm -thick cross-sections. Cryosections were placed on superfrost glass slides and stored at -80°C until assayed. When used, frozen tissue sections were first air dried (for 15 min) and then washed in phosphate-buffered saline (PBS). This investigation was approved by The Veterinary Administration of the Ministry for Agriculture, Forestry and Food, Republic of Slovenia (permit 326-07-285/99).

Single Immunoperoxidase Staining of MHCs on Multiple Tissue Cross-Sections

Four serial cryosections, 10 μm thick, were used for single immunoperoxidase staining of MHC protein isoforms to reveal the presence of type 1, 2a, 2b [Schiaffino et al., 1989] or 2x [Lucas et al., 2000] in muscle fibers of normal and experimental EDL muscles. Monoclonal mouse antibodies BA-D5, SC-71, BF-F3 and 6H1 were used. The first three antibodies were produced in a local laboratory from corresponding hybridoma cell lines provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The antibody 6H1 was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa, USA) (table 1). Each antibody was applied separately on tissue sections. Control and experimental tissue cross-sections were always placed on the same glass slide. All antibodies were visualized by indirect immunoperoxidase staining technique using DAB as chromogen [Gorza, 1990]. Briefly, cryosections were air dried, washed with PBS and blocked with normal rabbit serum (Dako, Glostrup, Denmark) diluted in PBS (1:40) for 30 min at room temperature. After washing with saline, they were incubated with primary antibodies for 1 h at room temperature at the following dilutions: BA-D5 (1:100), SC-71 (1:100), BF-F3 (1:20) and antibody 6H1 (1:20). After washing, all tissue sections were incubated with

secondary HRP-conjugated rabbit anti-mouse antibodies IgG, IgM, IgA (1:100; Dako), except for tissue sections labeled with antibody 6H1, where HRP-conjugated goat anti-mouse antibody IgM (1:100; Millipore, Billerica, Mass., USA) was used for better staining result. All primary and secondary antibodies were diluted in PBS and washed before each step of the staining procedure. The HRP activity was visualized with the DAB solution containing 8.8 mM H₂O₂ (Sigma Aldrich, St. Louis, Mo., USA). Finally, sections were dehydrated, cleared and mounted. Negative controls were processed in the same way, except that the primary antibodies were omitted.

Triple Immunofluorescent Staining of MHCs on a Single Tissue Cross-Section

Single cryosections, 5 µm thick, in series with the previously single-stained cross-sections, were processed for triple immunofluorescent staining. After thawing and washing in PBS, the following mouse monoclonal antibodies were used as the primary antibodies: BA-D5 (isotype IgG2b), SC-71 (isotype IgG1) and BF-F3 (isotype IgM), all from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. As the linking secondary antibodies, fluorochrome-conjugated goat anti-mouse IgG2b isotype-specific antibodies (Alexa Fluor 350 -- A21140), fluorochrome-conjugated goat anti-mouse IgG1 isotype-specific antibodies (Alexa Fluor 546 -- A21123) and fluorochrome-conjugated goat anti-mouse IgM isotype-specific antibodies (Alexa Fluor 488 -- A21042) were used, all purchased from Invitrogen, Carlsbad, Calif., USA (table 2). All primary and secondary antibodies were diluted in PBS and washed before each step of the staining procedure. Nonspecific binding of the antibodies was blocked in normal goat serum (1:5) for 30 min before the first antibody incubation.

Initially, the immunofluorescent staining of MHCs was performed in three steps: (1) the first primary antibody BF-F3 (1:20) was applied over night at 4°C. After washing, the

first linking secondary antibody Alexa Fluor 488 (1:350) was applied for 1 h to visualize MHC-2b in green. (2) The second primary antibody SC-71 (1:100) was applied for 1 h at room temperature. After a thorough wash, the second linking secondary antibody Alexa Fluor 546 (1:350) was applied for 1 h to visualize MHC-2a in red. (3) The third primary antibody BA-D5 (1:100) was applied for 1 h at room temperature. After a thorough wash, the third linking secondary antibody Alexa Fluor 350 (1:350) was applied for 1 h at room temperature to visualize MHC-1 in blue.

This three-step staining protocol was replaced with a two-step staining protocol, where a cocktail of all three primary antibodies was applied to a muscle sample for 1 h (the same concentrations were used as in three-step staining). After thorough washing, a cocktail of all three fluorochrome-conjugated secondary antibodies was applied to a muscle sample for 30 min (the same concentrations were used as in three-step staining). The results were similar for both protocols (data not shown).

Negative controls were processed in the same way, except that the primary antibodies were omitted. A series of single-stained tissue sections, where MHC isoforms were visualized with immunoperoxidase technique, were used as a positive control.

Triple Immunofluorescent Staining of MHCs with Overlaid Immunoenzyme Staining of MHC Isoform 2x

A single tissue section was labeled with the monoclonal antibody 6H1 (dilution 1:20) directed to MHC-2x and visualized with the DAB immunoperoxidase staining technique (NovoLink Polymer Detection System). Subsequently, the triple immunofluorescent staining cocktail was applied on the same tissue section.

Triple Immunofluorescent Staining of MHCs with Overlaid Succinate Dehydrogenase Staining

A single tissue section was stained for succinate dehydrogenase (SDH) [Reichmann and Pette, 1982]. Subsequently, the triple immunofluorescent staining cocktail was applied on the same tissue section.

Triple Immunofluorescent Staining of MHCs with Overlaid Lectinfluorescent Staining of Capillaries

A single tissue section was fixed in cold acetone (--20°C) for 10 min. After washing in PBS, a cocktail of fluorescein (FL-1101, dilution 1:300) and rhodamine (RL-1102, dilution 1:300) labeled Griffonia simplicifolia lectin I (Vector Laboratories, Burlingame, Calif., USA) was applied for 2 h. Subsequently, the triple immunofluorescent staining cocktail was applied on the same tissue section.

Single Immunofluorescent Staining of MHC Isoform 2x

A single tissue section (successive to a cross-section where MHC-2x was visualized with immunoperoxidase staining technique) was labeled with the primary antibody 6H1 (directed against MHC-2x, dilution 1:20) and visualized with the fluorochrome-conjugated goat anti-mouse IgM isotype-specific antibodies (dilution 1:350, Alexa Fluor 488 -- A21042; Invitrogen).

Image Acquisition and Processing

Specimens were observed with the Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan) equipped with the digital camera DXM1200F (Nikon Corporation). Fluorescence was excited with a super-high-pressure mercury lamp, and the excitation and emission signals were filtered using specific Nikon optical filter blocks: G-2A (the green excitation optical filter combination), B-2A (the blue excitation filter combination) and UV-2A (the ultraviolet excitation filter combination). The optimal

exposure time was 167 ms for all three channels. Single-channel images, captured independently by separate optical filter blocks, were acquired with the Nikon objective Plan-Fluor Ph1, 20x (using dark light), and stored as a single RGB-TIFF image (resolution 1,232 × 972 pixels) of merged red (R), green (G) and blue (B) channels (RGB; fig. 1a--d).

On the same field of view, where the fluorescence RGB image was captured (fig. 2a, d, g), transmission (white light) microscopy was applied to show either muscle fiber outlines (in specimens without additional staining; fig. 2b), oxidative muscle fibers (in specimens combined with SDH staining; fig. 2e) or the muscle fibers expressing the 2x isoform (in specimens combined with positively stained 2x fibers; fig. 2h).

The RGB (dark light) and the greyscale (white light) images were flattened and saved as one image (fig. 2c, f, i). If necessary, contrast and opacity adjustments were applied to the entire image.

To improve visualization of muscle fibers 2x on the immunoperoxidase (DAB)-stained tissue section (fig. 2j), immunofluorescent staining was applied on a successive tissue section (fig. 2k) and the image was inverted (fig. 2l).

Triple staining of MHC isoforms can be additionally combined with double staining of capillaries using fluorescein and rhodamine-labeled lectin (fig. 3).

White light microscopy was applied on a series of successive tissue sections where DAB immunoperoxidase staining showed only one type of an MHC isoform in a single section (fig. 4).

LUCIA G/F software program (version 4.80; Laboratory Imaging, Praha, Czech Republic) and the commercial image analysis programs Ellipse (ViDiTo, Košice, Slovakia) or Adobe Photoshop (version 7.0; USA) were used for image acquisition, browsing or color and opacity adjustments.

Results

The simultaneous immunofluorescent staining of the three MHC isoforms identified all muscle fiber types of control and experimental rat EDL muscles. In the RGB fluorescent image, pure type 1, 2a and 2b fibers are clearly visualized as vivid blue, red and green fibers, respectively, and pure type 2x fibers are unstained (black fibers) (fig. 1d, 4a--c). Hybrid fibers are more numerous in the experimental muscles and are visible in different color shades (fig. 4a--c). The accuracy of the fiber type classification from triple-stained single sections was verified by the staining intensity of defined muscle fiber types on immunoperoxidase-stained tissue sections (fig. 4a--o). The color shade for each specific muscle fiber type was the same in all samples (table 3).

Combining SDH and triple fluorescent staining significantly reduces the fluorescence luminosity but may still be useful to determine the oxidative potential of muscle fiber types (fig. 2d--f). Combined immunoperoxidase staining of MHC-2x with the triple fluorescent staining provides more visual information about the fibers expressing MHC-2x (fig. 2g--i). Combined staining of capillaries by fluorescein and rhodamine-labeled lectin with triple staining of MHC isoforms demonstrates the same number of capillaries in the composite (multiple stained), red and green single-channel images but not in the blue single-channel image (fig. 3a--d).

Discussion

There are several advantages of the triple fluorescent staining method over single peroxidase staining of MHC isoforms in muscle cells.

(1) The multicolor image of triple staining is very informative and provides quick identification of pure and hybrid muscle fiber types. In addition, the overall distribution of

all fiber types can be visualized simultaneously because each type has a recognizable color or color shade.

(2) The triple staining technique makes the sizing of specific fiber types easier and quicker than using a set of serial cross-sections, because all of the necessary morphometrical data can be obtained by segmentation of a single colored image. Therefore, quantitative measurements of fiber types are also less susceptible to variable staining and tissue shrinking conditions.

(3) The greyscale images derived from single red, green or blue channel images are of good contrast and are convenient for automatic image segmentation. By applying the threshold function, the area ratios of muscle fibers containing MHC type 1, 2a or 2b can be quickly assessed. Greyscale single-channel images can also facilitate the determination of fiber type.

(4) The muscle fiber outlines are visible in a fluorescently stained cross-section under transmission white light. Therefore, transmission white light images of muscle fiber outlines could also be used for automatic image segmentation, similarly to the method proposed by Hantaï and coworkers [1984], where images of fluorescently labeled muscle fiber borders were used for automatic analysis of muscle biopsies.

Identification of hybrid muscle fiber types, from multiple successive tissue cross-sections, is difficult, especially if the number of polymorphic fibers expressing two or more MHC isoforms is as high as in experimental muscles. The main advantages of our indirect immune staining method over the previously reported ones are as follows.

(1) More MHC fiber types, especially of the hybrid type, were visualized simultaneously in a single muscle tissue cross-section compared with previous reports [Raheem et al., 2009; Bloemberg and Quadrilatero, 2012]. Our method can identify

hybrid muscle fibers that simultaneously express 1/2a, 2a/2x, 2a/2x/2b or 2x/2b MHC isoforms [Andersen et al., 1999; Talmadge et al., 1999; Cebasek et al., 2007].

(2) Multiple staining of a single fiber cross-section can be performed with commercially available labeled antibodies [Gregorevic et al., 2008].

(3) Additional visual information can be derived if triple staining is combined with SDH staining (to assess the oxidative potential of the fiber) or double lectin staining (for counting the capillaries).

In summary, our triple fluorescent staining allows rapid determination of pure or hybrid MHC muscle fiber types in a single cross-section of rat EDL muscle. The technique should be applicable to all muscles in the rat hind limb and possibly to other species.

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Appendix after References (Editorial Comments)

Legend(s)

Fig. 1. Greyscale single-channel images (**a--c**) and merged red, green and blue channels in single RGB image (**d**) captured from triple-stained single section of reinnervated rat EDL. Scale bar = ■■.

Fig. 2. **a--c** RGB (dark light) image (**a**) and greyscale (white light) image (**b**) are overlaid and merged (**c**) to show better delineation of triple-stained single section of control EDL. **d--f** RGB (dark light) image (**d**) and greyscale (white light) image (**e**) of combined SDH and triple-stained single section of control EDL are overlaid and merged to show SDH positively stained (oxidative) fibers as overlaid blue fibers (**f**). **g--i** RGB (dark light) image (**g**) and greyscale (white light) image (**h**) of combined immunoperoxidase (for MHC-2x) and immunofluorescence (for MHC-1, MHC-2a and MHC-2b) stained single section of control EDL are overlaid and merged to show all four MHC isoforms: type 1 (blue), type 2a (red), type 2x (grey) and type 2b (green) (**i**). **j--l** Immunoperoxidase (**j**) and immunofluorescent (**k**) staining of MHC-2x performed in two successive tissue sections of reinnervated EDL using primary antibodies 6H1. Inverted fluorescent image improves visualization of muscle fibers expressing MHC-2x (**l**). Scale bars ■■ ■■.

Fig. 3. Multiple-stained single section of reinnervated EDL with combined double staining of capillaries (by fluorescein and rhodamine-labeled lectin) and triple staining of MHC isoforms. Capillaries and muscle fibers are simultaneously visible in an RGB

image **(a)**, red **(b)** and green **(c)** single-channel images, but not in the blue single-channel image **(d)**. Scale bar = ■■.

Fig. 4. **a--c** Triple-stained single cross-sections of control **(a)**, denervated **(b)** and reinnervated **(c)** rat EDL muscles. Primary antibodies BA-D5, SC-71 and BF-F3 and secondary isotype-specific antibodies IgG2b conjugated to Alexa Fluor 350, IgG1 antibodies conjugated to Alexa Fluor 546 and IgM antibodies conjugated to Alexa Fluor 488 were used to show MHC isoform type 1, 2a or 2b. Pure muscle fiber types are blue (type 1), red (type 2a) and green (type 2b), and unlabeled fibers are black (type 2x). Hybrid muscle fiber types express different MHC isoforms simultaneously and are visualized in violet (type 1/2a), dark red (type 2a/2x), red with green-yellow grains (type 2a/2x/2b) and black with green grains (type 2x/2b). The same primary antibodies that were used for triple fluorescent staining of a single tissue section were used also for conventional single immunoperoxidase staining performed on a series of successive tissue sections. Antibody BA-D5 was used to visualize fibers expressing MHC-1 (**d--f**), antibody SC-71 for MHC-2a (**g--i**) and antibody BF-F3 for MHC-2b (**m--o**). Antibody 6H1 is additionally used to visualize fibers expressing MHC-2x (**j--l**). Scale bar = 100 μ m.

Table(s)

Footnote(s)

Abbreviations used in this paper

DAB	3,3'-diaminobenzidine tetrachloride
EDL	extensor digitorum longus
HRP	horseradish peroxidase
MHC	myosin heavy chain
PBS	phosphate-buffered saline
RGB	merged red, green and blue channels
SDH	succinate dehydrogenase

Table 1. List of primary antibodies for MHC staining

Antibody name and type	Antigen species	Host species	Isotype	Specificity	Dilution	Supplier/reference
BA-D5 Monoclonal mouse anti-bovine	bovine	mouse	IgG2b	MHC-1	1:100	DSMZ/Schiaffino et al., 1989
SC-71 Monoclonal mouse anti-bovine	bovine	mouse	IgG1	MHC-2a	1:100	DSMZ/Schiaffino et al., 1989
6H1 Monoclonal mouse anti-rabbit	rabbit	mouse	IgM κ light chain	MHC-2x	1:20	DSHB/Lucas et al., 2000
BF-F3 Monoclonal mouse anti-bovine	bovine	mouse	IgM	MHC-2b	1:20	DSMZ/Schiaffino et al., 1989

DSHB = Developmental Studies Hybridoma Bank (University of Iowa); DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Table 2. List of secondary antibodies for MHC staining

Antibody name and type	Antigen species	Host species	Label or dye	Specificity	Dilution	Supplier
Polyclonal rabbit anti-mouse immunoglobulins/HRP Polyclonal rabbit anti-mouse	mouse	rabbit	HRP DAB	IgG, IgM, IgA	1:100	Dako (P0260)
Goat anti-mouse IgM (μ chain) HRP conjugate Polyclonal goat anti-mouse	mouse	goat	HRP DAB	IgM	1:100	Merck Millipore Chemicon (12-489)
NovoLink Polymer Detection System Polymer conjugated with anti-mouse and anti-rabbit antibody	mouse rabbit	–	HRP DAB	IgG	undiluted	Leica Microsystem Novocastra (RE 7150-CE)
Alexa Fluor 488 goat anti-mouse IgM (μ chain) Polyclonal goat anti-mouse	mouse	goat	Alexa Fluor 488	IgM	1:350	Invitrogen (A21042)
Alexa Fluor 546 goat anti-mouse IgG1 (y1) Polyclonal goat anti-mouse	mouse	goat	Alexa Fluor 546	IgG1	1:350	Invitrogen (A21123)
Alexa Fluor 350 goat anti-mouse IgG _{2b} (y2b) Polyclonal goat anti-mouse	mouse	goat	Alexa Fluor 350	IgG2b	1:350	Invitrogen (A21140)

Table 3. Correlation between the color shades of muscle fiber types visible in the fluorescent RGB image of the triple-stained tissue section and the staining intensity of muscle fiber types visible in the white light images of immunoperoxidase single-stained tissue sections

	Muscle fiber type color in RGB image							
	1 blue	2a red	2x black	2b green	1/2a violet	2a/2x dark red	2a/2x/2b red green yellow	2x/2b black green
BA-D5	++	–	–	–	++/+	–	–	–
SC-71	–	++	–	–	++/+	++/+	+	–
6H1	–	–	++/+	–	–	++/+	–	++/+
BF-F3	–	–	–	++	+	–	++/+	+

Symbols for staining intensity of muscle fiber types: ++ = a lot of antibodies (BA-D5, SC-71, 6H1 or BF-F3) are bound to the muscle fiber (dark brown-stained fibers); + = a few antibodies (BA-D5, SC-71, 6H1 or BF-F3) are bound to the muscle fiber (light brown-stained fibers); – = no antibodies are bound to the muscle fiber (unstained fibers).