

A novel triple immunoenzyme staining enables simultaneous identification of all muscle fiber types on a single skeletal muscle cryosection from normal, denervated or reinnervated rats

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Abstract

Triple immunofluorescence staining has recently been developed to simultaneously identify all muscle fibers on a single cryosection which is helpful for clinical and basic research, but it has disadvantages such as fast photobleaching and unclear outlines of muscle fibers. Triple immunoenzyme staining (TIE) is likely to avoid these disadvantages. In this study, we aimed to establish a sensitive and specific TIE technique to identify fiber types in normal, denervated, and reinnervated rat muscles, and to develop a systematic sampling method for muscle fiber quantification. Tibialis anterior and soleus from normal, denervated, and reinnervated Lewis rat hind limbs were used. Five consecutive cryosections were cut from each muscle, including one for TIE and four for single immunoenzyme staining (SIE). The TIE was performed using the polymerized reporter enzyme staining system for the first two antigens (A4.74 for MyHC-IIA, BA-F8 for MyHC-I) and alkaline phosphatase staining system for the third antigen (BF-F3 for MyHC-IIB), followed by corresponding detective systems and respective chromogens. The type of muscle fibers was quantified by systematic sampling at 12.5%, 25%, 33% and 50% of all muscle fibers, and was compared with that acquired from counting all the fibers (100%). All muscle fiber phenotypes, including pure and hybrid, could be simultaneously identified on a single TIE cryosection with clear outlines. The fiber types on TIE slides matched well with their respective counterpart on the consecutive SIE slides with a 95% match rate. Systematic sampling of 12.5% fibers could represent the true fiber type distribution of the entire muscle section. Our results suggest that novel TIE can effectively visualize fiber types in normal, denervated or reinnervated rat muscles.

Key Words: nerve regeneration; muscle fiber phenotyping; immunohistochemistry; triple immunoenzyme staining; myosin heavy chain; rats; neural regeneration

Introduction

Skeletal muscles of adult rats are composed of four types of pure muscle fibers including type I, IIA, IIB, IIX, and several types of hybrid muscle fibers such as type I/IIA, IIA/IIX and IIB/IIX (Rivero et al., 1998; Smerdu and Soukup, 2008; Tulloch et al., 2011; McMillan and Quadriatero, 2011). Each muscle fiber type is characterized by a specific myosin heavy chain isoform (MyHC-I, IIA, IIB, IIX, I/IIA, IIA/IIX and

IIB/IIX, respectively) (Lucas et al., 2000), contraction speed, metabolic and oxidative properties (Rivero et al., 1998). The fastest muscle fibers (type IIB) mainly locate at the superficial part while the slowest fibers (type I) mainly locate at the deep part of tibialis anterior (Armstrong and Phelps, 1984). The distribution and proportion of muscle fibers change extensively after exercise, disuse, denervation, and reinnervation (Michel et al., 1996; Bigard et al., 1997; Bobinac et al.,

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2000; Jergovic et al., 2001; Raheem et al., 2010). Therefore, muscle fiber phenotyping is of paramount importance to understand the muscle states in a wide range of fields including neurology, neurosurgery, orthopedics, sports medicine and aging researches (Meunier et al., 2010), and also to detect the extent of denervation or regeneration of muscle fibers.

Myofibrillar adenosine triphosphatase (mATPase) and immunohistochemical staining are universally used to identify muscle fiber phenotypes. mATPase staining has been widely used for over 40 years (Guth and Samaha, 1969). It has some limitations: is sensitive to pH value and temperature change; weakens over time, cannot specifically stain capillaries; is hard to work on severely atrophic and hybrid muscle fibers (Jergovic et al., 2001). Immunohistochemical staining can avoid the limitations of mATPase staining. Single immunostaining with specific antibody can recognize one pure muscle fiber type. Monoclonal antibodies including anti-myosin BA-F8, A4.74, BF-F3 and BF-35 have been described in detail in rats (Schiaffino et al., 1989; Rivero et al., 1998; Bobinac et al., 2000; Smerdu and Soukup, 2008; McMillan and Quadrilatero, 2011). They are specifically immunoreactive with type I (Michel et al., 1996; Bobinac et al., 2000), type IIA (Schiaffino et al., 1989; Smerdu and Soukup, 2008; Soukup et al., 2009), type IIB (Schiaffino et al., 1989; Rivero et al., 1998; Smerdu and Soukup, 2008), all but type IIX fibers (Schiaffino et al., 1989; Rivero et al., 1998; Smerdu and Soukup, 2008), respectively. Serial consecutive sections are needed to identify all muscle fibers by single immunostaining, which is laborious.

It is more complicated for immunohistochemical staining to identify hybrid fibers which contain multiple MyHC isoforms, whose staining properties are intermediate and largely depend on the inner proportion of various MyHC isoforms (Tulloch et al., 2011). There are only rare hybrid fibers in normal muscles, but abundant in denervated and reinnervated muscles, due to the conversion of muscle fibers from one type to another after denervation and reinnervation (Gorza, 1990; Tulloch et al., 2011). Therefore, identification of muscle fiber phenotypes in denervated and regenerated muscles is much more complicated than that in normal muscles.

Multiple immunohistochemical staining was first described by Nakane (1968) and has been universally used when investigators need to demonstrate multiple antigens simultaneously on a single section (Claassen et al., 1986; van der Loos et al., 1987; Gorza, 1990; Rivero et al., 1998; Smerdu and Soukup, 2008). Whereas triple immunostaining for muscle fiber phenotyping was not established until recently because of the complexity of muscle fiber phenotyping and coexistence of various muscle fiber types in the hybrid fibers. Triple immunofluorescence staining (TIF) was recently introduced by which the identification and comparison of all four types of pure muscle fibers on a single section were possible (Tulloch et al., 2011; McMillan and Quadrilatero, 2011). Triple immunoenzyme staining (TIE) has some advantages over TIF. It shows better morphologic patterns and tissue structures. The chromogen based immunoreactivity signals would not fade for a long time, making re-analysis of

the staining results possible. The immunostaining results can be analyzed under the common light microscopy instead of requiring fluorescence microscope in the dark room. It can also potentially remedy the limitation of TIF where basement membranes of muscle fibers cannot be clearly seen, particularly when there is a cluster of type IIX adjacent to each other (McMillan and Quadrilatero, 2011).

To co-localize all muscle fibers in a single section, a novel TIE staining method was developed in this study. We chose three primary antibodies with different subtypes of immunoglobulins (anti-myosin A4.74 IgG1 for MyHC-IIA, anti-myosin BA-F8 IgG2 for MyHC-I, anti-myosin BF-F3 IgM for MyHC-IIB) to perform the TIE in order to avoid the cross-labeling seen in previous publications (Tulloch et al., 2011; Bloemberg and Quadrilatero, 2012). The basement membranes were clearly demarcated, while little cross-labeling was seen in our novel TIE stained sections. In order to confirm the specificity of TIE, comparison with respective single immunoenzyme staining (SIE) on consecutive frozen sections was also carried out.

Materials and Methods

Muscle specimen harvest and cryopreservation

All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC protocol# A10611). All animal studies were performed in compliance with Association for Assessment and Accreditation of Laboratory Animal Care International and under the supervision of the Institutional Animal Care and Use Committee. Eighteen female Lewis rats, weighing 200 ± 5 g, aged 3–4 months (Harlan Laboratories, Inc., Indianapolis, IN, USA) were used in this study. Tibialis anterior and soleus muscles were harvested from the left hind limb of nine normal control rats, four rats whose left sciatic nerve had been transected at mid-thigh level for various intervals (1, 4, 8, and 12 weeks, respectively), and five rats whose left sciatic nerve was repaired with 4 to 5 intermittent 10-0 monofilament sutures (Ethilon[®], Ethicon US, LLC) after different delay intervals (0 week, 1 week, 4 weeks, 6 weeks and 8 weeks, respectively). This constituted 18 normal muscles, 8 muscles at varying stages of denervation, and 10 muscles of varying degrees of reinnervation. The harvested muscle specimen (the soleus muscle and tibialis anterior muscle) was pinned at a resting length on a piece of cork and then quickly frozen in isopentane cooled with liquid nitrogen. Frozen muscle specimens were stored in a -80°C freezer before cryosectioning and immunostaining.

Immunohistochemical staining

Frozen muscle specimens were transferred to the -20°C cryostat (Leica CM3050S; Leica Microsystems, Wetzlar, Germany) on dry ice. Each specimen was cut transversely into two parts at the midpoint of the muscle belly. One of the two parts was mounted on the cryostat such that five $10\ \mu\text{m}$ thick consecutive crosssections were cut from the midpoint of the muscle. The cryosections were then fixed in precooled absolute acetone at 4°C for 5 minutes. Four cryosections were

used for SIE with mouse monoclonal primary antibodies anti-myosin A4.74 IgG1, anti-myosin BA-F8 IgG2b, anti-myosin BF-F3 IgM and anti-myosin BF-F35 IgG1, respectively (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). One cryosection was used for TIE with three aforementioned primary antibodies against A4.74, BA-F8 and BF-F3.

For SIE, cryosection was incubated with the primary antibody (5 µg/mL) for 1 hour at room temperature. After washing, the slide with A4.74, BA-F8, or BF-F35 primary antibody was incubated in the polymerized peroxidase conjugated horse anti-mouse secondary antibody which was included in the ImmPRESS REAGENT Anti-Mouse IgG kit (Vector Lab, Burlingame, CA, USA) without dilution as per manufacturer's instruction and then in respective detection kit (Vector Lab, Burlingame, CA, USA) as follows: DAB (3,3'-diaminobenzidine) (brown) for A4.74 and BF-F35, SG kit (grey) for BA-F8. The slide with BF-F3 primary antibody was incubated in the goat anti-mouse secondary antibody from Alkaline Phosphatase labeled anti-mouse IgM kit (Jackson ImmunoResearch Inc, West Grove, PA, USA) diluted at 1:500 with a final working concentration of 1.6 µg/mL and visualized using the Vulcan fast red chromogen kit 2 (red) (Biocare Medical, Concord, CA, USA). All staining procedures using the detection kits were performed according to manufacture instructions. TIE was performed using the ImmPRESS polymerized reporter enzyme staining system for the first two antibodies (A4.74 and BA-F8) and alkaline phosphatase staining system for the third antibody (BF-F3) as per manufacture instructions. After fixation in acetone, the sections were first incubated with monoclonal anti-myosin A4.74 IgG1 primary antibody (5 µg/mL) for 1 hour. The sections were incubated with secondary antibody for 30 minutes and then treated with DAB substrate which produces a dark brown reaction. After PBS washes, the sections were incubated with the second primary antibody anti-myosin BA-F8 IgG2b (5 µg/mL) for 1 hour. Then the sections were incubated with secondary antibody for 30 minutes and treated with an SG detection kit which produces a grey reaction. The sections were then incubated with the third primary antibody, anti-myosin BF-F3 IgM (5 µg/mL) for 1 hour, alkaline phosphatase labeled anti-mouse IgM secondary antibody for 1 hour and treated with Vulcan fast red chromogen kit 2 that produces a red reaction. All the staining steps were done at room temperature. Subsequently, the sections were mounted and stored at room temperature for future imaging and analysis. The antibodies and detection reagents are summarized in **Table 1**.

In some SIE slides, hematoxylin staining (nuclear counterstaining) or laminin immunostaining (cell membrane counterstaining) were performed after muscle fiber immunoenzyme staining to improve visibility.

Imaging and quantification

All the slides were scanned using a Nanozoomer Digital Pathology 2.0 HT scanner (Hamamatsu Inc, Houston, TX, USA) at 20× magnification to acquire images of the muscle

cross-sections. The distribution, relative position and morphology of various muscle fibers on the TIE section were compared with those on the four corresponding consecutive SIE sections to determine the validity of TIE.

For quantitation of the proportion of each fiber type on a given TIE section, WebSlide Enterprise software was used. The scanned image was divided into grids. Then the grid of interest was enlarged and imported to Image J software for each fiber type to be manually tallied. The number of fibers stained positive for a given fiber phenotype was the sum of the tally of that fiber type acquired from all the sampled grids. This number was then divided by the total number of fibers tallied regardless of phenotype and determined as the proportion (percentage) of that given fiber phenotype. For each TIE stained section, muscle fibers from all the grids were tallied first for the various fiber phenotypes and their respective proportions were calculated and considered as true values. Then we systematically sampled every other, every third, every fourth and every eighth grid (50%, 33%, 25% and 12.5% sampling frequency, respectively), tallied and calculated the fiber phenotype proportions. The proportions of each muscle fiber type acquired at 50%, 33%, 25% and 12.5% sampling frequencies were divided by the true values (acquired at 100%). The ratio was recorded as relative rate. The closer was the relative rate to 1.0, the truer was the statement that results obtained at that sampling frequency reflect the whole picture.

Statistical analysis

The relative rates of all muscle fiber types between varying sampling frequencies were compared and analyzed by JMP 10 (SAS Institute Inc, Cary, NC, USA). One-way analysis of variance (ANOVA) was used for the normally distributed data in this study. Tukey's *post hoc* test was performed for multiple comparisons. Statistical significance was defined as $P < 0.05$.

Results

TIE for rat muscle fibers

All muscle fiber types including pure and hybrid fibers, could be identified in our novel TIE sections of normal, denervated or reinnervated tibialis anterior and soleus muscles. The TIE results of tibialis anterior are shown in **Figure 1a** (normal tibialis anterior), **Figure 2a** (1-week denervated tibialis anterior), **Figure 3a** (8-week denervated tibialis anterior), **Figure 4a** (12-week denervated tibialis anterior) and **Figure 5a** (reinnervated tibialis anterior). Their corresponding four consecutive SIE results are shown in panels **b–e** of each figure.

Type I, IIA, IIB, IIX fibers were distinguishable as dark grey, dark brown, dark red, unstained or very slight brown staining, respectively, in TIE sections of tibialis anterior (**Figures 1a, 2a, 3a, 4a, 5a**). Type I and IIA fibers were distinguishable as dark grey and dark brown staining, respectively, in TIE sections of soleus. Hybrid type IIA/IIX and IIB/IIX fibers were distinguishable as mild to moderate brown staining and mild to moderate red staining, respectively, in TIE

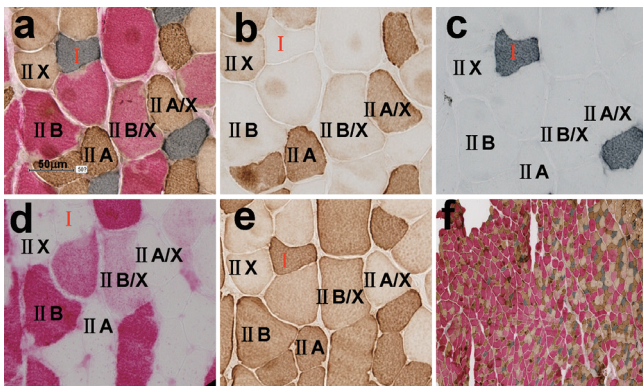


Figure 1 Triple immunoenzyme staining (TIE) and four corresponding single immunoenzyme staining (SIE) results in a normal tibialis anterior muscle.

TIE at 20× (a) and 5× magnification (f). SIE with antibody A4.74 (b), BA-F8 (c), BF-F3 (d) and BF-F35 (e) at 20× magnification. I, IIA, IIB, IIX, IIA/X and IIB/X represent muscle fiber type I, IIA, IIB, IIX, IIA/X and IIB/IIX, respectively. Fiber phenotyping on TIE (a) matched well with phenotyping on the other four SIEs (b, c, d, e).

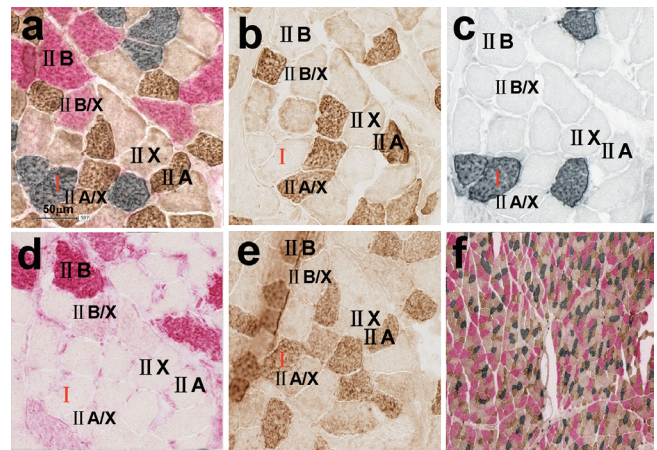


Figure 2 Triple immunoenzyme staining (TIE) and four corresponding single immunoenzyme staining (SIE) results in a tibialis anterior muscle subjected to 1 week denervation.

TIE at 20× (a) and 5× magnification (f). SIE with antibody A4.74 (b), BA-F8 (c), BF-F3 (d) and BF-F35 (e) at 20× magnification. I, IIA, IIB, IIX, IIA/X and IIB/IIX, respectively. Fiber phenotyping on TIE (a) matched well with phenotyping on the other four SIEs (b, c, d, e).

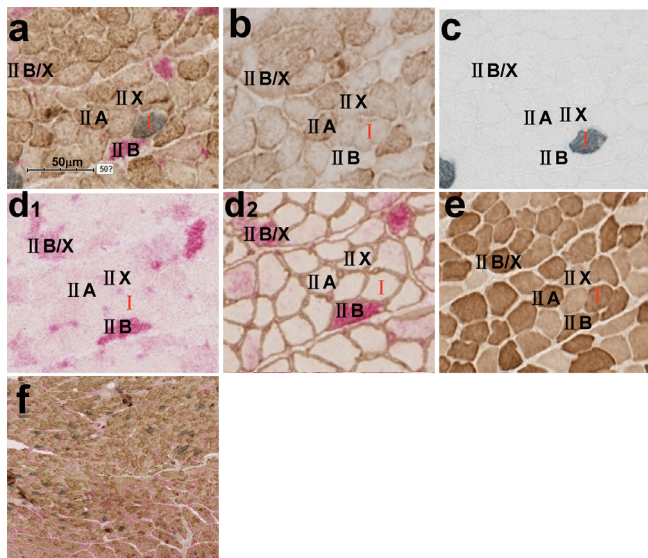


Figure 3 Triple immunoenzyme staining (TIE) and four corresponding single immunoenzyme staining (SIE) results in a tibialis anterior muscle subjected to 8-week denervation.

TIE at 20× (a) and 5× magnification (f). SIE with antibody A4.74 (b), BA-F8 (c), BF-F3 (d), BF-F3 + laminin (d₂) and BF-F35 (e) at 20× magnification. I, IIA, IIB, IIX and IIB/X represent muscle fiber type I, IIA, IIB, IIX and IIB/IIX, respectively. Fiber phenotyping on TIE (a) matched well with phenotyping on the other four SIEs (b, c, d, e). Laminin immunostaining (cell membrane counterstaining) was added in d₂ to improve the visibility of severely atrophied muscle fibers.

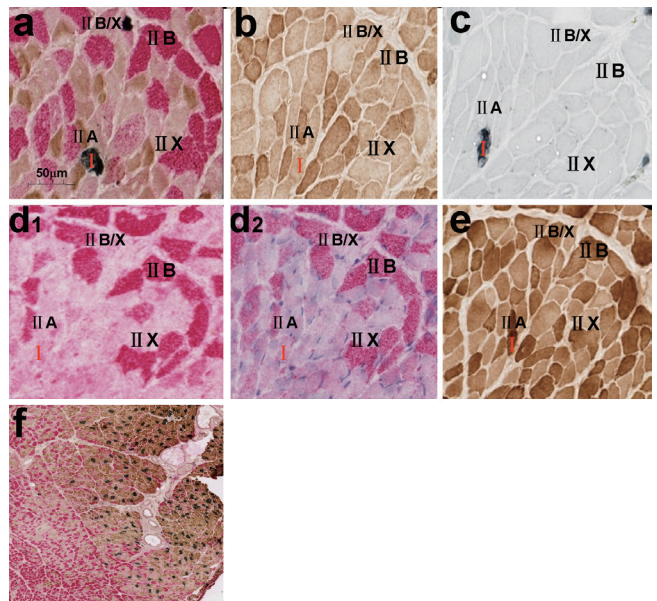


Figure 4 Triple immunoenzyme staining (TIE) and four corresponding single immunoenzyme staining (SIE) of a tibialis anterior muscle that was denervated for 12 weeks.

TIE at 20× (a) and 5× magnification (f). SIE with antibody A4.74 (b), BA-F8 (c), BF-F3 (d), BF-F3 + hematoxylin (d₂) and BF-F35 (e) at 20× magnification. I, IIA, IIB, IIX and IIB/X represent muscle fiber type I, IIA, IIB, IIX and IIB/IIX, respectively. Fiber phenotyping on TIE (a) matched well with phenotyping on the other four SIEs (b, c, d, e). Hematoxylin staining (nuclear counterstaining) was added in d₂ to improve the visibility of severely atrophied muscle fibers.

sections of tibialis anterior (Figures 1a, 2a, 3a, 4a, 5a). Hybrid type IIC (I/IIA) fibers could be distinguishable as mild to moderate brown staining, or as mild to moderate grey staining in tibialis anterior (Figure 5a).

The exact same areas on consecutive SIE-stained sections that matched the areas on TIE-stained section were shown to verify the color schemes of the triple stain. Type IIA fibers were stained dark brown in Figures 1b, 2b, 3b, 4b, 5b) (SIE staining with monoclonal anti-myosin A4.74 IgG1 antibody).

Type I fibers were stained dark grey in Figures 1c, 2c, 3c, 4c, 5c) (SIE staining with monoclonal anti-myosin BA-F8 IgG2b antibody). Type IIB fibers were stained dark red in Figures 1d, 2d, 3d, 4d, 5d) (SIE staining with monoclonal anti-myosin BF-F3 IgM antibody). Type I, IIA and IIB fibers were stained dark brown, while type IIX fibers were unstained or

Table 1 Antibodies and detection systems used in the single and triple immunoenzyme staining

Primary antibodies (concentration)	Fiber type specificity	Secondary antibodies	Chromogen (color)
Anti-BA-F8 IgG2b (5 µg/mL)	Type I	Polymerized peroxidase conjugated anti-mouse IgG	SG (grey)
Anti-A4.74 IgG1 (5 µg/mL)	Type IIA	Polymerized peroxidase conjugated anti-mouse IgG	DAB (brown)
Anti-BF-F3 IgM (5 µg/mL)	Type IIB	Alkaline phosphatase anti-mouse IgM	Vulcan fast red (red)
Anti-BF-F35 IgG1 (5 µg/mL)	All but type IIX	Polymerized peroxidase conjugated anti-mouse IgG	DAB (brown)

IgG: Immunoglobulin G; SG: slate grey; DAB: 3,3'-diaminobenzidine.

Table 2 Establishment of systematic sampling for muscle fiber phenotyping quantifications

Muscles	Sampling frequency (%)	N ^a	Relative rate (mean ± SD)	Relative rate (95% CI)	F value	P value
Normal tibialis anterior ^b	50.0	36	0.990±0.060	0.970–1.011	0.253	0.859
	33.0		1.010±0.116	0.971–1.049		
	25.0		0.993±0.107	0.957–1.029		
	12.5		1.009±0.184	0.947–1.072		
Abnormal tibialis anterior ^c	50.0	36	1.005±0.069	0.982–1.028	0.245	0.865
	33.0		1.001±0.123	0.959–1.043		
	25.0		0.985±0.123	0.944–1.027		
	12.5		0.981±0.214	0.908–1.054		
Normal Soleus ^d	50.0	18	1.012±0.056	0.985–1.040	0.541	0.656
	33.0		1.017±0.064	0.985–1.049		
	25.0		1.047±0.120	0.988–1.107		
	12.5		1.063±0.240	0.944–1.183		
Abnormal Soleus ^e	50.0	18	1.028±0.091	0.983–1.073	0.910	0.441
	33.0		0.968±0.109	0.914–1.022		
	25.0		0.986±0.119	0.926–1.045		
	12.5		1.005±0.136	0.937–1.073		

^aThe total number of tallied datasets for all the muscle fiber types in the group of muscle sections collectively. For example, there were nine normal tibialis anterior muscle specimens. Each tibialis anterior muscle had four fiber types. Therefore, there were 36 tallied numbers (9 times 4). There were nine abnormal soleus muscle samples (four denervated and five reinnervated). Each soleus had two muscle fiber types. Therefore there were 18 tallied numbers (9 times 2). ^bNormal tibialis anterior muscle. ^cTibialis anterior muscles with varying degrees of denervation or reinnervation. ^dNormal soleus muscle. ^eSoleus muscles with varying degrees of denervation or reinnervation.

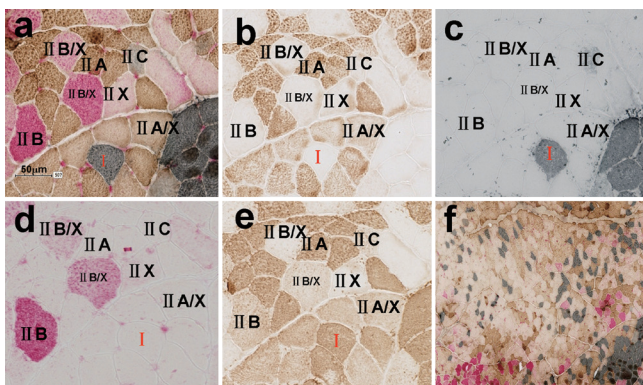


Figure 5 Triple immunoenzyme staining (TIE) and four corresponding single immunoenzyme staining (SIE) results in a reinnervated tibialis anterior muscle (16 weeks after 4-week delayed nerve repair).

TIE at 20 × (a) and 5× magnification (f). SIE with antibody A4.74 (b), BA-F8 (c), BF-F3 (d) and BF-F35 (e) at 20× magnification. I, IIA, IIB, IIX, IC, IIA/X and IIB/X represent muscle fiber type I, IIA, IIB, IIX, I/IIA, IIA/IIX and IIB/IIX, respectively. Fiber phenotyping on TIE (a) matched well with phenotyping on the other four SIEs (b, c, d, e).

very lightly stained in **Figures 1e, 2e, 3e, 4e, 5e** (SIE staining with monoclonal anti-myosin BF-35 IgG1 antibody). Hybrid type IIA/IIX fibers were stained mild to moderate brown in

Figures 1b, 2b, 3b, 4b, 5b, while hybrid type IIB/IIX fibers were stained mild to moderate red in **Figures 1d, 2d, 3d, 4d, 5d**. Hybrid type IIC (I/IIA) fibers were distinguishable as mild to moderate grey in **Figure 5c**. All hybrid fibers were stained mild to dark brown or unstained in **Figures 1e, 2e, 3e, 4e, 5e**, depending on the inner composition of the hybrid. Hematoxylin staining (nuclear counterstaining) and laminin immunostaining (cell membrane counterstaining) were performed in **Figure 4d2** and **Figure 3d2**, respectively, to improve the visibility.

TIE specificity

At least 200 adjacent muscle fibers were randomly selected from TIE and its corresponding consecutive SIE images. The relative position, immunostaining properties and morphology of these muscle fibers on the TIE image were compared to those on SIE images. Results showed that muscle fiber phenotypes identified on the TIE section matched well (95% match rate) with their counterparts that were identified on SIE sections which were stained individually with antibody for one phenotype (**Figures 1a–1e, 2a–2e, 3a–3e, 4a–4e, and 5a–5e**), confirming the specificity of the TIE method. The TIE test was repeated twice and reproducible results were obtained.

Table 3 Percentages (%) of various muscle fiber types in normal tibialis anterior and soleus muscles in Lewis rats

Muscles	n	I	IIA	IIB	IIX	I/IIA	IIA/IIX	IIB/IIX
Tibialis anterior	9	1.5±0.7	17.2±3.3	64.3±4.3	10.9±5.5	0.03±0.04	4.40±2.1	1.67±0.5
Soleus	9	90.3±3.1	8.6±2.7	0	0	1.1±0.7	0	0

All data except the number of rats are expressed as the mean ± SD. N is the number of included rats. I, IIA, IIB, IIX, IIC, IIA/X and IIB/X represent muscle fiber type I, IIA, IIB, IIX, I/IIA, IIA/IIX and IIB/IIX, respectively.

Systematic sampling

We found that the relative rates at the varying sampling frequencies for both normal and abnormal tibialis anterior and soleus muscles were very close to 1.0. No statistically significant differences were detected for the relative rates of all muscle fiber types among the 50%, 33%, 25% and 12.5% sampling frequencies (Table 2).

Systematic sampling of 12.5% of the entire muscle section represented the truth (100%) as well as the other three higher sampling frequencies did. A sampling frequency of 12.5% was hence determined for future studies of muscle fiber phenotyping quantification, making this task less labor intensive.

Muscle fibers in normal, denervated or reinnervated rat muscles

We found that the majority of the fastest fibers (type IIB) were located in the superficial part, while the majority of the slowest fibers (type I) were located in the deep part of the normal tibialis anterior muscle. After denervation and reinnervation of tibialis anterior muscle, the superficial fast fiber area decreased and the deep slow fiber area increased. Normal tibialis anterior muscle (fast fiber dominant muscle) was mainly constituted of type II (type IIB, type IIX and type IIA) muscle fiber types, a few type I muscle fibers and rarely hybrid fibers (type I/IIA, IIA/IIX, IIB/IIX). Normal soleus (slow fiber dominant muscle) was mostly constituted of type I muscle fiber, a few type IIA muscle fiber and rarely hybrid (type I/IIA) muscle fibers without type IIB and type IIX muscle fibers (Table 3).

Discussion

A novel, reproducible, sensitive and specific TIE staining method that enables simultaneous identification of all muscle fiber phenotypes on a single cryosection of normal, denervated or reinnervated rat muscle has been successfully established. A systematic sampling method for muscle fiber phenotype quantification has also been verified. Systematic sampling of 12.5% of the entire muscle cross section was found to be enough to accurately represent the true fiber phenotypes of the normal, denervated or reinnervated tibialis anterior and soleus muscles. The specimens that were analyzed comprised normal muscles, muscles of varying degrees of denervation atrophy, and muscles at varying stages of reinnervation. They represented a wide range of muscle fiber phenotyping conditions. Therefore, this systematic sampling method is suitable for analyzing not only normal rat muscles, but also muscles denervated or reinnervated at

different extents.

The proportion of muscle fibers in normal, denervated or reinnervated muscles in our study is consistent with the finding from previous studies. The proportion of each muscle fiber type was uniform with a small standard deviation in normal tibialis anterior and soleus muscles from nine rats (Table 3) which reflected the specificity and reliability of this novel TIE staining method. The average proportion of type I and type IIA fibers was 90.3% and 8.6% respectively, in the normal soleus muscle in our study, comparable to the data, 87–100% and 0–13%, respectively, reported in a study by Novak et al. (2010). The average proportion of type I, type IIA, type IIB and type IIX muscle fibers was 1.5%, 17.2%, 64.3% and 10.9%, respectively, in the normal tibialis anterior muscles in our study. These numbers were also consistent with those reported in other studies (Laughlin and Armstrong, 1982; Armstrong and Phelps, 1984; Gorza, 1990). In the denervated and reinnervated tibialis anterior muscles, the proportion of the fastest fibers (type IIB) decreased dramatically, whereas that of slower fibers (type I, type IIA, type IIX) obviously increased. With the total number of muscle fibers being unchanged, this finding suggested the conversion of faster fibers to slower ones which is also consistent with the findings from previous studies (Pette and Vrbova, 1985; Michel et al., 1996; Bigard et al., 1997; Windisch et al., 1998; Bobinac et al., 2000; Kostrominova et al., 2005). These confirmations added credibility to this novel TIE staining technique.

One of the major challenges of multiple immunostaining is the identification of hybrid muscle fibers. The immunostaining properties of hybrid fibers are intermediate between the corresponding pure fibers, depending on the inner proportion of each MyHC isoform in hybrid fibers (Tulloch et al., 2011). Hybrid type IIA/IIX muscle fibers that mildly stained with A4.74 antibody and unstained with BF-35 antibody contain mostly MyHC-IIX and a low proportion of MyHC-IIA, while those contain more proportions of MyHC-IIA were moderately stained with A4.74 (Smerdu and Soukup, 2008). Hybrid type IIB/IIX muscle fibers with more proportion of MyHC-IIB have stronger immunoreactions with BF-F3 antibody, resulting in darker red reaction on TIE sections (Figure 5a) and SIE sections stained with BF-F3 antibody (Figure 5d) and darker brown reaction on SIE sections with BF-F35 (Figure 5e). Hybrid type IIC (I/IIA) muscle fibers, whose staining property is intermediate between the muscle fiber type I and type IIA, were stained mild to moderate brown on TIE sections of the soleus, or mild to moderate grey as shown on TIE and SIE sections of

reinnervated tibialis anterior (**Figure 5a, c**). Some publications maintained that type IIA and hybrid type IIA/X could not be distinguished by multiple immunostaining, because anti-myosin A4.74 antibody was reported to immunoreact with both type IIA and IIX which makes the differentiation of hybrid type IIA/X from type IIA and IIX difficult (Raheem et al., 2010). In our study, we found that type IIX fibers were very slightly stained or unstained, type IIA fibers were deeply stained, while hybrid type IIA/X fibers were intermediately stained on TIE sections. It was possible to distinguish these three phenotypes with this novel staining method.

There are circumstances when hybrid fibers could not be well identified. First, the immunoreaction magnitude of hybrid fibers depends on the relative proportions of various MyHC isoforms. If the proportions of MyHC isoforms in hybrid fibers only differ very slightly from pure fibers, the difference in staining magnitude is not readily discernible by the naked eye. Second, cross reaction of secondary antibodies with primary antibodies may hinder identification of hybrid fibers (Raheem et al., 2010). Third, the conversion of muscle fiber phenotype from one type to another according to Gorza's pattern (Gorza, 1990) after denervation or reinnervation occurs such that even the same type of hybrid fiber has different constitutions of MyHC isoforms, making identification of these hybrid fibers much more complicated.

The key technical point of successfully established TIE method is to reduce the cross-reactions. Our protocol takes advantage of the fact that the antibodies against MyHC isoforms belong to different subtypes of immunoglobulins: anti-myosin A4.74 IgG1 for MyHC-IIA, anti-myosin BA-F8 IgG2b for MyHC-I, anti-myosin BF-F3 IgM for MyHC-IIB. In general, any immunohistochemical multiple-staining technique is a combination of several individual antigen visualization methods. A successful triple immunohistochemical staining protocol needs to overcome two main problems: (1) determining how to prevent cross-reactions between several individual detection methods; (2) distinguishing the color combinations that provide the best contrast between several individual colors and a mixed color at the sites of colocalization. Many investigators have tried to find solutions to overcome these problems, and developed several concepts and color combinations to perform successful multiple immunostaining. Most of these concepts are based on the differences between primary antibodies including animal species, Ig isotypes or IgG subclasses, conjugates, or concentrations. Additionally, different chromogens are selected to show different colors for different antigens in tissues (van der Loos, 2008).

The advantages of our novel and effective TIE staining method include: (1) Instead of using single immunohistochemical staining on multiple muscle sections, the TIE enables simultaneous identification of all muscle fibers on a single cryosection, which saves time and tissue sections. (2) Compared to TIF staining, the TIE staining is less expensive, much more convenient for visualization and imaging, and outlines muscle fibers better. (3) The TIE technique has been proven to work well not only on normal muscles, but also

on muscles of varying degrees of denervation atrophy, and muscles at varying stages of reinnervation. This warrants its broader use in future studies.

Conclusions

A novel, reproducible, sensitive and specific TIE method has been successfully established in this study which enables simultaneous identification of all muscle fiber types on a single cryosection. It works well in normal, denervated or reinnervated rat muscles. It enables the visualization of muscle fiber basement membranes, which facilitates the accurate identification of fiber type and morphology. Meanwhile, a systematic sampling method has been validated, making the manual counting and analysis of muscle fiber phenotyping more accurate and less laborious.

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Research ethics: All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC protocol# A10611). All animal studies were performed in compliance with Association for Assessment and Accreditation of Laboratory Animal Care International and under the supervision of the Institutional Animal Care and Use Committee.

Data sharing statement: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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