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Chapter 15

Muscle Fibre Phenotyping from a Single Section: Is It as Informative as from Serial Sections?

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1. Introduction

Major sarcomeric thick filaments' protein of mammalian skeletal muscles is myosin. The thick myosin filament is composed of hexameric myosin molecules consisting of 2 myosin heavy chain (MyHC) subunits and 4 myosin light chain subunits (2 pairs of nonidentical light chains). The globular, amino terminal head domain, of MyHC has binding sites for actin and ATP. The elongated, alpha-helical coiled-coil carboxyl-terminal rod domain of MyHC exhibits filament forming properties, for review see (1). Several distinct MyHC isoforms exist. Human limb and trunk muscles contain muscle fibres expressing skeletal muscle-specific MyHC isoform IIA (MyHC-IIA) encoded by MYH2 gene on chromosome 17, MyHC isoform IID/IIX (MyHC-IIX) (2-4) encoded by MYH1 gene on chromosome 17 and an isoform that is expressed in both skeletal and cardiac muscle MyHC-β or MyHC-I encoded by MYH7 gene on chromosome 14 (5, 6). Two developmental myosin heavy chain isoforms (MyHC- embryonic and MyHC- perinatal) may be present in adult muscles, particularly in regenerating muscle fibres. MyHC family in humans includes also cardiac specific isoform MyHC-α and specialized isoform expressed in extraocular muscles (MyHC-extraocular) (6, 7). The forth skeletal muscle-specific isoform MyHC-IIB, is expressed in mice and rat skeletal muscles and is thought not to be expressed in adult human muscles at the protein level; however MyHC-IIB mRNA has been detected in specialized human muscles (8, 9) and in limb muscles of patients suffering from Duchenne muscular dystrophy (10), suggesting that MyHC-IIB gene in humans can be reactivated in skeletal muscles undergoing profound degeneration/regeneration.

In humans MyHC isoforms could be expressed either as single isoforms (in pure fibres-I, IIA and IIX) or co-expressed in different proportions in hybrid fibres (mainly -I/IIA, -IIA/IIX and -I/IIA/IIX). Hybrid fibres could reflect either a state of transition toward an isoform that is stimulus dependent or a state of stability (11). In normal conditions the proportion of hybrid

fibres depends on age, gender and physical activity: The studies of Williamson et al. (11, 12) showed that in young untrained women the proportion of hybrid fibres was 30%, in young untrained man the proportion was 40% (12) and in older untrained man the proportion was about 30% (11). In young and old the proportion of hybrid fibres decreased with progressive resistance training (11, 12) (though differences exist with respect to the type of MyHC upregulation).

Muscle fibre typing which includes subclassification of fast fibres (distinction between IIA and IIX fibres) and identification of hybrid fibres by the type of MyHC isoform or by the activity of myofibrillar ATP-ase is technically quite a demanding process, since it requires serial muscle sections, is time consuming and expensive. Besides, identification of hybrid fast fibres by ATP-ase activity is generally not accurate (11).

Identification of muscle fibre types on a single muscle section applying specific antibodies to MyHC isoforms could enhance and simplify the phenotyping (13, 14). Gregorevic et al. (13) presented on animal muscles sequential staining of a single section with three different MyHC specific antibodies (MyHC-I, MyHC-IIA, MyHC-IIB) labelled with different fluorophores. Raheem et al. (14) applied on human muscles sequential immunohistochemical staining of a muscle section with antibodies against two MyHC isoforms (MyHC-I and MyHC-IIA), where first antigen was visualized with peroxidase and the second with alkaline phosphatase-conjugated secondary antibodies. Neither Gregorevic et al. (13) nor Raheem et al. (14) identified IIX fibres with antibodies specific to MyHC-IIX, but only by exclusion, i.e. fibres not immunoreactive with antibodies to MyHC-I, MyHC-IIA and MyHC-IIB in animals or MyHC-I and MyHC-IIA in humans were identified as IIX fibres.

In this study we compare methods for muscle fibre phenotyping on a single section with those on serial sections and discuss the advantages and limits of muscle phenotyping on a single section. In addition we demonstrate that A4.74 antibody, otherwise declared to be specific for MyHC-IIA in humans (14, 15), cross-immunoreacts with IIX fibres in humans, consistent with Smerdu and Soukup (16).

2. Methods

Muscle fibre phenotyping was performed according to the expression of myosin heavy chain isoforms (MyHC) by indirect immunoperoxidase method using anti-mouse immunoglobulins as secondary antibodies (P260, DAKO) as described previously (4, 16), except for IIX fibres where Novolink polymer detection system (Novocastra secondary antibodies, Leica Microsystems) was used as recommended by the manufacturer. Briefly *slow* fibres were demonstrated by BA-D5 antibody immunoreactive with β/slow MyHC-I in rats (2) and humans (5) in a dilution 1:100; *fast* fibres were demonstrated by A4.74 antibody (former Alexis Biochemicals, now Enzo Life Sciences) immunoreactive according to the product data sheet in rats and humans with MyHC-IIA, but according to (16) with MyHC-IIA and MyHC-IIX in humans and in dogs (17). A dilution of A4.74 was 1:100. Immunoreaction using A4.74 antibody was developed either with peroxidase-conjugated

anti-mouse immunoglobulins (P260, DAKO) or with alkaline phosphatase-conjugated anti-mouse immunoglobulins (D486, DAKO). IIX fibres were demonstrated by 6H1 antibody developed by Lucas (18) in a dilution 1:3000. BF-35 antibody which stains all muscle fibres, except fibres expressing MyHC-IIX (2) was used undiluted. Muscle fibre phenotyping on serial muscle sections was compared with two double immunoenzyme staining methods (i) described by Raheem et al. (14) and (ii) our own modification. Briefly, in (i) a single muscle section was successively treated with antibodies against MyHC-I (BAD5) and antibodies declared as specific against MyHC–IIA (A4.74). First primary antibody was visualized with peroxidase and the second with alkaline phosphatase. BCIP/NBT (Sigma) was used as a substrate for alkaline phosphatase. In the second modification (ii) we successively applied antibodies against MyHC-IIX (6H1) and antibodies declared as specific against MyHC–IIA (A4.74). Autopsy samples of vastus lateralis muscle of two healthy males who died suddenly were obtained within 12 hours after death. Muscle sampling was approved by the National Medical Ethical Committee of the Republic of Slovenia.

3. Results

Consistent with (16) we demonstrated that antibody A4.74 reacts in human vastus lateralis muscle with both MyHC-IIA and MyHC-IIX fibres (Fig. 1 and Fig. 2).

Higher dilutions of A4.74 antibodies did not eliminate staining of IIX fibres at dilutions at which IIA fibres could be stained reasonably well (results not shown).

Comparison of fibre phenotyping with the method of Raheem et al. (14) and fibre phenotyping on serial sections is presented in Fig. 3.

Double immunoenzyme staining method (Fig. 3a) demonstrates well muscle fibres expressing MyHC-I (slow fibres), all fast fibres (IIA and IIX fibres) as one group, as well as hybrid fibres IIA/I in which MyHC-IIA predominates (IIA>I). However the relative proportion of individual MyHC isoforms can not be estimated from a single section. Hybrid fibres IIA/I with similar contributions of MyHC-IIA and MyHC-I (IIA~I) (upper fibre with white asterisk in Fig. 3) and hybrid fibres with higher proportion of MyHC-I as MyHC-IIA (I>IIA) (lower fibre with white asterisk in Fig. 3) can not be recognised on a single section but only on serial sections. Hybrid IIA/IIX fibres, expressing MyHC-IIA and MyHC-IIX (fibres labelled with black dots (•) in Fig. 3) can be identified on serial sections only, because with the double staining method (and with A4.74 antibodies – Fig. 3b) they can not be distinguished from IIA fibres.

Distinguishing between slow and fast fibres can be successfully accomplished already with a single type of antibodies as BAD5 antibodies (stain all slow fibres) or A4.74 antibodies (stain all fast fibres) in humans. Unstained fibres in either case belong to other main group of muscle fibres (fast – Fig. 3c or slow – Fig. 3b).

Subtyping of fast fibres is more difficult. Antibodies to both, MyHC-IIA and MyHC-IIX have to be used. To see, whether it is possible to determine hybrid IIX/IIA fibres on a single

section, we successively applied antibodies against MyHC-IIX (6H1) and antibodies against MyHC-IIA + MyHC-IIX (A4.74) and compared the results with subtyping of fast fibres on serial sections and with double immunoenzyme staining method of Raheem et al. (14) (Fig. 4).

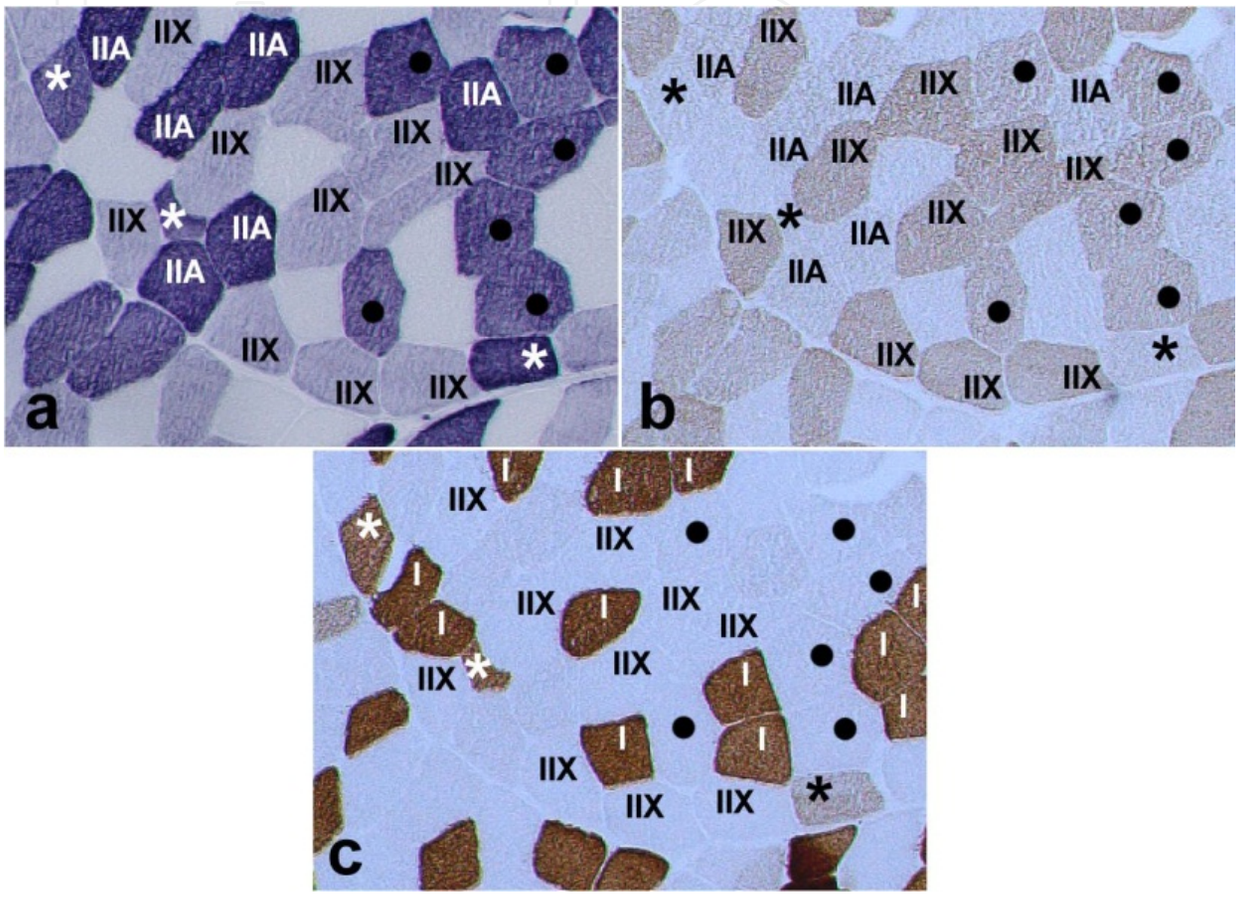

Figure 1. Immunoreactivity of A4.74 antibodies compared to immunoreactivity of 6H1 and BAD-5 antibodies on serial sections of human vastus lateralis muscle.
(a): A4.74 antibodies were declared as specific for MyHC-IIA, however A4.74 antibodies render an intense immunoreactivity in IIA fibres, in hybrid IIA/IIX fibres and a *moderate* immunoreactivity in *IIX* fibres. They do not stain slow fibres. (b): 6H1 antibodies specific for MyHC-IIX have relatively intense immunoreactivity in IIX fibres and hybrid IIA/IIX fibres. They do not stain IIA fibres and slow fibres. (c): BAD-5 antibodies specific for MyHC-I have intense immunoreactivity in I fibres. They do not stain IIA, IIX and hybrid IIA/IIX fibres.
Fibres labelled with dots (•): hybrid fibres IIA/IIX which express MyHC-IIA (a) and MyHC-IIX (b) and do *not* express MyHC-I (c). Fibres labelled with asterisks (*): hybrid fibres IIA/I which express MyHC-IIA (a) and MyHC-I (c) and do *not* express MyHC-IIX (b). IIA fibres: intense immunoreactivity with A4.74 antibodies, negative with 6H1 antibodies and BAD-5 antibodies. IIX fibres: relatively intense immunoreactivity with 6H1 antibodies (b), moderate immunoreactivity with A4.74 antibodies (a) and negative with BAD-5 antibodies (c). Slow or I fibres: intense immunoreactivity with BAD-5 antibodies (c), negative with A4.74 and 6H1 antibodies.

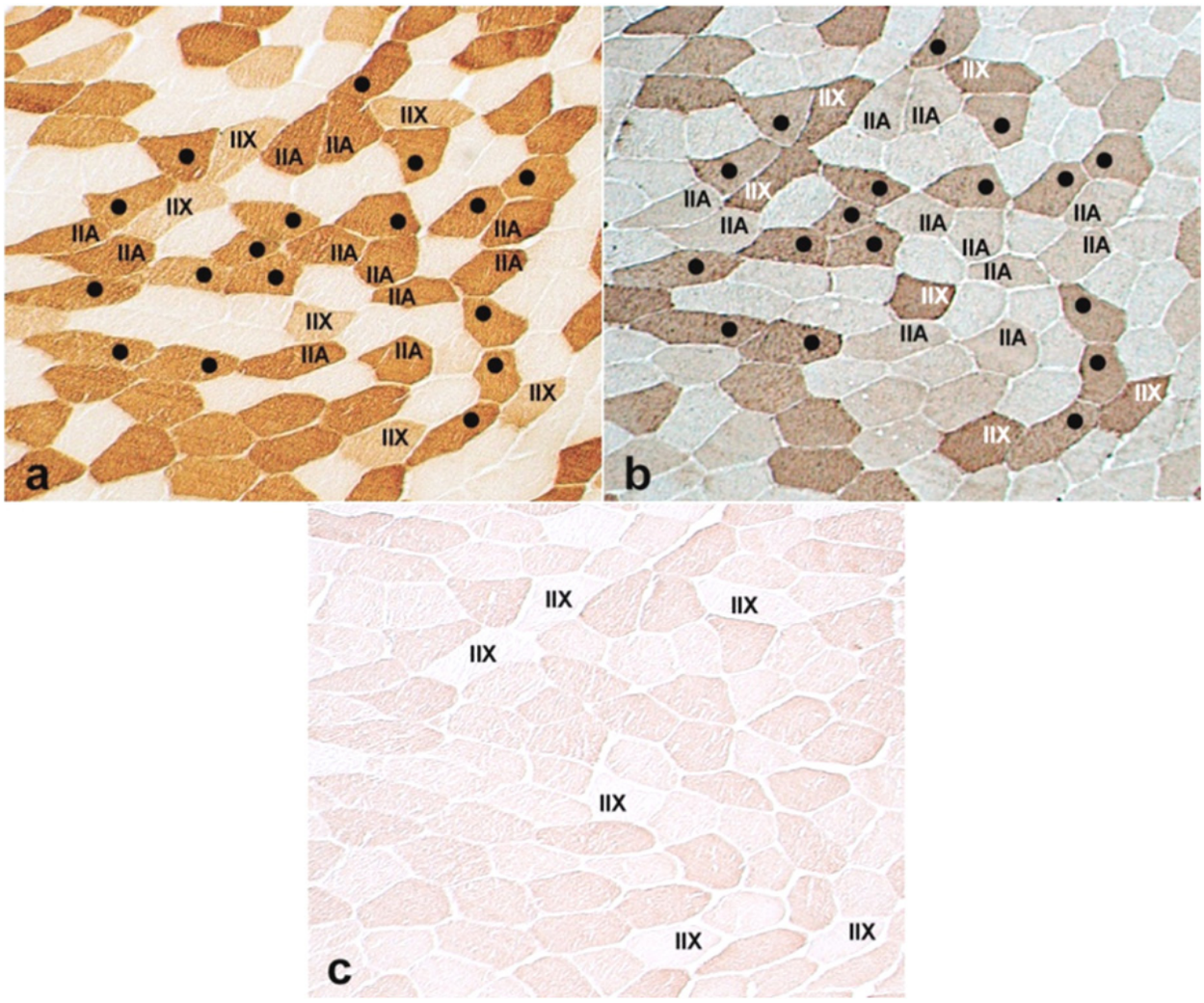

Figure 2. Immunoreactivity of A4.74 antibodies compared to immunoreactivity of 6H1 and BF-35 antibodies on serial sections of human vastus lateralis muscle.
(a): A4.74 antibodies show an intense immunoreactivity in IIA fibres and hybrid IIA/IIX fibres and also a *moderate immunoreactivity in IIX fibres*. (b): 6H1 antibodies have intense immunoreactivity in IIX fibres and hybrid IIA/IIX fibres. (c): BF-35 antibodies stain all muscle fibres except "pure" IIX fibres which do not co-express other MyHC isoforms. Fibres labelled with dots (•): hybrid IIA/IIX fibres which express MyHC-IIA (a) and MyHC-IIX (b) and are all immunoreactive with BF-35 antibody (c). IIX fibres: intense immunoreactivity with 6H1 antibodies (b), moderate immunoreactivity with A4.74 antibodies (a) and negative with BF-35 antibodies (c).

Due to cross-immunoreactivity of A4.74 antibodies with pure IIX fibres separation of IIX and other fast fibres (IIA fibres and IIA/IIX fibres as a group) is possible already with A4.74 antibodies where unstained fibres are slow fibres (Fig. 1a, Fig. 2a, Fig. 3b, Fig. 4b). A4.74 antibodies alone can not distinguish between "pure" IIA fibres and hybrid IIA/IIX fibres. With double immunostaining of IIA and IIX fibres (Fig. 4c) no additional information is provided. Hybrid fibres IIA/I can not be visualised with this method (fibres labelled with arrows and white asterisks in Fig. 4).

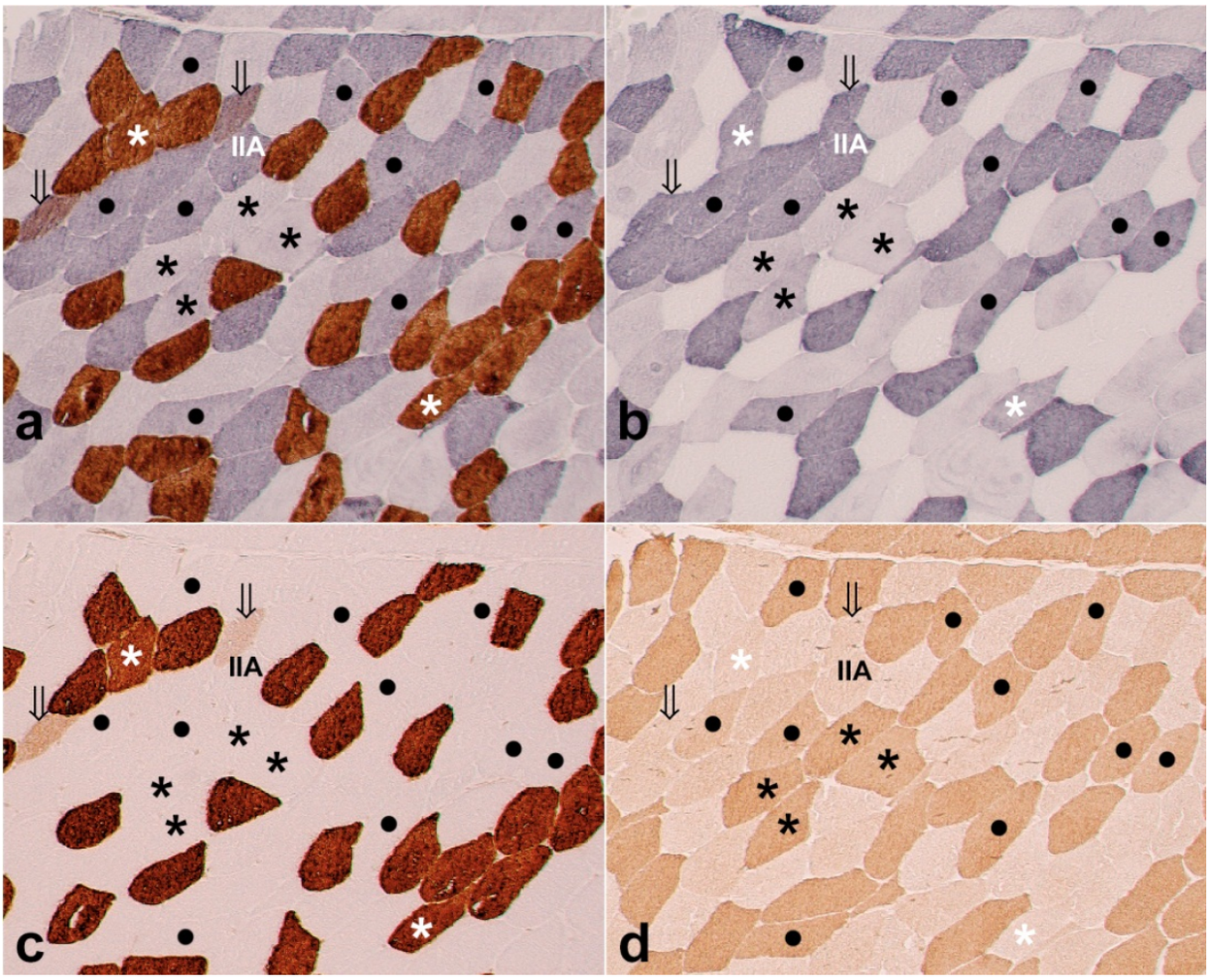

Figure 3. Phenotyping of muscle fibres with the double immunoenzyme staining method according to Raheem et al. (14) on a single section compared to phenotyping of muscle fibres on serial sections. (a): Double immunoenzyme staining – applying antibodies against MyHC-I (BAD5) and antibodies against MyHC-IIA + MyHC-IIX (A4.74). (b-d): Immunohistochemical reactions with individual antibodies. (b): against MyHC-IIA + MyHC-IIX (A4.74) visualized with alkaline phosphatase. (c): against MyHC-I (BAD-5) visualized with horseradish peroxidase. (d): against MyHC-IIX (6H1) visualized with horseradish peroxidase. Fibres labelled with *black* asterisks (*) are IIX fibres. Fibers labelled with black dots (•) are hybrid IIA/IIX fibres. Fibres labelled with arrows are hybrid IIA/I (IIA>I) fibres. Fibres labelled with *white* asterisks (*) are hybrid IIA/I (IIA~I) or (I>IIA) fibres (upper and lower fibre).

Neither double staining method identifies hybrid fibres co-expressing MyHC-IIA and MyHC-IIX (IIA/IIX). Hybrid fibres IIA/IIX can not be separated either form IIA fibres (in double immunostaining with BAD-5 and A4.74 antibody) ((Fig. 3a, Fig. 4a)) or from IIX fibres (in double immunostaining with 6H1 and A4.74 antibody) (Fig. 4c).

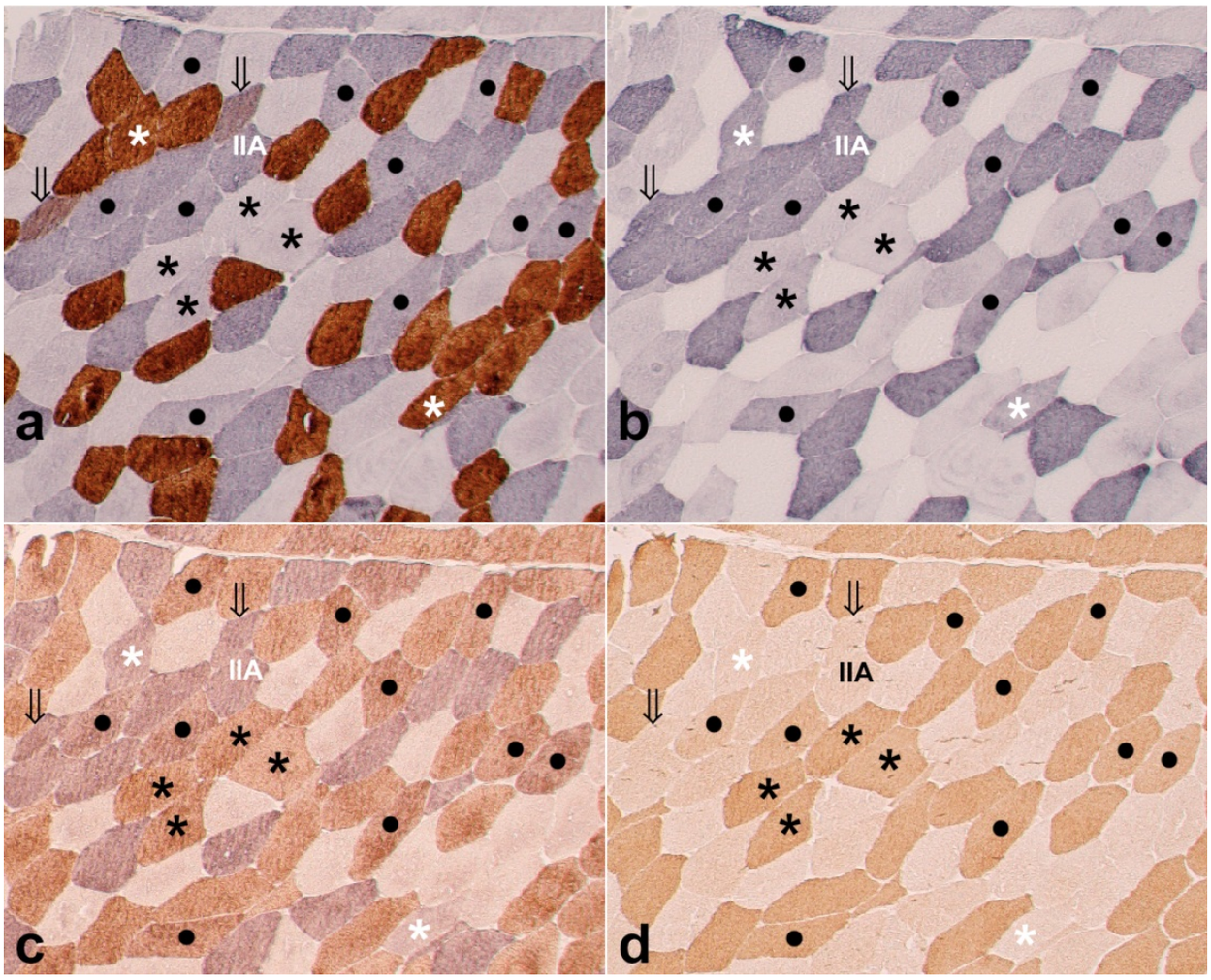

Figure 4. Double immunoenzyme staining according to Raheem et. al. (14) and double immunoenzyme staining of fast (IIA and IIX) fibres compared to phenotyping of muscle fibres on serial sections. (a): Double immunoenzyme staining method according to Raheem et al. (14) successively applying antibodies against MyHC-I (BAD5) and antibodies against MyHC-IIA+ MyHC-IIX (A4.74). (c): Double immunoenzyme staining of fast fibres successively applying antibodies against MyHC IIX (6H1) and antibodies against MyHC-IIA+ MyHC-IIX (A4.74). (b) and (d) are immunohistochemical reactions with individual antibodies. (b): against MyHC-IIA+ MyHC-IIX (A4.74) visualized with alkaline phosphatase. (d): against MyHC-IIX (6H1) visualized with horseradish peroxidase. Fibres labelled with *black* asterisks (*) are IIX fibres. Fibres labelled with black dots (•) are hybrid IIA/IIX fibres. Fibres labelled with arrows are hybrid IIA/I (IIA>I) fibres. Fibres labelled with *white* asterisks (*) are hybrid IIA/I (IIA~I) or (I>IIA) fibres (upper and lower fibre).

4. Discussion

Similarly to Smerdu and Soukup (16) we have demonstrated in human vastus lateralis muscle that A4.74 antibodies which should be specific for muscle fibres expressing MyHC-IIA weakly stain also MyHC-IIX expressing fibres. The latter were not hybrid IIX/IIA fibres, but pure IIX fibres, since the corresponding fibres were negative with BF-35 antibodies, which stain all muscle fibres, except fibres expressing MyHC-IIX (2, 19). Smerdu and

Soukup (16) showed that muscle fibres stained weakly with A4.74 antibodies and strongly with antibodies specific for MyHC-IIX (6H1) in humans expressed exclusively MyHC-IIX mRNA, which further confirms that weakly stained fibres are pure IIX fibres. Recently, Bloemberg and Quadrilatero (20) showed that SC-71 antibody, another antibody directed against MyHC-IIA, also cross-reacts with IIX fibres, as already also described by Smerdu and Soukup (16). A4.74 antibodies thus stain dark fibres expressing MyHC-IIA (fibres IIA and hybrid fibres IIA/IIX) and stain intermediate fibres expressing MyHC-IIX (Fig.1a, Fig. 2a, Fig. 3b, and Fig. 4b).

Both techniques for phenotyping of muscle fibres on a single section, triple immunofluorescent method (13) and double immunoenzyme staining method (14) did not directly identify IIX fibres (by antibodies to the MyHC-IIX). We have demonstrated that hybrid fibres IIX/IIA are missed by double immunoenzyme staining method (14) as they are misclassified as IIA fibres (Fig. 3a). Similar would hold also for the triple immunofluorescence method mentioned above, as antibodies to the MyHC-IIX were not used. The colour of the reaction product in hybrid IIX/IIA fibres, which are not recognised as such, does not change (remains blue) during superposition on immune reaction with antibodies directed against MyHC-I, since MyHC-I are not expressed in these hybrid fibres. We have clearly demonstrated (Fig. 3) that the staining intensity of MyHC-IIA in hybrid fibres can be as intensive as in pure IIA fibres, but to prove that such a fibre is a hybrid fibre, serial sections including immunohistochemical reaction to MyHC-IIX must be analysed.

The hybrid fibres IIA/I can be demonstrated with this method, if the proportion of MyHC-IIA predominates (IIA>I) in hybrid fibres (fibres labelled with arrows in Fig. 3), but if proportions are similar (IIA~I) or MyHC-1 predominates (I>IIA), such fibres are missed (fibres labelled with white asterisks in Fig. 3).

We conclude that hybrid fibres can be reliably demonstrated only on serial muscle sections. They may be demonstrated on a single section, if staining with antibodies directed to both MyHC expressed in hybrid fibres are used, but this may not be valid for all proportions of individual MyHC in hybrid fibres. By no means can hybrid fibres co-expressing three types of different MyHC be demonstrated by the double staining method. Nevertheless, the method of Raheem et al. (14) was not developed for demonstration of hybrid fibres, but as an alternative to routine ATP-ase staining in diagnostics of myopathies and can be particularly useful for demonstrating of advanced atrophic fibres in conditions as myotonic dystrophy type 2 (14) which justifies it as a good method for routine diagnostics.

The double immunoenzyme staining method (14) in addition presents well slow fibres expressing MyHC-I and all fast fibres, expressing either MyHC-IIA or MyHC-IIX, but IIA and hybrid IIA/IIX fibres can not be distinguished. The distinction between fast and slow fibres can be accomplished already by staining of muscle section with antibodies to MyHC-I alone. Double immunoenzyme staining offers more clear presentation of fast fibres' contours, which may be as “unstained” fibres difficult to present in sections stained only with antibodies to MyHC-I (Fig. 3c), especially in clusters of fast fibres. Alternatively, antibodies to MyHC-IIA, as A4.74 and SC-71, which also cross-react with MyHC-IIX (16, 20)

can also be used to separate fast and slow fibres on a single section. In this case slow fibres are unstained (Fig. 3b or Fig. 4b). Obviously the advantage of the double immunoenzyme staining of Raheem et al. (14) is in (i) the clear simultaneous presentation of fast and slow fibres and (ii) presentation of "pure" IIX fibres (not co-expressing other MyHC isoforms); the latter is possible due to cross-reactivity of antibodies to fast fibres (A4.74 and SC-71) as mentioned above, but identification of hybrid fibres is unsatisfactory and therefore also the separation of IIA fibres and hybrid fibres co-expressing MyHC-IIA and MyHC-IIX.

In our own modification of double immunoenzyme staining method in which we superimposed two immunohistochemical reactions for demonstration of fast fibres (IIA and IIX), we demonstrated that IIX fibres can not be reliably distinguished from hybrid fibres IIX/IIA, since the differences in colours were negligible (Fig. 4c). We consider this as an additional proof that demonstration of hybrid fibres on a single section is tricky, even if two corresponding antibodies to MyHC isoforms expected to be co-expressed in hybrid fibres are used. The mixed colour of the two reaction products may simply reflect the dominance of one colour and may not be correlated to the proportions of MyHC isoforms. In Fig. 4c in hybrid IIX/IIA fibres the brownish colour of MyHC-IIX isoforms completely covered the blue colour of the MyHC-IIA isoforms. Similar is valid also for the demonstration of hybrid fibres I/IIA (I~IIA and I>IIA), labelled with white asterisks in Fig. 3, in which strong brown colour of the reaction product of MyHC-I also completely covered the blue colour of MyHC-IIA. In addition, with double immunoenzyme staining technique it is assumed, that first primary antibody (in our case BAD-5) is completely coupled with the secondary antibodies (in our case peroxidase-conjugated secondary antibodies). However, the cross-immunoreactivity of secondary phosphatase-conjugated antibodies with the primary antibodies (BAD-5) can not be excluded. As expected we could not identify hybrid IIA/I fibres by superimposing two immunohistochemical reactions to fast fibres, as antibodies to MyHC-I were not used.

Recently Bloemberg and Quadrilatero (20) reported on rapid determination of myosin heavy chain expression on a single section using multicolour immunofluorescent method on animal and human muscle. In human muscle they used three different primary antibodies against different MyHC isoforms, including antibody to MyHC-IIX (18). The method takes the advantage of the fact that antibodies against MyHC isoforms, belong to different subtypes of immunoglobulins: IgG2b (against MyHC-I), IgG1 (against MyHC-IIA) and IgM (against MyHC-IIX). Muscle fibres immunoreactive with particular antibody can be visualised with secondary antibodies specific for only one subclass of immunoglobulins and labelled with different fluorophores. The authors were able to demonstrate major fibre types and hybrid I/IIA and IIX/IIA fibres. However, the identification of hybrid IIX/IIA fibres on merged images is not straightforward, but hybrid fibres can be recognised, if analysing all single channel images (see their Fig. 5). Also the reported percentage of hybrid fibres in this study (20) is much lower than in the previous studies using single fibre electrophoresis (11, 12). It seems that the advantage of multicolour fluorescent method is the possibility to analyse single channel images, that is similar to analysis of individual serial sections, to

identify especially hybrid fibres with two MyHC isoforms (20) and possible also with three MyHC isoforms, if in doubts on merged images.

If different fluorophores are directly coupled to primary antibodies more than three labelled antibodies can be used on the same section (13) and antibodies as MyHC-neonatal and MyHC-embryonic, which are expressed during development and regeneration, can be demonstrated on the same section with other myosin antibodies. In addition when using multicolour immunofluorescent method correlation between metabolic profile of muscle fibres and contractile characteristics can be studied more easily (20). Obviously metabolic profile can be identified in next two consecutive muscle sections where tracing of muscle fibres is easier than in more distant muscle sections. Adjustments of orientations of muscle profiles in distant sections are sometimes necessary with specialised computer programs for image analysis as (21). Theoretically total of six emission/detection channels can be used (13), if primary antibodies are directly coupled with fluorophores which have very narrow emission spectrum. Besides different antibodies to MyHC isoforms antibodies to other proteins labelled with distinct fluorophores may be included and the results correlated to contractile profiles of muscle fibres. However, when analysis is based on mixed colours full attention must be paid to possible misinterpretation of staining artefacts as hybrid fibres.

5. Conclusion

A good agreement was found between muscle fibre phenotyping on serial muscle sections and muscle fibre phenotyping on a single section by double immunohistochemical staining method, if muscle fibres expressed a single MyHC isoform. The identification of hybrid fibres expressing two different MyHC isoforms requires the superposition of both antibodies directed against MyHC isoforms which are expressed in hybrid fibres. Even then, the identification of hybrid fibres may not be successful: it depends on relative proportions of individual MyHC isoforms. In addition cross reactivity of secondary antibodies with primary antibodies may hinder identification of hybrid fibres. Hybrid fibres expressing three different MyHC isoforms can not be presented with the double immunohistochemical staining method.

Muscle fibre phenotyping on serial muscle sections was definitely superior to the double staining method performed on a single section, when *hybrid* fibres were present.

The multicolour immunofluorescent method applying three different antibodies to major adult MyHC isoforms in human skeletal muscle (MyHC-I, MyHC-IIA, and MyHC-IIX) on a single muscle section may be a promising tool in simplifying muscle phenotyping and in identifying hybrid fibres. Primary antibodies to MyHC isoforms directly labelled with fluorophores enable simultaneous use of larger number of antibodies against MyHC as mentioned above. Presentations of MyHC isoforms and other proteins on the same section would also be an interesting application of the multicolour fluorescent method in further studies. Nevertheless, combination of more reactions on a single section means also combination of all artefacts that accompany individual procedures. Therefore all combined

reaction procedures must be applied with full precaution; otherwise they might turn into misinterpretations of artefacts.

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