

## PLASTICITY OF SKELETAL MUSCLE STUDIED BY STEREOLOGY

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### ABSTRACT

The present contribution provides an overview of stereological methods applied in the skeletal muscle research at the Institute of Anatomy of the Medical Faculty in Ljubljana. Interested in skeletal muscle plasticity we studied three different topics: (i) expression of myosin heavy chain isoforms in slow and fast muscles under experimental conditions, (ii) frequency of satellite cells in young and old human and rat muscles and (iii) capillary supply of rat fast and slow muscles. We analysed the expression of myosin heavy chain isoforms within slow rat soleus and fast extensor digitorum longus muscles after (i) homotopic and heterotopic transplantation of both muscles, (ii) low frequency electrical stimulation of the fast muscle and (iii) transposition of the fast nerve to the slow muscle. The models applied were able to turn the fast muscle into a completely slow muscle, but not vice versa. One of the indicators for the regenerative potential of skeletal muscles is its satellite cell pool. The estimated parameters, number of satellite cells per unit fibre length, corrected to the reference sarcomere length ( $N_{sc}/L_{fib}$ ) and number of satellite cells per number of nuclei (myonuclei and satellite cell nuclei) ( $N_{sc}/N_{nucl}$ ) indicated that the frequency of M-cadherin stained satellite cells declines in healthy old human and rat muscles compared to young muscles. To access differences in capillary densities among slow and fast muscles and slow and fast muscle fibres, we have introduced Slicer and Fakir methods, and tested them on predominantly slow and fast rat muscles. Discussing three different topics that require different approach, the present paper reflects the three decades of the development of stereological methods: 2D analysis by simple point counting in the 70's, the disector in the 80's and virtual spatial probes in the 90's. In all methods the interactive computer assisted approach was utilised.

Keywords: capillaries, myosin heavy chains, plasticity, satellite cells, skeletal muscle, stereology.

### WHAT IS THE BASIS OF SKELETAL MUSCLE PLASTICITY?

Skeletal muscle is composed of muscle fibres of several types that are responsible for muscle contraction. The proportion of different fibre types determines the speed of contraction. Capillary network supplies muscle fibres with nutrients and oxygen: The more energy the muscle fibres can take from oxygenation the more fatigue resistant they are. Skeletal muscle is a very plastic structure, which means that it is able to adapt its structure and function to different workloads imposed on it. Different types of training transform fast contracting fast fatigable muscle fibres into slower contracting fatigue resistant fibres or else slow contracting fatigue resistant fibres into faster contracting fast fatigable fibres. Intensive training or different diseases can result in muscle injury and degeneration. However, skeletal muscle is a very plastic structure with a great regenerative potential provided by satellite cells, which are the

only cells in skeletal muscle that can multiply and provide new muscle fibres. They play an essential role in muscle regeneration and repair.

The aim of this study was to find out how different experimental conditions affect regeneration and repair of different muscle fibre types and whether it is possible to cause transformation of a slow contracting muscle into a fast contracting muscle and vice versa.

In the present paper we present our methodological approach to answer the following questions:

1. Is it possible to transform a slow contracting muscle into a completely fast contracting muscle and vice versa?
2. Is the satellite cell pool reduced during ageing?
3. What is the difference in the capillary network among slow and fast muscles and among slow and fast muscle fibres, respectively? Which is the best approach to study the remodelling of the capillary network in experiment and disease?

## IS IT POSSIBLE TO TRANSFORM A SLOW CONTRACTING MUSCLE INTO A COMPLETELY FAST CONTRACTING MUSCLE AND VICE VERSA?

To answer this question we have studied the expression of specific contractile protein myosin heavy chain (MyHC) and its isoform pattern within individual muscle fibres, which determines the contraction velocity of individual fibre types. The contraction velocity declines gradually along the following pathway: MyHC-2b ↔ MyHC-2x/d ↔ MyHC-2a ↔ MyHC-1(β slow). MyHC-2b expressing fibres are the fastest and MyHC-1(β slow) expressing fibres are the slowest (Botinnelli *et al.*, 1994). Different workloads, training, electrical stimulation or inactivity, provoke fibre type transformations that occur along the previously mentioned pathway in both directions (Pette and Staron, 1997). During the process of fibre type transitions, hybrid fibres appear, *i.e.*, fibres that express two or more MyHC isoforms (*e.g.*, MyHC-2a/MyHC-1 etc.) (Pette and Staron, 1997). Volume density of different pure and hybrid fibres gives an insight into the adaptation of skeletal muscle to the changed physiological or experimental conditions.

All experiments were done with female Wistar rats on predominantly slow soleus and predominantly fast extensor digitorum longus muscles. Muscles were injured by treatment with the myotoxic agent bupivacaine that caused a complete degeneration of skeletal muscle and left intact only satellite cells (Mauro, 1961). Satellite cells multiply, provide myoblasts that fuse and form new muscle fibres. The following experimental models were applied:

1. Homotopic and heterotopic transplantation, *i.e.*, transplantation of the slow or the fast muscle into its own location (homotopic) and transplantation of the slow muscle into the bed of the fast muscle or fast muscle into the bed of the slow muscle (heterotopic transplantation).
2. Continuous low frequency electrical stimulation (10 Hz, 24 hrs per day, 60 days).
3. Transposition of the fast nerve to the slow soleus muscle.
4. Non treated control muscles served as control.

The experimental design is in detail described elsewhere (Snoj-Cvetko *et al.*, 1996a, b; Eržen *et al.*,

2000). In short, myosin heavy chain (MyHC) isoforms (β slow, 2a, 2x/d and 2b) were detected in 10 μm transverse sections by the indirect immunoperoxidase method, applying monoclonal antibodies BAD5, SC71, BF35 and BFF3 (Schiaffino *et al.*, 1986). The reaction was visualized by the brown reaction product of diaminobenzidine.

Figs. 1a,b show MyHC isoform expression in control slow soleus muscle, where MyHC-1 predominates. Expression of MyHC isoforms in the fast extensor digitorum longus muscle is presented in Figs. 1c-f.

We counted points hitting individual MyHC isoform positive fibres (Weibel, 1979) within the whole cross-section in the middle of the muscle belly applying a computer. In every model at least five muscles (from five rats) were analysed within a control or experimental group. Area density of fibres expressing different MyHC isoforms was calculated. Area density of fibres expressing different MyHC isoforms showed that the ratio among MyHC isoforms was changed in different models.

We succeeded to transform the fast muscle into a complete slow one, however, transformation of the slow muscle into a fast one was never complete.

The direction of transformation was different in different models. Transformation towards slower fibre types (Fig. 2) was achieved after (i) transplantation of the fast muscle into the bed of the slow muscle and in (ii) low frequency electrical stimulation. However, after (i) transplantation of the slow muscle into the bed of the fast muscle and (ii) transposition of the fast nerve to the slow muscle, transitions towards faster fibre types occurred (Fig. 3) (Snoj-Cvetko *et al.*, 1996a, b; Eržen *et al.*, 1999; 2000).

Since the fibre type transition from fast to slow was almost complete, but not vice versa, we assume that the satellite cell pool is either different in fast and slow muscles or our experiments did not trigger slow to fast transformation.

The stereological approach that we applied in experimental models is a valuable tool for quick evaluation of the remodelling and transformation of fibre types. It provides an insight into the direction of fibre type transition which reflects the adaptation of skeletal muscle to changed functional demands.

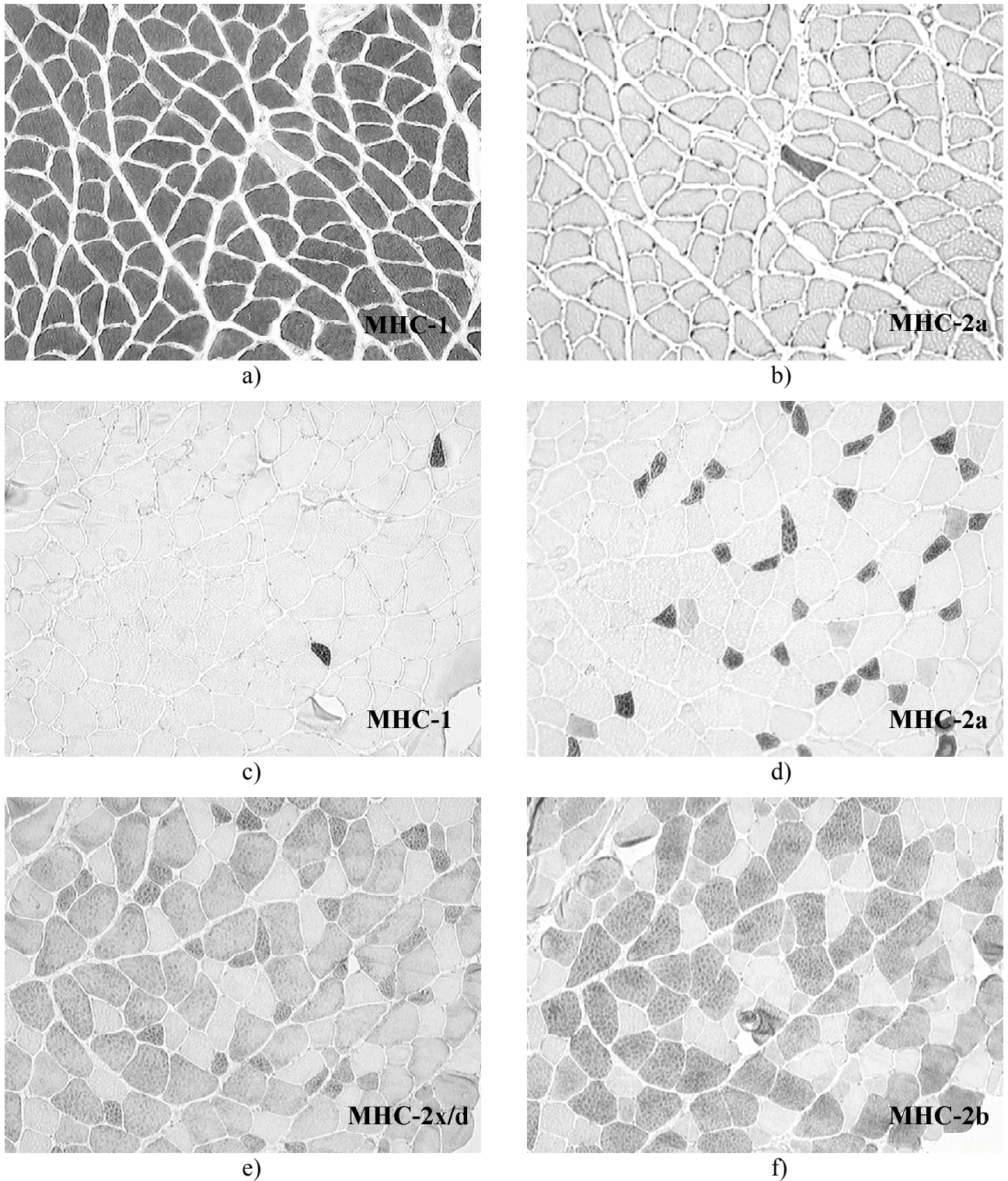


Fig. 1. Expression of myosin heavy chain isoforms (MyHC) in the rat slow soleus (a,b) and fast extensor digitorum longus muscle (c-f). MyHC-1 (a,c), MyHC-2a (b,d), MyHC-2x/d (e), MyHC-2b (f).

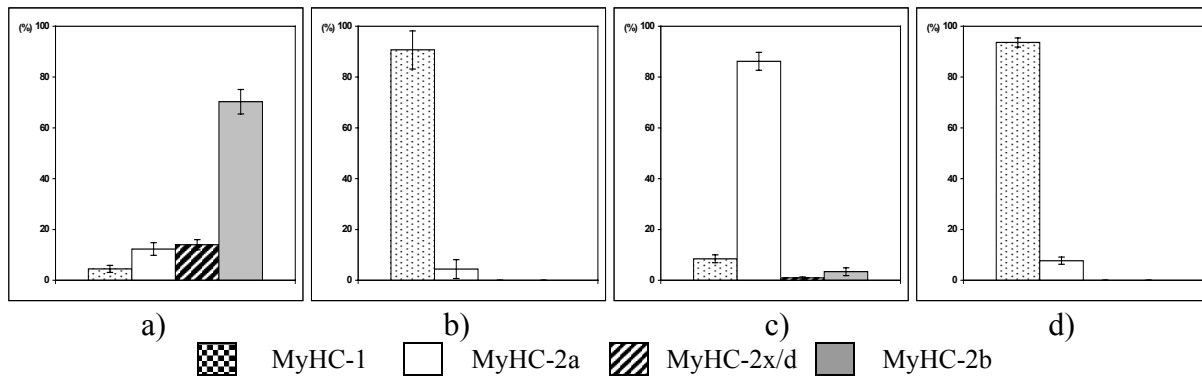


Fig. 2. Transformation of the fast extensor digitorum longus (EDL) muscle into a muscle with slower characteristics: a) control EDL muscle, b) bupivacaine injected EDL muscle transplanted into the location of the soleus muscle, c) bupivacaine injected low frequency stimulated EDL muscle, d) control soleus muscle.

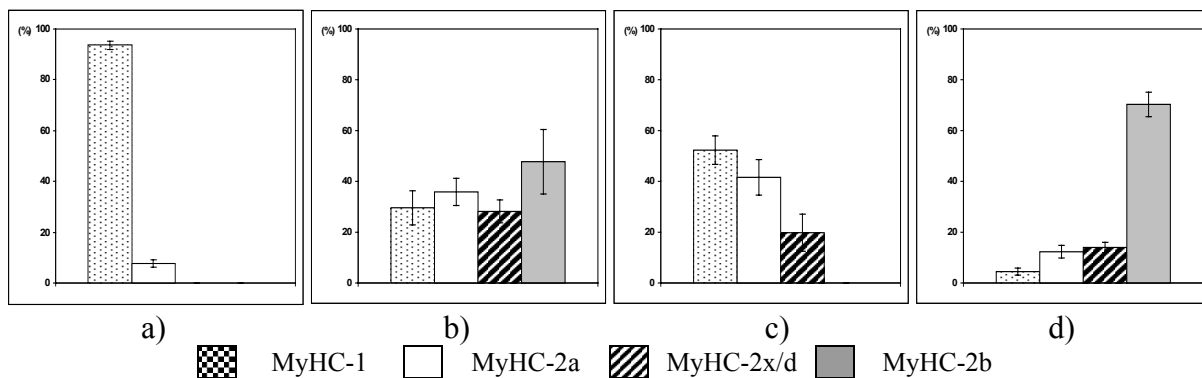


Fig. 3. Transformation of the slow soleus (SOL) muscle into a muscle with faster characteristics: a) control SOL, b) bupivacaine injected SOL transplanted into the location of the fast EDL muscle, c) transposition of the 'fast' nerve to the bupivacaine injected SOL, d) control EDL.

## IS THE SATELLITE CELL POOL REDUCED DURING AGEING?

Satellite cells are quiescent cells positioned between the plasmalemma and the basal lamina of skeletal muscle fibres. They are the only cells in skeletal muscle that can divide and give rise to new muscle fibres. However, the number of divisions that each satellite cell can undergo during its life span is limited. Finally, the capability of the muscle to regenerate depends not only on the number of satellite cells but also on the number of divisions that are still left.

To answer the question whether satellite cell number declines during ageing, autopsy samples of vastus lateralis muscles of six young (aged  $28.7 \pm 2.3$  years) and six old men (aged  $70.8 \pm 1.3$  years) and five young and five old rat (three months and two years) were systematically sampled (see Sajko *et al.*, 2002 for

details). Satellite cells were visualised by binding of M-cadherin antibody marked with fluorescent fluorochromes. Stacks of perfectly registered optical images were captured by a confocal microscope.

Satellite cells and nuclei were counted by the disector method (Gundersen *et al.*, 1986; Sterio, 1989), applying the DISECTOR software (Tomori *et al.*, 2001) (Fig. 4).

In this research we introduced a new parameter, number of satellite cells per unit fibre length corrected to a reference sarcomere length (Nsc/Lfib), a parameter that is not influenced either by the fibre diameter and volume or by the contraction state of the muscle, therefore it can be compared among different muscles under different physiological, experimental and pathological conditions. The frequency of satellite cells was additionally estimated by the number of satellite cells per number of myonuclei (Nsc/Nnucl),



number of satellite cells per unit muscle volume ( $N_{sc}/V_m$ ) and number of satellite cells per fibre number ( $N_{sc}/N_{fib}$ ) within a  $10\ \mu\text{m}$  transverse section. The latter is nothing but  $N_{sc}/L_{fib}$  where  $L_{fib}$  was  $10\ \mu\text{m}$ . We used this parameter for the comparison with other studies describing changes in satellite cell frequency and frequency of myonuclei in physiological, pathological or experimental conditions (*e.g.*, Kadi *et al.*, 2000) where the authors counted profiles instead of particles. Parameters  $N_{sc}/L_{fib}$  and  $N_{sc}/N_{nucl}$  were corrected to a reference sarcomere length. The estimator  $N_{sc}/N_{fib}$ , however, was not corrected. Compared to previously published studies in this field, we collected our data with systematic sampling on sufficiently large samples and unbiased design based methods for counting satellite cells and nuclei were applied. The methodology was in detail described in Sajko *et al.* (2002).

The main issue of this study was that the frequency of M-cadherin stained satellite cells estimated by  $N_{sc}/L_{fib}$  and  $N_{sc}/N_{nucl}$ , declines significantly ( $p < 0.01$ ) during ageing in rat (Rudež *et al.*, 2004) and in human (Table 1) whilst the number of myonuclei remains unchanged. However, the remaining regenerative potential of the aged muscle depends not only on the satellite cell frequency but also on the number of the remaining divisions (*cf.* Sajko *et al.*, 2004).

We assume that our data on the frequency of satellite cells in human vastus lateralis muscle as well in rat extensor digitorum longus muscle, will serve as standard for further studies on the regenerative potential of skeletal muscles. Number of satellite cells per  $\text{cm}^3$  of muscle tissue, which roughly corresponds to number of satellite cells per gram of muscle tissue is an important information for those working with tissue cultures.

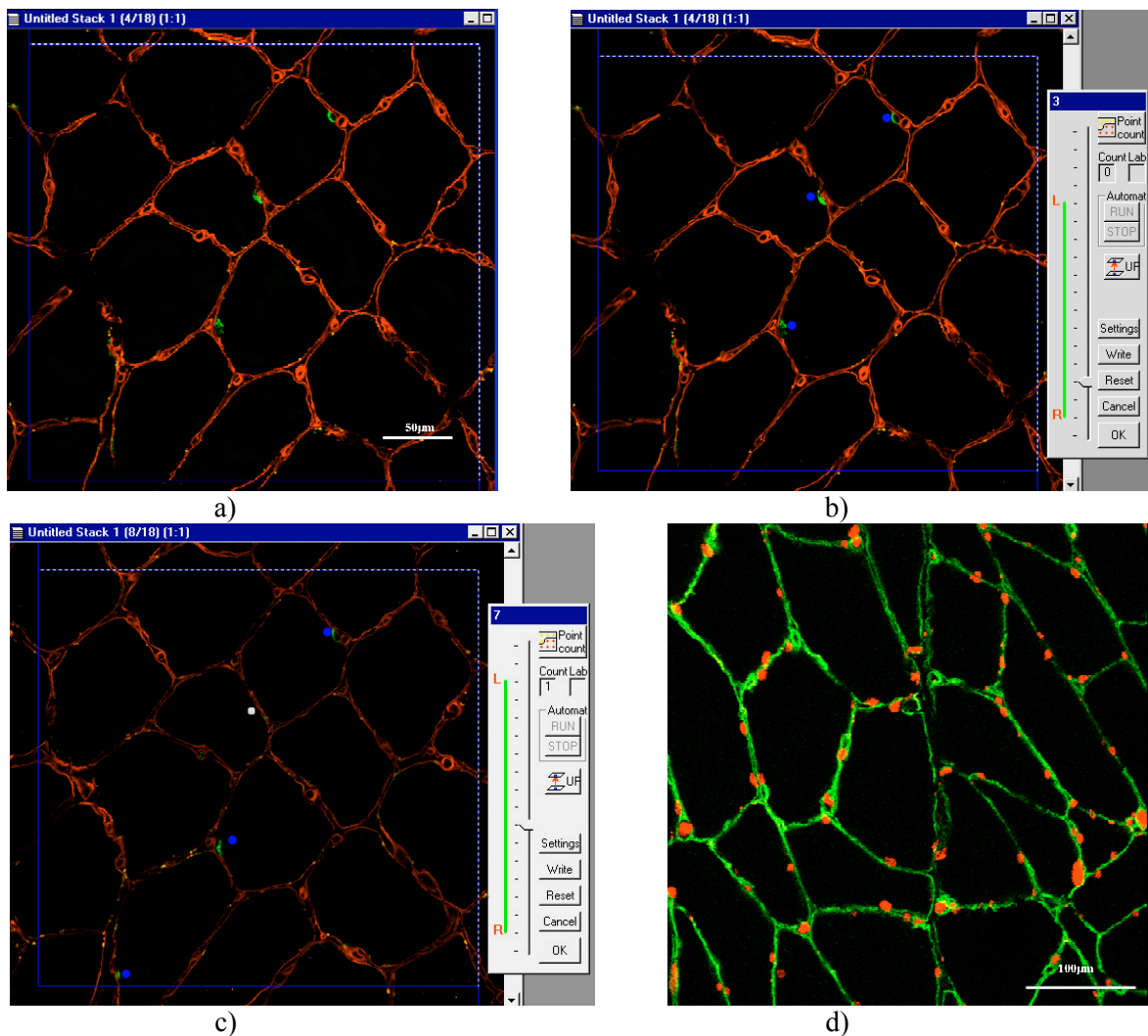


Fig. 4. Counting of satellite cells and nuclei with the DISEKTOR software. a) satellite cells stained with M-cadherin (green) and basal lamina of muscle fibres with laminin (red), b) satellite cells are marked (blue) in an optical section within a stack of images, c) the satellite cell that disappeared from the field of view is marked (white) and counted. d) Myonuclei (red) were counted under the basal lamina (green) of each fibre.

Table 1. *The frequency of satellite cells in human vastus lateralis muscle during ageing.*

		Age	Nsc/Lfib (mm <sup>-1</sup> )	Nsc/Nnucl %	Nsc/Nfib % × 10 <sup>-2</sup>	Nsc/Vm cm <sup>3</sup> × 10 <sup>5</sup>
Young N = 6	Mean	28.7	1.85*	2.02*	2.40	5.08
	SEM	2.3	0.23	0.33	0.40	1.02
Old N = 6	Mean	70.8	1.19*	1.09*	1.60	3.02
	SEM	1.3	0.06	0.12	0.10	0.39

\*p ≤ 0.05

## WHAT IS THE DIFFERENCE IN THE CAPILLARY NETWORK AMONG SLOW AND FAST MUSCLE?

Capillaries supply skeletal muscle with nutrients and oxygen. The more oxygen the muscle needs for its work the denser is its capillary network. The question is what does the expression ‘dense capillary network’ mean and which is the best parameter to describe this feature? Moreover, skeletal muscles are usually a mosaic of muscle fibres with different contractile and metabolic characteristics. Very often, especially in muscles of experimental animals and in patients suffering from different neuromuscular diseases or disorders, muscle fibres exhibit a large span of fibre diameters. Additionally, skeletal muscles are very versatile and plastic structures. They are able to adapt to different workloads imposed on them by remodelling their pattern of fibre types. Slow fibres transform to fibres with more pronounced fast characteristics and vice versa, giving rise to hybrid fibres. More glycolytic fibres change the metabolic profile toward more oxidative and vice versa. Angiogenesis is presumably more pronounced around some fibres than around the others. Finally, estimators that do not distinguish among fibre types blur the variability in fibre type composition of individual muscles.

Capillary supply to the muscle fibres has been evaluated either by counting capillary profiles per muscle cross-sectional area, per fibre number or around a fibre. Further, specific capillary per fibre ratio (sharing factor) (*i.e.*, number of fibres that share one capillary or number of capillaries that share one fibre) was determined (*cf.* Bennet *et al.*, 1991). Better parameters consider fibre area or perimeter, like local capillary to fibre ratio (*i.e.*, area supplied by one capillary or capillary domain), local capillary density (*i.e.*, capillary domain normalized by the fibre size) (Egginton and Ross, 1989), and capillary to fibre contact (*i.e.*, percent of muscle fibre perimeter in contact with the capillary membrane) (Sullivan and Pittman, 1987).

Since skeletal muscle is an anisotropic tissue where muscle fibres running more or less parallel dictate the preferential axis and all the other tissue constituents more or less follow this preferential axis, to obtain reliable results it is necessary either to randomise sections or randomise test systems. The most often suggested methods for randomising sections are orientator (Mattfeldt *et al.*, 1990) or isector (Nyengaard and Gundersen, 1992). Providing high quality muscle sections by the both methods is quite a hard task. Moreover, in the isotropic sections it is not easy to evaluate the relation of capillaries to the muscle fibres as all kind of muscle profiles, from cross-sectioned to more or less oblique can be found.

Another approach is sectioning of the same specimen in two perpendicular planes and subsequent correction for anisotropy, as suggested by Mathieu *et al.* (1983) (see also Mattfeldt and Mall, 1984). Estimation of Lv and Sv is also possible from sections cut in three perpendicular directions, applying ortrips (orthogonal triplet probes) suggested by Mattfeldt *et al.* (1985). In routine diagnosis and in research, skeletal muscle tissue is usually kept frozen. This is a precondition for demonstrating the activity of different enzymes which is crucial for diagnostic purposes. Cutting frozen muscle tissue in two or three perpendicular directions, however, as required in the former procedures, is quite demanding and many profiles can be lost.

In contrast to the most previous reports on the capillary density that are based on the data obtained from 2-D studies, in our research 3-D virtual probes, introduced by Larsen *et al.* (1998), were superimposed to stacks of perfectly registered optical images captured by a confocal microscope.

Applying the ‘POINT GRID’, ‘FAKIR’ and ‘SLICER’ plugins of the Ellipse programme system (Tomori, 2002) we estimated fibre volume and surface area of individual fibre tube as well as the length of capillaries supplying individual fibres. Details of the methods are explained elsewhere (Kubínová and Janáček, 1998; Kubínová *et al.*, 2001; Čebašek *et al.*, 2004).

In our first studies we evaluated the capillary network in the rat soleus muscle (SOL), a slow contracting predominantly oxidative muscle and in extensor digitorum longus muscle (EDL), a fast contracting predominantly oxidative-glycolytic muscle.

Capillaries were recognised by immunofluorescent staining of fibronectin (Eržen and Maravić, 1992).

The length of capillaries ( $L(cap)$ ) adjacent to individual fibres was estimated by counting transsections of capillaries with three parallel equidistant virtual test planes, perpendicular to each other, applying the SLICER method (Fig. 5). The surface area of each

muscle fibre in the given stack ( $S_i(fib)$ ) was estimated by counting intersections of the fibre surface with a cubic spatial grid consisting of three mutually perpendicular FAKIR probes (Fig. 6), *i.e.*, parallel test lines resembling nails of a fakir bed piercing the surface (Kubínová and Janáček, 1998). In both fakir and slicer methods, isotropic uniform random (IUR) orientation of the virtual spatial grid consisting of fakir or slicer probes, respectively, was guaranteed by a random generation of the grid origin and a random rotation of the grid (for details see Kubínová *et al.*, 2002) using special FAKIR and SLICER programmes.

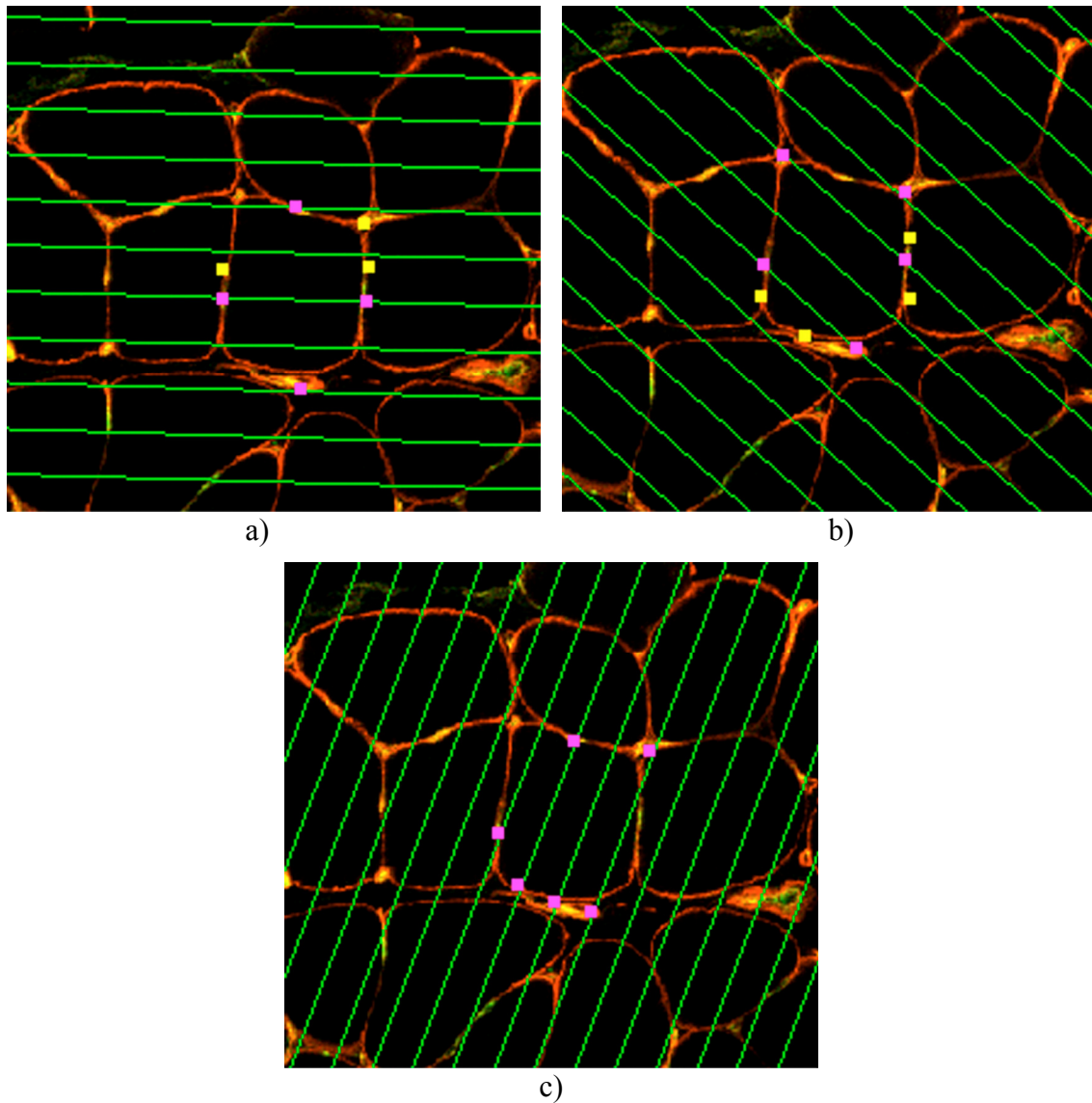


Fig. 5. Estimating capillary length of muscle fibres by counting transsections with virtual test plane in three mutually perpendicular directions (a, b and c).

The volume of each muscle fibre in the given stack ( $V_i(\text{fib})$ ) was estimated using a cubic spatial grid of points which is a modification of the Cavalieri principle (Fig. 7).

We have proved that the SOL muscle has larger length of capillaries per fibre length ( $L(\text{cap})/L(\text{fib})$ ) than the EDL muscle. On the other hand, these two muscles have a similar ratio of capillary length to fibre surface area ( $L(\text{cap})/S(\text{fib})$ ) and volume ( $L(\text{cap})/V(\text{fib})$ ) (Kubínová *et al.*, 2001) (Table 2).

We assume that the developed immunohistochemical (Čebašek *et al.*, 2004) as well as stereo-

logical methods (FAKIR and SLICER) and parameters can successfully be applied in our further studies on the remodelling of the capillary network of muscle fibres in slow and fast skeletal muscles. The methods introduced enable estimation of different variables within skeletal muscle tissue, muscle fibres or muscle fibre types as the reference space. Additionally, the acquisition of perfectly registered stacks of successive optical images generated by the confocal microscope, provides a basis for further 3-D reconstruction of capillaries and muscle fibres or capillaries and muscle fibre types. Nevertheless, all

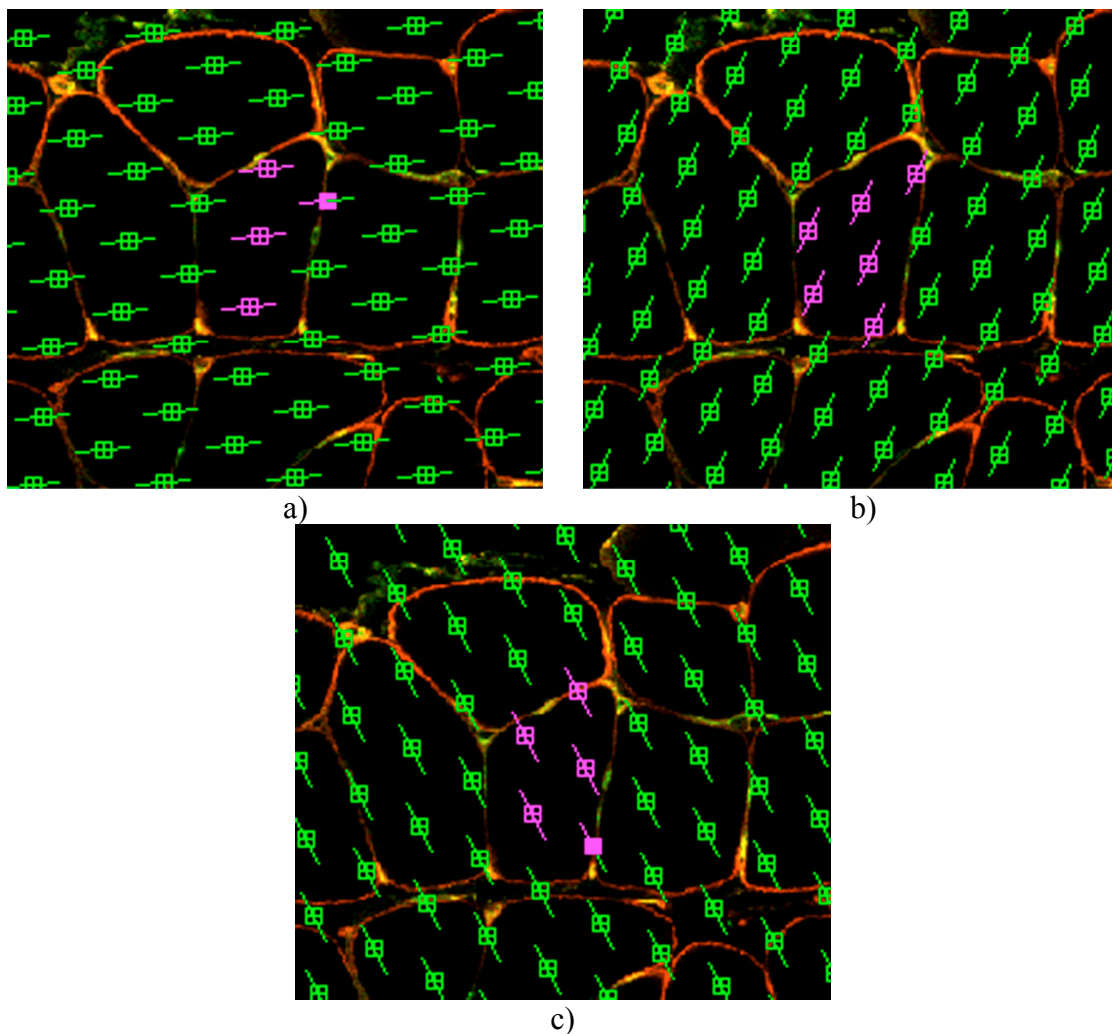


Fig. 6. Estimating muscle fibre surface area by counting intersections with virtual Fakir probes in three mutually perpendicular directions (a, b and c).

Table 2. Capillary supply in the rat soleus (SOL) and extensor digitorum longus (EDL) muscles (mean  $\pm$  SD).

	soleus	extensor digitorum longus
$L(\text{cap})/L(\text{fib})$	$5.772 \pm 1.103^*$	$3.808 \pm 0.38^*$
$L(\text{cap})/V(\text{fib})$ ( $\text{mm}^{-2}$ )	$2884 \pm 829$	$3708 \pm 873$
$L(\text{cap})/S(\text{fib})$ ( $\mu\text{m}^{-1}$ )	$0.0341 \pm 0.008$	$0.0309 \pm 0.005$

\* $p \leq 0.05$



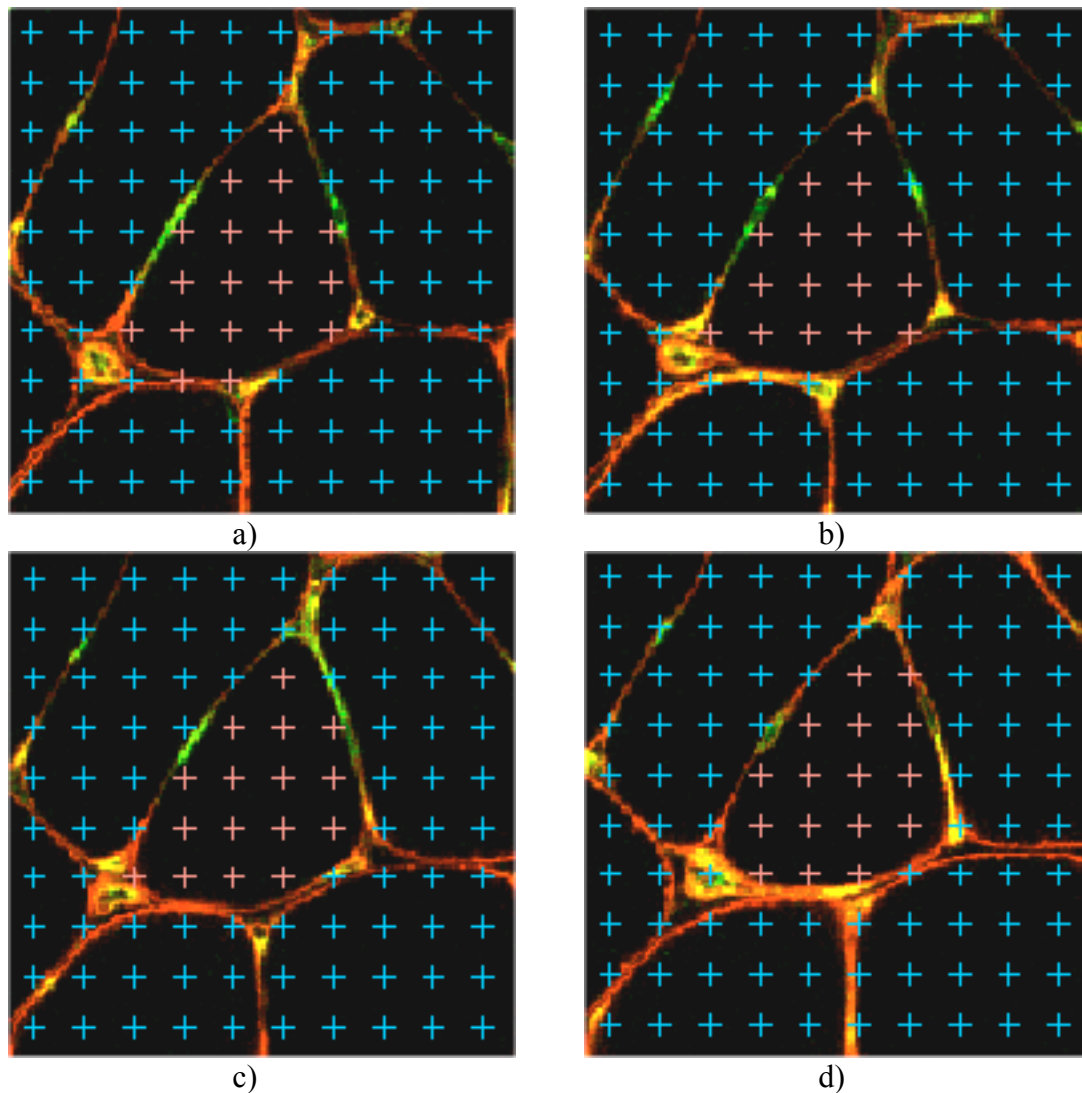


Fig. 7. Defining the absolute volume of a muscle fibre from four levels (a-d) applying the Cavalieri principle.

the introduced variables need to be tested by studying remodelling of the capillary network in different physiological, pathological and experimental conditions.

We are convinced that in skeletal muscles the capillary density and topology change differently under various conditions of hypoxia or workloads. This remodelling is more reflected in other capillary characteristics than just the capillary length per tissue volume ( $L_{cap}/V_{tissue}$ ).

In conclusion, stereological methods are still not sufficiently recognized in skeletal muscle research and little attention is paid to proper sampling design. In this paper we present a set of methods that we have found useful when studying plasticity of skeletal muscle.

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