A novel staining method for quantification and 3D visualisation of capillaries and muscle fibres

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The aim of this study was to introduce a combined fluorescent staining that clearly demonstrates capillaries and distinguishes them from the basal lamina of muscle fibres in skeletal muscle tissue. The triple staining with CD31, *Griffonia (Bandeira) simplicifolia* lectin (GSL I) and laminin efficiently distinguishes vascular endothelium from the basal lamina of skeletal muscle fibres in physiological and pathological conditions. The presented triple staining method has several advantages, which facilitate quantitative analysis of the capillary network, and its relation to individual muscle fibres.

Key words: 3D measurement, capillaries, confocal microscopy, immunohistochemistry, lectin histochemistry, skeletal muscle.

Capillaries are essential for the survival, normal function and adaptation of muscle tissue to changing workloads (Hudlicka 1991, Green et al. 1999). Traditionally, the morphometric characteristics of capillary network were measured in two dimensions (2D) from transverse or longitudinal tissue sections (Mathieu et al. 1983; Egginton and Ross, 1989).

The morphological analysis in 2D gives only a general description of the capillary bed since the capillaries form a network in three dimensions (3D). The precise topology and course of capillary network can only be described by 3D analysis of muscle tissue capillaries. Therefore, visualisation and analysis of capillaries in 3D are the methods of choice for detection of the early morphological changes in the capillary bed.

Clear visualization of capillaries is a prerequisite for quantitative analysis. Detailed studies of skeletal muscle capillary network are conventionally made from thin tissue sections. Histological methods like haematoxylin eosin or toluidine blue staining (Kiernan JA, 1990) require the presence of erythrocytes in the vessel lumen. Consequently, capillaries are often poorly recognisable because of erythrocytes being scarcely distributed in capillaries of skeletal muscle (Damon and Duling, 1984). Enzyme-histochemical methods like alkaline phosphatase (Gomori, 1941), dipeptidyl peptidase IV (Lojda, 1979), amylase periodic acid Schiff (PAS) reaction (Andersen, 1975) and ATPase (Sillau and Banchero, 1977; Rosenblat et al., 1987) visualise the enzyme product in the capillary wall. Although identification of capillaries is superior when compared to histological methods, the enzyme based capillary staining can provide inconsistent results due to different enzyme activity in control and pathological conditions (Hansen Smith et al., 1992b). Capillary wall was successfully and comparably visualised immuno-histochemically with antibodies against endothelium (von Willebrand factor

(vWF), CD31 (JC70A), CD34 (QBEnd 10) (Mukai et al., 1980; Delisser et al., 1993; Kuzu et al., 1992) or antibodies against basement membrane (fibronectin, collagen IV, laminin) (Barsky et al., 1983; Otsuki et al., 1990; Madsen and Holmskov, 1995). Different lectin-histochemical methods have also been proposed as good capillary staining techniques. Ulex europaeus agglutinin I lectin (UEA-I) proved to be a more sensitive and a more reliable human endothelium marker than vWF (Sehested and Hou-Jensen, 1981). Griffonia (Bandeira) simplicifolia I lectin (GSI lectin) selectively binds to capillaries in rat skeletal muscle (Hansen Smith et al., 1988; Smolkova et al., 2001) and identifies both perfused and nonperfused microvessels (Greene et al., 1990). The advantage of GSI lectin over enzyme histochemical techniques is that binding of GSI lectin remains unchanged after exposure to hypoxia (Hansen Smith et al., 1992b). Therefore, it was proposed as a suitable marker of capillaries in physiological studies of skeletal muscle (Hansen Smith et al., 1992a).

The most often used methods for the quantification require identification of capillary profiles in stained thin tissue sections. The parameters describing the capillary frequency are number of capillaries per unit section area, number of capillaries per fibre number, diffusion distance, sharing factor, etc (Snyder, 1987; Egginton and Ross, 1989; Bennett et al., 1989). However, the whole information is restricted to two dimensions only, while the third dimension of the capillary geometry is lost.

In our previous paper (Kubínová et al., 2001), we introduced a stereological method for three dimensional (3D) quantification of capillaries in skeletal muscle. The capillaries were visualised by immunofluorescent detection of fibronectin (Eržen and Maravić, 1992). Digital images of successive optical sections, were obtained by confocal microscope from intact volume of thick tissue sections. Capillaries were visible deep into the tissue section, but they were not clearly distinguished from other structures in skeletal muscle. Recently, we have been looking for a staining technique that would further improve the identification of capillaries. Disadvantages of the capillary detection based on fibronectin staining are the following: (i) it is not sufficiently specific because additionally to the basal lamina of capillaries it stains also the basal lamina of muscle fibres and the loose connective tissue among muscle fibres (Stenman and Vaheri 1978;

Sanes JR, 1994) and (ii) in experimental or pathological conditions which favour growth of connective tissue, e.g. after denervation and reinnervation (Salonen et al., 1985; Siironen et al., 1992), the extensive and possibly also overstained fibronectin could mask capillary profiles in the endomysium.

The aim of this study was to develop an improved staining method for quantification of capillaries in 3D. Such a method must fulfill the following conditions: (i) capillaries must be clearly distinguished from other structures in skeletal muscle, (ii) capillaries should be visualised with fluorochromes to enable confocal focusing through thick sections, (iii) antibodies should penetrate sufficiently deep into the tissue section, (iv) preparation of specimens must be handy and fast enough for processing of large numbers of muscle samples at once, and (v) the new staining should be suitable for the application of our previously developed stereological method. The triple staining with CD31, Griffonia (Bandeira) simplicifolia lectin (GSL I) and laminin seems to satisfy our requirements. To check the efficiency of this staining, we estimated capillary length to adjacent fibre length and compared the obtained results with our previous study, accomplished on fibronectin stained muscle sections (Kubínová et al., 2001).

Materials and Methods

All experimental work was performed on Wistar rats weighing between 200 to 250g.

Tissue pretreatment

Whole soleus (SOL) muscles were excised, placed in OCT embedding medium (Tissue-Tek), frozen in liquid nitrogen and stored at -80°C. Additionally, some muscles were fixed in cold acetone (-20°C) overnight at 4°C.

Preparation of tissue slices

The midsection of frozen muscles was cut with a cryostate (Reichert-Jung Frigocut 2800) at -20°C into 30 µm, 35 µm, 40 µm, 50 µm or 60 µm transverse sections. Sections were collected in PBS and subsequently mounted on ChemMate capillary Gap Microscope Slides previously coated with an adhesive (DAKO No. S 2024). They were air-dried and subsequently stored at –80°C.

Muscles fixed in acetone were cut with a razor blade into approximately 1 mm thick tissue sections and washed in PBS.

Staining protocols for capillaries and muscle fibres

Cryosections of fresh frozen muscles were fixed in cold acetone (-20°C) for 10 minutes and washed with phosphate-buffered saline (0.01M, pH 7.4) with 0.2% Triton X-100 (PBS-T) for 20 minutes. After preincubation in normal goat serum (1:5 in PBS) for 30 min, to reduce background staining, sections were incubated in succession, with two different primary antibodies. The first antibody against CD31 (BD PharMingen No. 555025) (1:3000 in PBS-T) (at 4°C for 3 days) was used to label endothelial cells in capillaries. After several washes in PBS-T Alexa Fluor 488 (Molecular Probes A-11001) (1:350) with addition of 3% normal rat blocking serum was applied for 2 hours to visualize endothelium in green colour. The second antibody against laminin (CHEMICON No. MAB 1920) (1:1000 in PBS-T) was applied for 3 days at 4°C to label the basal lamina of muscle fibres. After excessive washing (in PBS-T) Alexa Fluor 546 (Molecular Probes A-11003) at dilution 1:350 was applied for 2 hours to visualise basal laminas in red colour.

After thorough washing in PBS-T, sections were incubated overnight with fluorescein labelled Griffonia (Bandeira) simplicifolia lectin (GSL I) (Vector Laboratories No. FL 1101 (1:300, at 4°C), an additional marker of capillaries.

Muscles fixed in acetone were stained floating in the incubation medium. Incubation time for each antibody was adjusted to the section thickness: from 3-6 days for CD31 and laminin and from 24 hours to 3 days for lectin. Secondary antibodies were applied for 2 days.

Negative controls were processed in the same way, only that the primary antibodies were replaced with PBS-T.

Mounting

After staining, all tissue sections were mounted in an aqueous mounting medium, a mixture of 10g polyvinyl alcohol (PVA), 30 mL of glycerol and 70 ml of phosphate-buffered saline (PBS) (pH 7.2), with addition of small amount of sodium azide (NaN3). When mounting 1 mm thick tissue sections, silicone spacers were placed between the glass slides and glass coverslips (Molecular Probes P-18175).

Sampling for stereological measurements

Triple stained 35 μm thick transverse sections were selected for stereological measurements. Two sections from the right soleus muscle were obtained from five different rats. On average five fields per muscle were sampled by systematic uniform random sampling. A stack of 21 optical sections, 1 mm apart, was captured from every field by the two-channel Zeiss LSM 510 confocal microscope using a Zeiss Plan-Neofluar oil immersion 40x objective (numerical aperture 1.3).

Green and red fluorescences were excited with argon (488nm) and He/Ne (543nm) laser. Emission signal was filtered using a narrow band BP 505-530 nm and LP 560 nm filter, respectively. From each stack of digitized images on average 11 fibres were chosen within an unbiased sampling frame (Gundersen, 1977).

Stereological measurements

Captured images with x-y dimensions of 326 μm by 326 μm were analysed with the SLICER and FAKIR modules (Kubínová et al., 2001; Kubínová et al., 2002), running in Ellipse software environment (ViDiTo, Slovakia), for estimating length density of capillaries and fibre surface area. The results were expressed as the length of capillaries adjacent to a fibre within a 20 μm muscle section (Lcap) [μm], as capillary length per adjacent fibre length (Lcap/Lfib) [μm/μm] and capillary length adjacent fibre surface area (Lcap/Sfib) [μm/μm^2]. Our previous results (Kubínová et al., 2001) were recalculated to a 20 μm thick muscle section to enable easier comparison.

Statistics

Mean and standard deviation of the mean were calculated for each parameter. The mean values obtained with the two staining methods were compared with the paired, two tailed Student's t-test. A P value < 0.05 was considered to be statistically significant.

3D reconstruction

3D reconstruction of capillaries and muscle fibres was made from triple stained, thick sections applying the maximum projection algorithm to the stacks of confocal images in green and red channel and subsequent merging of these two projections.

Results

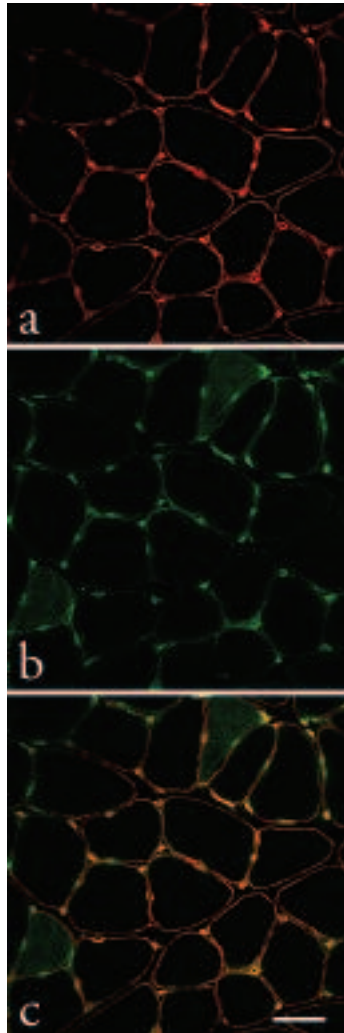The novel, triple fluorescent staining (CD31, laminin and lectin) provided a clear-cut distinction

Figure 2. 3D reconstruction of the capillary network in the rat soleus muscle. Bar: 50 µm.

between capillaries and muscle fibres in rat soleus muscle (Figure 1). In the merged images from the two channels of the confocal microscope, green or yellow-green capillary profiles were in clear contrast with the red outlines of muscle fibres (Figure 1c). The interior of capillary profiles was labelled twice, with an anti-rat CD31 and with *Griffonia (Bandeira) simplicifolia* lectin (GSL I), both visualized with green fluourochrome (Figure 1b). The basal lamina of capillaries and muscle fibres was labelled with an anti-rat laminin and visualized with a red fluorochrome (Figure 1a). Because of interference between green and red colour (i.e., collocalisation of antigens and emission interference of fluorochromes), capillary profiles in some places appear yellow (Figure 1c). Compared to the fibronectin staining the present triple staining evidently enhanced capillary identification. It took us about 5-10 min to measure the length of capillaries
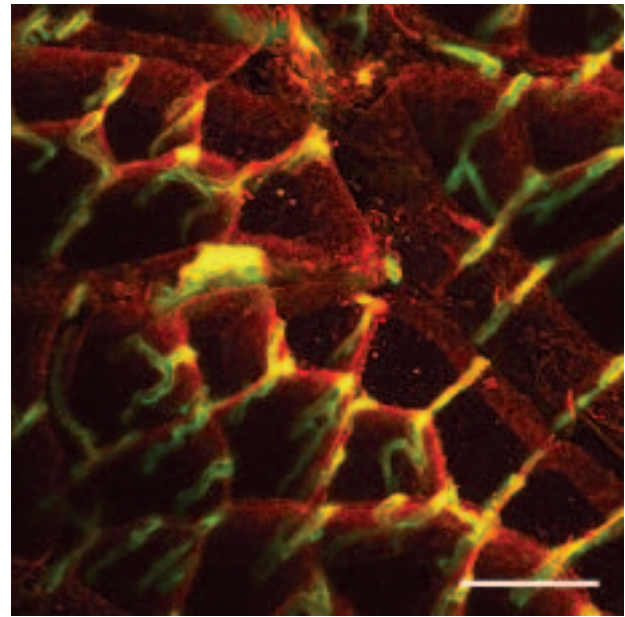
adjacent to one muscle fibre and additional 4 min for measurements of the fibre surface area.

It was difficult to achieve successful mounting of 1 mm thick tissue sections in an upright position, although we used silicone spacers to make chambers between the cover slip and glass slide. However, staining was efficient and some samples were suitable for 3D reconstructions (Figure 2).

The most reliable results were obtained from 35 µm thick transverse sections. Consistent and good penetration of antibodies could be achieved to the depth of 20 µm (Figure 3), penetration of antibodies, deeper than 30 µm, was inconsistent and insufficient.

Stereological analysis of the triple stained sections gave data comparable to the results of our previous study (Kubínová et al., 2001), where capillaries were stained with fibronectin (Table 1).

Discussion

In this study a novel, triple staining method was introduced for the detection of capillaries in skeletal muscle tissue. In merged images the staining provides green or yellow-green capillary profiles that are in clear contrast with the red outlines of muscle
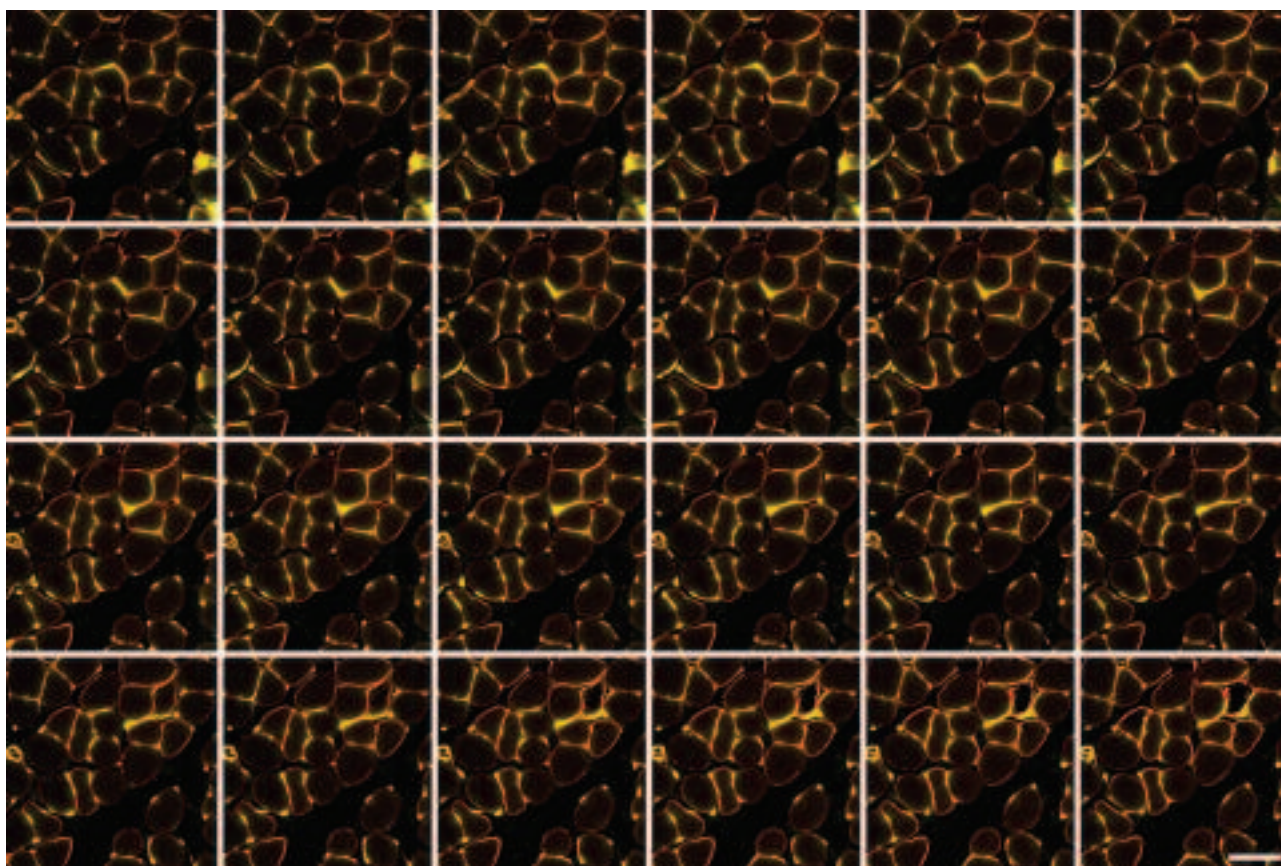
Figure 3. A stack of successive optical sections, 1 μm apart, of the rat soleus muscle stained with CD31, Griffonia (Bandeira) simplicifolia lectin, and laminin. Bar: 100 μm.

Table 1. Morphometric characteristics of capillaries in the rat soleus muscle.

Method	Lcap	Lcap/Lfib	Lcap/Sfib
Triple staining[a]	123,1±14	6,130±0,878	0,0272±0,0043
Fibronectin staining*[b]	115,4±22	5,772±1,217	0,0341±0,0083
a:b	NS	NS	NS

*Lcap [μm] - length of capillaries adjacent to a fibre within 20 μm thick muscle sections; Lcap/Lfib [μm/μm] capillary length to adjacent fibre length; Lcap/Sfib [μm/μm²] capillary length to adjacent fibre surface area (mean±SD). Capillaries were identified with a triple staining (CD31, Griffonia (Bandeira) simplicifolia lectin, and laminin) and with fibronectin staining. *Results are adapted from Kubínová et al. (2001); NS= not significant*

fibres. The staining has turned out to be superior to the fibronectin staining and, to our knowledge, to all other published staining techniques for the detection of capillaries in skeletal muscles. The advantages of the proposed staining are the following: (i) identification of capillaries and fibre outlines is quick and clear-cut; (ii) lectin binding to the capillary walls is in contrast to enzyme based stainings independent of hypoxia, which guarantees comparable staining under different experimental or pathological conditions; (iii) the staining distinguishes capillaries clearly from the surrounding tissue also in circumstances that favour growth of the connective tissue; (iv) the triple staining provides a reliable basis for potential semi-automatic and further automatic recognition of capillaries in skeletal muscle and (v) faster identification of capillaries speeds up the quantitative analysis.

The thickness of transverse tissue sections that can be stained with this new triple staining method was limited by the depth of antibody penetration. If 50 μm sections were incubated on slides, penetration of antibodies was incomplete. Several attempts to enhance penetration of antibodies into 50 μm sections, like prolongation of the incubation time, addition of Triton X100 (Hartman et al., 1972) and dehydration with subsequent rehydration (Costa et al., 1980) were not successful enough. Therefore, we

prepared 35 μm thick sections where penetration of the antibodies and fluorescein labelled lectin was optimal. In some cases, the depth of the penetration of the antibody against laminin was only 20 μm, therefore the quantitative analysis was performed within the stacks of images not thicker than 20 μm.

We considered the possibility that capillary staining in large muscle samples or in whole muscles could be improved by injection of dyes. Injection of India ink and Microfil does not guarantee the visualisation of all vessels and capillaries (Plyley and Groom, 1975; Willner and Groom, 1977; Renkin et al., 1981), especially in skeletal muscle, where spatial and temporal heterogeneity of capillary perfusion was described (Lindbom, 1983; Lindbom and Arfors, 1984; Ellis et al., 1990). Further, the injection techniques are applicable only in muscles from experimental animals, but not for potential quantitative analysis of the capillary network in human biopsy samples, which is one of our future goals.

Many techniques have been developed to study microvascular network, however, only few of them allow 3D quantification of capillaries in skeletal muscle. The methods of vascular corrosion casting have been successfully applied for light (Cheung, 1996) and electron microscopic examination (Potter and Groom, 1983). The micro-corrosion cast technique using scanning electron microscopy enables visualisation of capillaries, however, morphometrical measurements are very time consuming. The corrosion casting procedures also dissolve the surrounding tissue of vessels, which precludes studies on the relationship between casts and surrounding tissues. Beside this, some of the casting materials are too viscous to fill microvascular bed of skeletal muscle and some of the muscles (e.g. m. gracilis) can be uniformly perfused only when the limb is left free to move (Potter and Groom, 1983).

For 3D quantification of capillaries, sections of soleus muscles hand cut into about 1 mm thick physical sections and processed floating in the incubation medium are an alternative to thinner sections cut with a cryotome and further processed on glass slides. Penetration of the antibodies and lectin into thick floating sections was superior to their penetration into sections incubated on glass slides. However, maintaining a constant orientation of the mounted muscle sections and focusing through the muscle tissue under the confocal microscope was more difficult than in the latter method.

When the relationships between different tissue constituents within intact tissues are analyzed, confocal microscopy presents a useful tool for quantitative measurements of their structure and arrangement in 3D. Digital images of perfectly registered stacks of serial optical sections from thick specimens that can be recorded by confocal microscope represent suitable data also for computer 3D reconstructions that can be made without the necessity to solve the tedious problem of alignment of images in successive physical sections (Pawley, 1995).

The results of the analysis and visualisation of density of the capillary network, obtained in this study, were comparable with our previous results in the rat soleus muscles (Kubínová et al., 2001). However, the capillaries were more clearly visualized, which facilitated our measurements of capillary length. The presented triple staining method reduced the time of measurement by more than 50%, thus we are going to use it in our future studies of quantification and 3D visualization of capillaries and muscle fibres.

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