Chapter 11
Validation of a New Semi-Automated Technique to Evaluate Muscle Capillarization

Sam B. Ballak, Moi H. Yap, Peter J. Harding, and Hans Degens

Abstract The method of capillary domains has often been used to study capillarization of skeletal and heart muscle. However, the conventional data processing method using a digitizing tablet is an arduous and time-consuming task. Here we compare a new semi-automated capillary domain data collection and analysis in muscle tissue with the standard capillary domain method. The capillary density (1481 ± 59 vs. 1447 ± 54 caps mm^{-2}; R^2:0.99; $P < 0.01$) and heterogeneity of capillary spacing (0.085 ± 0.002 vs. 0.085 ± 0.002; R^2:0.95; $P < 0.01$) were similar in both methods. The fiber cross-sectional area correlated well between the methods (R^2:0.84; $P < 0.01$) and did not differ significantly (~8 % larger in the old than new method at $P = 0.08$). The latter was likely due to differences in outlining the contours between the two methods. In conclusion, the semi-automated method gives quantitatively and qualitatively similar data as the conventional method and saves a considerable amount of time.

Keywords Validation • Capillarization • Muscle • Capillary density • Fiber cross-sectional area

1 Introduction

An adequate blood supply to the muscle is not only important for delivery of oxygen to the working muscle, but also for the removal of metabolites and heat. This exchange between blood and muscle fibers takes place in the capillaries and an

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adequate muscle capillarization is thus crucial for muscle function. The capillary supply to a fiber is determined by the fiber its size, type, mitochondrial content and metabolic activity of surrounding fibers [1–3]. During hypoxia [4] and hypertrophy [2] capillary proliferation ensures adequate muscle oxygenation.

The method of capillary domains has been used to study the capillarization in skeletal [2, 3] and heart muscle [5]. The strengths of the method are that it not only provides measures of overall capillary supply, such as the capillary density (CD in caps mm^{-2}) and capillary to fiber ratio, but also the capillary supply to individual fibers. It is also unique in that it gives an indication of the heterogeneity of capillary spacing, which can have a significant impact on muscle oxygenation [6–9], and is an accurate method to estimate the oxygen supply areas of individual capillaries and is an indirect indicator of tissue oxygenation [10]. The data obtained with the method of capillary domain can be fed into models of tissue oxygenation [4, 6, 8]. The drawback of the method is, however, that data collection is a manual and time-consuming process. First, pictures have to be printed, fibers and capillaries manually traced on paper and then traced again on a digitizer. The coordinates of the capillaries and fiber outlines are then processed and analyzed with AnaTis (BaLoH Software, www.baloh.nl) [11].

Automation would significantly reduce the data processing time, potentially reduce human errors and improve the accuracy of the data processing. A semi-automated method would also provide the possibility to expand the analysis by introducing new parameters. Therefore, the aim of this study was to compare the conventional method with a new semi-automated Matlab® based software.

2 Methods

2.1 Immunohistochemistry

The left *m. plantaris* of ten 9-month-old C57BL/6j mice were excised, frozen in liquid nitrogen at optimal length and stored at −80 °C for further analysis. Sections of 10 μm were cut in a cryostat at −20 °C and stained with biotinylated lectin (*Griffonia simplicifolia*) to identify capillaries as described previously [3].

2.2 Analysis of Capillarization

The capillarization was analyzed first as described previously [2, 3]. In short, the coordinates of the capillaries and the outlines of the muscle fibers were manually delineated with a digitizing tablet (Summagraphics MM1201) and the data fed into AnaTis (BaLoH Software, www.baloh.nl) to calculate capillary domains. Domains are areas surrounding a capillary delineated from surrounding capillaries by

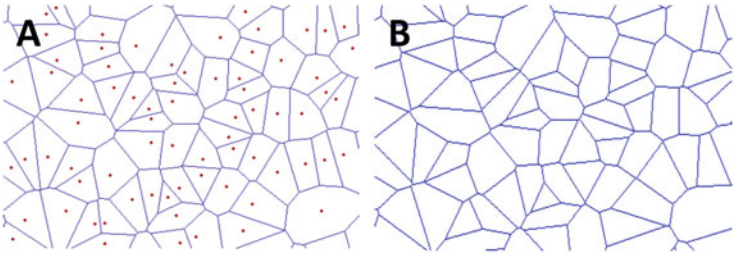

Fig 11.1 Calculated capillary domains using Voronoi tessellations in the conventional (**a**) and new (**b**) method

equidistant boundaries [5]. It also gives an index of the heterogeneity of capillary spacing as the logarithmic standard deviation of the domain radii (Log_RSD) and overall indices of capillarization, such as capillary density (CD; cap mm^{-2}). In addition, the program calculates the fiber cross-sectional areas (FCSA) and provides indices of the capillary supply to individual fibers: the local capillary to fiber ratio (LCFR). It is calculated as the sum of the fractions of the capillary domains overlapping a given fiber. The capillary fiber density (CFD; cap mm^{-2} of a given fiber) is the LCFR divided by the FCSA of that fiber. We developed a semi-automated program for capillary domain analysis, as originally described by Hoofd et al. [5] based on Voronoi tessellations. Two annotation tools were developed using Matlab® libraries. The first annotation tool was used to select the capillary and border coordinates. The second annotation tool delineated the fiber outlines. Subsequently, a data analysis function was implemented to calculate the capillary domain (Fig. 11.1). Capillary domains or fiber outlines crossing the border were considered border domains or fibers. The domain size, CD, FCSA, LCFR and CFD were calculated with custom Matlab® functions. All statistics and metrics were compiled from these data.

2.3 Statistical Analysis

The data of the two methods were compared with a paired Student's *t* test and correlations (R^2). Data are presented as mean ± standard error of the mean (SEM). A value of $P < 0.05$ was considered significant.

3 Results

Figure 11.2a shows a comparison of the CD obtained with the conventional and new method. The CD in the conventional and the new method (1481 ± 59 vs. 1447 ± 54 cap mm^{-2}) are highly correlated (R^2:0.99, $P < 0.01$). The same

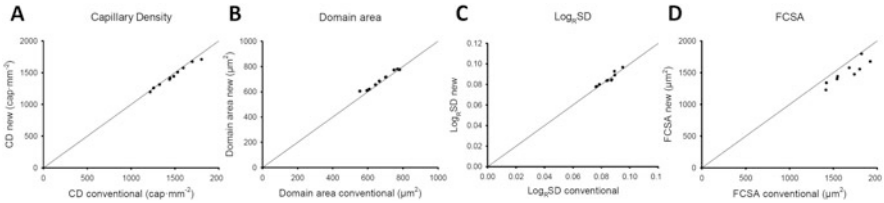

Fig 11.2 The correlation between the old and new method for CD (cap mm^{-2}) ($R^2 = 0.99$; $P < 0.01$) (**a**), domain area (μm^2) ($R^2 = 0.97$; $P < 0.01$) (**b**), Log_RSD ($R^2 = 0.95$; $P < 0.01$) (**c**) and FCSA (μm^2) ($R^2 = 0.87$; $P < 0.01$) (**d**) compared between the conventional (x-axis) and new method (y-axis). The line represents the line of identity

Table 11.1 Correlations and *P*-values of indices of capillary supply to individual fibers (*LCFR* local capillary to fiber ratio, *CFD* capillary fiber density) and % connective tissue (%CT) between the two methods in *m. plantaris* of 9-month-old male C57Bl/6j mice

	Conventional method	New method	R^2	P-value
%CT (%)	11.5 ± 0.6	16.7 ± 1.4	0.43	0.01
LCFR	2.503 ± 0.076	1.998 ± 0.070	0.80	0.00
CFD (mm^{-2})	1564 ± 56	1252 ± 46	0.86	0.00

applied to the capillary domain areas (R^2:0.97, $P < 0.01$; Fig. 11.2b). Figure 11.2c shows the heterogeneity of capillary spacing (Log_RSD) for the conventional and the new method (0.085 ± 0.002 vs. 0.085 ± 0.002). The Log_RSD for both methods are strongly correlated (R^2:0.95, $P < 0.01$). The correlation for the FCSA between the two methods is strong (R^2:0.87, $P < 0.01$; Fig. 11.2d) but the new method gives consistently, but not significantly lower FCSAs than the old method (1693 ± 66 vs. 1531 ± 55 μm^2; $P = 0.08$).

Table 11.1 shows that the percentage of connective tissue (%CT) did correlate between the two methods (R^2:0.43, $P = 0.01$). Although the LCFR and the CFD correlated strongly (R^2:0.80 and 0.86; $P < 0.01$), the new method gave consistently lower values for both LCFR and CFD than the old method ($P < 0.01$ and $P = 0.01$).

4 Discussion

The main finding of this study is that a newly developed Matlab® based semi-automatic method of capillary domains provides a quantitatively and qualitatively similar outcome for parameters of overall capillary supply as the traditional manual method. Although the FCSA is underestimated in the new method, affecting quantitatively also indices of the capillary supply to individual fibers, this is a systematic underestimate. Therefore, the qualitative outcome is similar for the two methods and the newly developed method can be readily used for comparative studies on changes in muscle or cardiac capillary supply.

The CD and Log_RSD obtained by the two methods were virtually identical. This indicates that the coordinates recorded for each capillary were comparable between the methods. It also indicates that the calculation of the capillary domains is comparable between the two methods, since for the calculation of the Log_RSD the surface areas of the individual domains and the radius of circles with corresponding surface areas have to be calculated.

There was, however, a small difference in the way the fiber outlines were traced; the FCSA was systematically underestimated in the new method. In the conventional method, accidental fiber overlap could be accounted for, while in the Matlab® based software fibers overlapping each other are joined and assessed as one large fiber. Even with good tracing skills, a researcher may avoid tracking the borders too close to each other to prevent this fusion of two separate fibers; in other words, the experimenter may intentionally draw the fibers slightly too small, resulting in a smaller fiber area. On the other hand, the conventional method could lead to a small overestimation of the size of the fibers since overlapping areas will be counted twice. Together, this stresses the importance of good tracing skills in both the new and the old method.

The difference in fiber size, caused by the difference in tracing the outlines of the fibers, also has an impact on the %CT, which was significantly higher in the new method. The differences in fiber size also work through in the LCFR and CFD, which were lower in the new than in the old method, even though the correlation between the two methods was strong. Thus, while there is a systematic qualitative difference between the two methods, the new method is readily applicable in comparative studies.

We are currently working on the tracing algorithm to adjust the systematic underestimation of the FCSA in the Matlab® based software. The main aim of this study was to evaluate the validity of this technique before looking into that issue.

It is clear that the method of capillary domains has limitations, as it does not consider the three-dimensional structure of the capillary network, differences in flow between capillaries or decrements in oxygen content of the blood from the arteriolar to the venous side of the capillary. Other investigators have tried to address this issue [12, 13], but this was outside the scope of the present study. Nevertheless, the method of capillary domains has successfully been used to calculate the domain size in venous and arterial capillaries [14] and using assumptions of the decrement in capillary oxygen tension from the arteriolar to venular side of the capillary, estimates can be made of the oxygenation in successive planes of tissue [15]. In addition, in a mathematical model also the impact of flow heterogeneity on tissue oxygenation can be incorporated [16] illustrating the usefulness of the collected data in the two dimensional plane to get an estimate of tissue oxygenation under different conditions.

The new Matlab® based program saves on average about 1.5 h per section/photo. This will increase even more when additional analyses are automated further. In addition, the Matlab® based program makes analysis more flexible and allows the implementation of new variables in the future. This new time-sparing method is the

first step in fully automating the process and assessment of capillary domains. Future research should focus on fully automating the sampling of capillary and fiber outline coordinates.

5 Conclusion

In conclusion, the new semi-automated Matlab® based method is highly comparable to the standard method of capillary domain analysis when considering indices of overall muscle capillarization. However, due to differences in the way of tracing the fiber outlines there are small difference in FCSA, which also affect indices of capillary supply to individual fibers. Together, this new method appears to be a valid way to qualitatively and quantitatively analyze the capillarization in cardiac and skeletal muscle.

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