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**Two new reliable immunohistochemical methods for simultaneous  
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human skeletal muscle**

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## Abstract

Capillary network of skeletal muscle has a crucial role in oxygen supply and is strongly associated with the phenotype and metabolic profile of muscle fibers. Abundant literature has explored capillarization of skeletal muscle in different populations and in response to different interventions. Capillary and fiber type identification techniques have considerably evolved over the last decades, but to the best of our knowledge, no validated immunohistochemical method has yet been developed to simultaneously identify capillaries (using CD31), the three different muscle fiber types and basal lamina. Nine human muscle biopsies of *vastus lateralis* were stained using 5 different methods to test: the reliability of different CD31 antibodies for capillary identification, the reliability between single section or serial sections methods, and the intra-experimenter reproducibility in visual detection of capillaries. High reliability for the different antibodies directed against capillaries was observed for capillary contacts (CC) measurements (intra-class correlations (ICC) [ICC<sub>95%</sub>] of 0.89 [0.72;0.96] for type I fibers, 0.93 [0.81;0.97] for type IIA fibers, 0.88 [0.71;0.96] for type IIX fibers, 0.95 [0.86;0.98] for all fiber types) as well as a high level of similarity between single and serial sections methods. A strong similarity in capillary analysis between the different methods was obtained for each sample measurements. Analysis of Lin's concordance correlation coefficients and Bland and Altman's graphics showed a strong intra-experimenter reproducibility. This article proposes two time- and tissue-sparing immunohistochemical methods to accurately assess a complete fiber typing (type I, IIA, and IIX) along with muscle capillarization on a single muscle section.

## Introduction

Human muscle is highly heterogeneous and composed of different types of fibers: slow oxidative fibers (type I fibers), fast oxidative glycolytic fibers (type IIA fibers) and fast glycolytic fibers (type IIX fibers) (Schiaffino and Reggiani 2011). This classification of muscle fiber types relied on different methods. Since the '70s, it has been assessed using the myofibrillar adenosinetriphosphatase (mATPase) method (Brooke and Kaiser 1970; Johnson et al. 1973; Essén et al. 1975; Denis et al. 1986; Proctor et al. 1995; Gavin et al. 2015), based on mATPase sensitivity to pH. In the '90s, the development of the first monoclonal antibodies directed against myosin heavy chains (MHC) gradually replaced mATPase staining (Schiaffino et al. 1989; Gorza 1990; Kadi et al. 1998). In 2012, Bloemberg & Quadrilatero performed a fine muscle fiber typing (type I, IIA, and IIX fibers) on a single muscle section (Bloemberg and Quadrilatero 2012). Furthermore, capillary supply is also a major determinant of muscle phenotype, and techniques of capillaries identification have also progressed a lot. First techniques to identify capillaries with the combination of amylase and periodic acid schiff staining (Andersen 1975) have been progressively superseded by the use of the *Ulex europaeus* agglutinin 1 (UEA 1) lectin, anti-collagen type IV, anti-von Willebrand factor (Parsons et al. 1993; Madsen and Holmskov 1995; Qu et al. 1997), and then by the CD31 antibody (Charles et al. 2006; Merlet et al. 2019) which recognizes the platelet endothelial cell adhesion molecule-1 (PECAM-1) – a transmembrane glycoprotein expressed by vascular endothelial cells. Capillary supply is commonly assessed through global indexes independent of the fiber type (such as the capillary density (CD) or the capillary to fiber ratio (C/F)) but is also frequently assessed through local indexes in a fiber-type specific manner (such as capillary contacts per fiber (CC), CC per fiber cross-sectional area (CAFA) or capillary to fiber perimeter exchange index (CFPE)) (Hepple et al. 1997; Hepple 1997; Harris 2005; Charles et al. 2006). The capillary network can even be further analyzed using morphometric indexes such as the capillary tortuosity index (CapTor) (Vincent et al. 2010; Ravelojaona et al. 2015) or the ratio between the length of contact with the muscle fiber (LC) to the perimeter of muscle fiber (LC/PF) (Charifi et al. 2004; Merlet et al. 2019). To date,

analyses of local capillary indexes require serial cross-sectioning, which present several limitations. First, serial cross-sectioning is tissue-consuming while human tissues are difficult to obtain and most of the time collected in reduced quantity. Serial cross-sectioning analyses are also time-consuming as the same fibers must be clearly identified among hundreds of others over several cross-sections. This also presents risks of bias due to the changes of fiber types and capillaries along muscle fibers. From these limitations, the need for simultaneous identification of fiber types and capillaries had already been raised more than thirty years ago (Rosenblatt et al. 1987; Paljärvi and Naukkarinen 1990; Eržen and Maravić 1993), even suggesting that immunohistochemical techniques could provide further possibilities. In more recent publications, new immunofluorescence techniques succeeded to stain capillaries, type I fibers, type II fibers, and laminin (a protein component of basal lamina) on a single muscle section (Snijders et al. 2016, 2017; Nederveen et al. 2016, 2018; Tan et al. 2018; Moro et al. 2019). However, these techniques did not distinguish type IIA from type IIX muscle fibers. Type IIX fibers present a specific phenotype – for review see (Schiaffino and Reggiani 2011). For instance, they present a lower capillarization than type IIA fibers (Andersen 1975; Larsson et al. 1999; Campos et al. 2002; Gavin et al. 2015), and in response to training their proportion decreases, contrary to the proportion of type IIA fibers, which sometimes increases (Gavin et al. 2015; Kosek et al. 2006; Mohr et al. 1997). Therefore, in training studies, it is of high interest to separately analyze type IIA from type IIX fibers. To the best of our knowledge, only one publication reported the staining of the three muscle fiber types and basal lamina using immunohistochemical techniques along with the staining of capillaries in humans (Al-Shammari et al. 2019) – but using UEA 1 instead of CD31 antibody, which is known to be one of the most specific and sensitive markers of endothelial cells (Duscha et al. 1999). Thus, we aimed to propose accurate and reliable immunohistochemical methods identifying basal lamina, capillaries with CD31 antibody, as well as type I, IIA and IIX muscle fibers, on a single muscle section.

## **Materials and methods**

The present study used muscle samples from a previous protocol (Galusca et al. 2018) that was conducted in accordance with the Helsinki Declaration and approved by the local research and ethics committee of Saint-Étienne – France. All subjects gave written informed consent prior to inclusion in the study and the protocol was registered at ClinicalTrials.gov as NCT01224561.

### **Muscle biopsies**

Biopsies from the *vastus lateralis* were performed under local anesthesia by a specialized surgeon in nine participants. A biopsy of approximately 140 mg of muscle was collected at one-third of the distance from the upper margin of patella to the anterior superior iliac spine with Weil-Blakesley forceps. A part of the sample containing well-identified fascicles was well oriented, included in an embedding medium (Cryomount, Histolab, Göteborg, Sweden), frozen in liquid nitrogen-precooled isopentane, and stored either in an ultra-low temperature freezer or in a liquid nitrogen tank. Serial 10- $\mu$ m thick transverse sections were cut at -18°C using a cryostat (CM1950, Leica biosystems, Wetzlar, Germany), mounted on five serial glass slides, air-dried at room temperature, and stored at -20°C.

### **Immunohistochemical stains**

As displayed in Tab.1, five slides were stained with 5 different cocktails of antibodies, respectively referred to as method 1 (M1), method 2 (M2), method 3 (M3), method 4 (M4), and method 5 (M5). M1 and M3 aimed to identify on a single muscle section: capillaries, complete fiber type distribution, and laminin. M1 consisted of a two-step procedure using a mouse monoclonal CD31 antibody (clone JC70A, IgG1 isotype, Dako, Agilent Technologies, Santa Clara, USA) whereas M3 consisted of a single-step procedure using a rabbit polyclonal CD31 antibody (Ab28364, IgG isotype, Abcam, Cambridge, UK) (see Tab.2). The two-step M1 procedure consisted of CD31 application in a first step, followed by the application of a cocktail of the other antibodies in a second separate step (Tab.2). The single-step M3

procedure consisted of the application of CD31 in the same cocktail as the other antibodies. M2 and M4 aimed to identify capillaries and laminin (without fiber type staining), both using a single-step procedure. M2 used the mouse monoclonal CD31 antibody (Clone JC70A, Dako) whereas M4 used the rabbit polyclonal CD31 antibody (Ab28364, Abcam) for identification of capillaries. M5 only aimed to identify fiber type distribution, using a single-step procedure adapted from Bloemberg and Quadrilatero's protocols (Bloemberg and Quadrilatero 2012). All the staining procedures were verified using negative controls to ensure appropriate staining specificity. For all details about staining procedures of the different methods, see Tab.1 and Tab.2.

**Tab.1:** Information on primary and secondary antibodies used in the different methods

Design		Primary antibody							Secondary antibody					
Protocol	Method	Identification	Antibody	Reference	Species	Clonality	Isotype	Dilution	Source	Reference	Species	Isotype	Source	Dilution
Two-step	M1	Capillaries	Anti-CD31	Anti-CD31 – JC70A	Mouse	Mono	IgG1	1:40	Dako	546 – A-21123	GaM	IgG1	ThermoFisher Alexa Fluor®	1:300
		Type I fibers	Anti-MHCI	BA-F8 – AB_10572253	Mouse	Mono	IgG2b	1:100	DSHB	350 – A-21140	GaM	IgG2b		
		Type IIA fibers	Anti-MHC (all but IIX)	BF-35 – AB_2274680	Mouse	Mono	IgG1	1:100	DSHB	488 – A-21121	GaM	IgG1		
		Laminin	Anti-laminin	2E8 – AB_2134060	Mouse	Mono	IgG2a	1:100	DSHB	633 – A-21136	GaM	IgG2a		
Single-step	M2	Capillaries	Anti-CD31	Anti-CD31 – JC70A	Mouse	Mono	IgG1	1:40	Dako	546 – A-21123	GaM	IgG1	ThermoFisher Alexa Fluor®	1:300
		Laminin	Anti-laminin	2E8 – AB_2134060	Mouse	Mono	IgG2a	1:100	DSHB	633 – A-21136	GaM	IgG2a		
Single-step	M3	Capillaries	Anti-CD31	Anti-CD31 – Ab28364	Rabbit	Poly	IgG	1:30	Abcam	546 – A11035	GaR	IgG	ThermoFisher Alexa Fluor®	1:300
		Type I fibers	Anti-MHCI	BA-F8 – AB_10572253	Mouse	Mono	IgG2b	1:100	DSHB	350 – A-21140	GaM	IgG2b		
		Type IIA fibers	Anti-MHC (all but IIX)	BF-35 – AB_2274680	Mouse	Mono	IgG1	1:100	DSHB	488 – A-21121	GaM	IgG1		
		Laminin	Anti-laminin	2E8 – AB_2134060	Mouse	Mono	IgG2a	1:100	DSHB	633 – A-21136	GaM	IgG2a		
Single-step	M4	Capillaries	Anti-CD31	Anti-CD31 – Ab28364	Rabbit	Poly	IgG	1:30	Abcam	546 – A11035	GaR	IgG	ThermoFisher Alexa Fluor®	1:300
		Laminin	Anti-laminin	2E8 – AB_2134060	Mouse	Mono	IgG2a	1:100	DSHB	633 – A-21136	GaM	IgG2a		
Single-step	M5	Type I fibers	Anti-MHCI	BA-F8 – AB_10572253	Mouse	Mono	IgG2b	1:100	DSHB	350 – A-21140	GaM	IgG2b	ThermoFisher Alexa Fluor®	1:300
		Type IIA fibers	Anti-MHC (all but IIX)	BF-35 – AB_2274680	Mouse	Mono	IgG1	1:100	DSHB	488 – A-21121	GaM	IgG1		

DSHB: Developmental studies hybridoma bank, GaM: Goat anti-Mouse, GaR: Goat anti-Rabbit, MHC: Myosin heavy chain



**Tab.2:** Protocols of the single- and two-step procedures

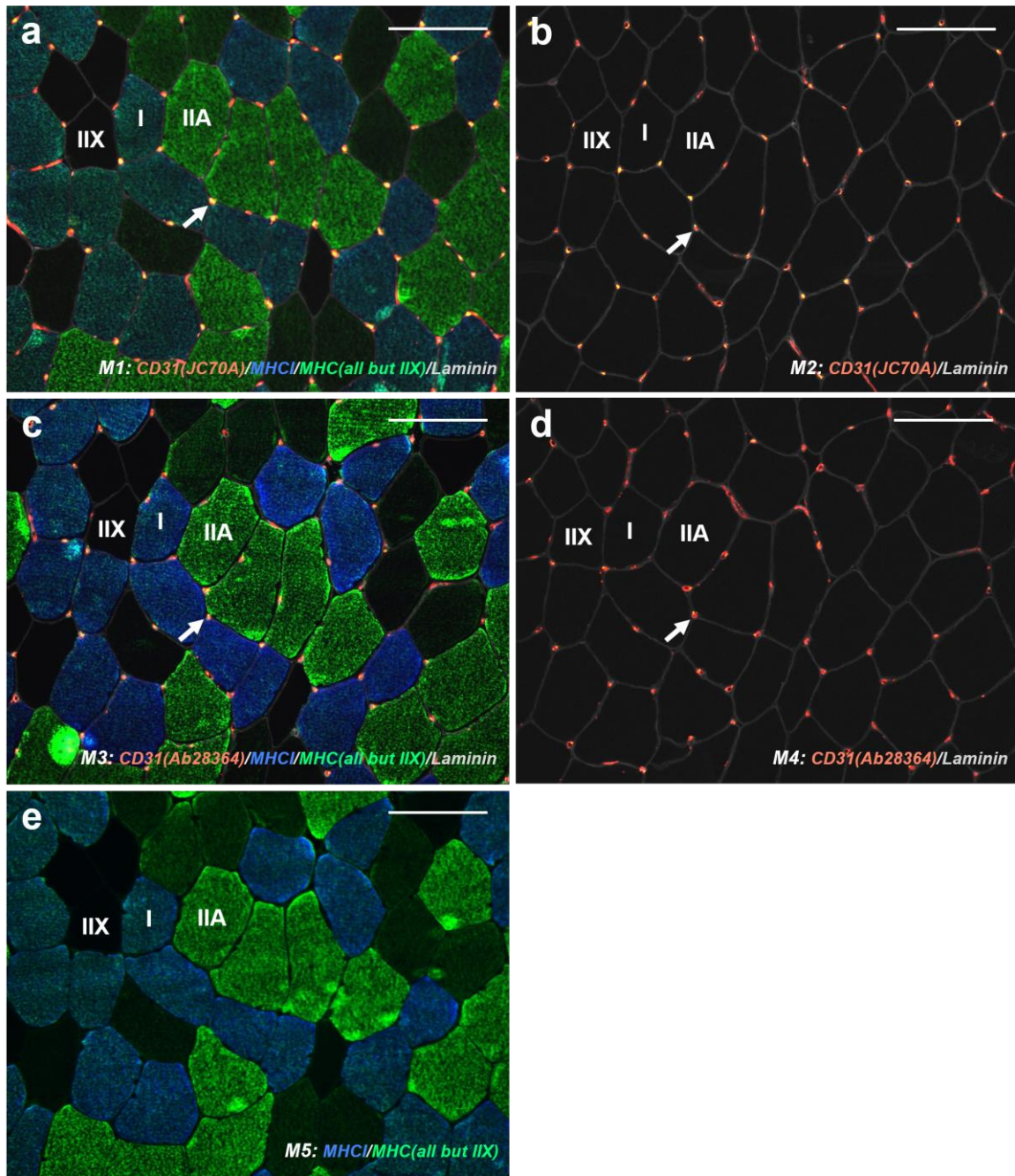
<b>M1: two-step procedure (CD31 application followed by the cocktail of the other antibodies in a second step)</b>	<b>Time</b>	<b>M2, M3, M4, and M5: single-step procedure (CD31 directly applied in the cocktail of the other antibodies)</b>	<b>Time</b>
Air dry sections at room temperature	40 min	Air dry sections at room temperature	40 min
Fixation in acetone bath	15 min	Fixation in acetone bath	15 min
Sections encircling with hydrophobic pen	10 min	Sections encircling with hydrophobic pen	10 min
PBS wash	3×5 min	PBS wash	3×5 min
Blocking with PBS and GS 10% + BSA 10%	20 min	Blocking with PBS and GS 10% + BSA 10%	20 min
Apply primary antibody: CD31 JC70A	60 min	Apply first antibody cocktail: BA-F8 and/or BF-35 and/or 2E8 and/or CD31 Ab28364 and/or CD31 JC70A (See Tab.1)	60 min
PBS wash	3×5 min	PBS wash	4×5 min
Blocking with PBS and GS 10% + BSA 10%	20min	Blocking with PBS and GS 10% + BSA 10%	30 min
Apply appropriate secondary antibody: 546 IgG1	60 min (dark)	Apply appropriate secondary antibody cocktail: 350 IgG2b and/or 488 IgG1 and/or 546 IgG1 and/or 546 IgG and/or 633 IgG2a (See Tab.1)	60 min (dark)
PBS wash	4×5 min	PBS wash	3×5 min
Blocking with PBS and GS 10% + BSA 10%	30 min	Mounting with ProLong™ Gold Antifade	
Apply primary antibody cocktail: BA-F8 + BF-35 + 2E8	60 min (dark)		
PBS wash	4×5 min		
Blocking with PBS and GS 10% + BSA 10%	30 min		
Apply appropriate secondary antibody cocktail: 350 IgG2b + 488 IgG1 + 633 IgG2a	60 min (dark)		
PBS wash	3×5 min		
Mounting with ProLong™ Gold Antifade (P36934 ThermoFisher Scientific, Waltham, USA)			
<b>TOTAL</b>	<b>8 hours 10 min</b>		<b>4 hours 45 min</b>

BSA: Bovine serum albumin, GS: Goat serum, M: Method, PBS: Phosphate buffered saline

### Image acquisition and analysis

Muscle sections were observed under optical microscopy (DM2000, Leica microsystems, Wetzlar, Germany) with a ×20 objective, connected to a 5 Megapixels camera with active cooling (DFC450 C, Leica microsystems, Wetzlar, Germany). Leica software (LAS version 4.3.0, Leica microsystems, Wetzlar, Germany) was used for image acquisition. The microscope was equipped with a fluorescence illumination system (Lumen 200, Prior Scientific, Cambridge, UK), and with blue (Excitation: bandpass

filter (BP): 360/40 nm, Emission: BP 470/40 nm), green (Excitation: BP 480/40 nm, Emission: BP 527/30 nm), orange-red (Excitation: 545/30 nm, Emission: BP 610/75 nm), and deep red (Excitation: BP: 620/60 nm, Emission: BP 700/75 nm) filter cubes. ImageJ software (NIH, Bethesda, USA) was used to merge channels and to perform image analysis. Nine regions on each muscle section were captured in order to be representative of a large part of the muscle section. Thus, a significant number of muscle fibers could be identified on each muscle biopsy. Owing to serial cross-sectioning, the exact same muscle fibers were analyzed between the 5 different methods, for each muscle sample (see Fig.1). The number of capillary contacts per fiber (as previously defined as “CC” (Hepple et al. 1997; Harris 2005)) was assessed for M1, M2, M3, and M4 – given that M5 was only used for fiber typing but not for direct capillaries identification. For further details on CC measurements, see the review of Harris that reported the different capillary indexes used in literature (Harris 2005). CC analysis was performed on 50 type I, 50 type IIA and 50 type IIX fibers - except for two muscle biopsies for which only 34 and 35 type IIX fibers were analyzed due to their low proportion. Analyses of CC were performed on single merged images for M1 and M3. For M2 and M4 that only stained capillaries and basal lamina, CC analyses required the use of a serial cross-section to identify muscle fiber type of the cells in contact with the capillaries, therefore we used M5 staining in this case to assess muscle fiber type. The same experimenter repeated twice all measurements of each image to assess intra-experimenter reproducibility of capillaries identification. The two measurements were spaced in time to avoid any memory bias of the experimenter.



Immunohistochemical staining using M1 (Method 1) a), M2 (Method 2) b), M3 (Method 3) c), M4 (Method 4) d), and M5 (Method 5) e). Type I fibers are blue (BA-F8), type IIA fibers are green (BF-35), type IIX fibers are black (unstained), and initially deep red laminin (2E8) has been colored in grey. Capillaries are orange red (arrow points) using JC70A antibody from mouse on a) and b), and using Ab28364 antibody from rabbit on c) and d) M: Method, MHC: Myosin heavy chain, the arrows point to a capillary, scale bars: 100  $\mu$ m

**Fig.1** Immunohistochemical staining of human skeletal muscle

## **Statistics**

Statistical analyses and graphics were carried out using Stata software, Version 15 (StataCorp, College Station, USA) and GraphPad software (version 5.0, Prism, San Diego, USA). Continuous data were expressed as mean  $\pm$  standard deviation (SD). The assumption of normality was assessed using the Shapiro-Wilk test. To measure the inter-methods and intra-experimenter agreement, intra-class correlation coefficients (ICC) and Lin's concordance correlation coefficients (CCC) were estimated, and were interpreted according to usual rules of thumbs (Altman 1991):  $<0.2$  (negligible),  $0.2-0.4$  (low/weak consistency),  $0.4-0.6$  (moderate agreement),  $0.6-0.8$  (substantial/good agreement), and  $>0.8$  (excellent agreement). The results were also presented using Bland and Altman's plots (Fig.3).

## **Results and discussion**

We aimed to propose new reliable time- and tissue-sparing immunohistochemical staining protocols to accurately identify capillaries, muscle fiber types (I, IIA, IIX), and basal lamina, on a single muscle section. Based on works from the last decade (Bloemberg and Quadrilatero 2012; Snijders et al. 2016, 2017; Nederveen et al. 2016, 2018; Tan et al. 2018; Moro et al. 2019), we proposed here two new staining methods: M1 and M3 (as detailed in Tab. 1 and Tab.2).

First, the concordance between JC70A and Ab28364 antibodies both directed against PECAM-1 had to be estimated. JC70A is a mouse monoclonal antibody and Ab28364 is a rabbit polyclonal antibody, developed by different companies and with different isotypes (see Tab.1 and Material and methods). Thus, differences in capillaries identification could have been expected. From a visual point of view, Fig.1 shows slightly sharper staining with JC70A monoclonal antibody compared to Ab28364 polyclonal antibody. This observation led us to question a potential overestimation of capillaries using the polyclonal antibody, even if the experimenters who are familiar with capillary analyses should probably not confound capillaries with artefacts. An analysis of ICC was performed to assess

concordance between CC measurements from these different antibodies. Tab.3 showed high values of ICC between methods for type I fibers (ICC [ICC<sub>95%</sub>]: 0.89 [0.72;0.96]), type IIA fibers (ICC [ICC<sub>95%</sub>]: 0.93 [0.81;0.97]), type IIX fibers (ICC [ICC<sub>95%</sub>]: 0.88 [0.71;0.96]), and for all fiber types (ICC [ICC<sub>95%</sub>]: 0.95 [0.86;0.98]); thus demonstrating a high degree of similarity in capillaries identification. Observation of individual values may also be of clinical interest. As displayed in Fig.2, a strong similarity in CC measurements was observed for each muscle sample using the different methods. This reinforces again the strong similarity in capillaries identification using JC70A or Ab28364 antibodies, even at an individual level.

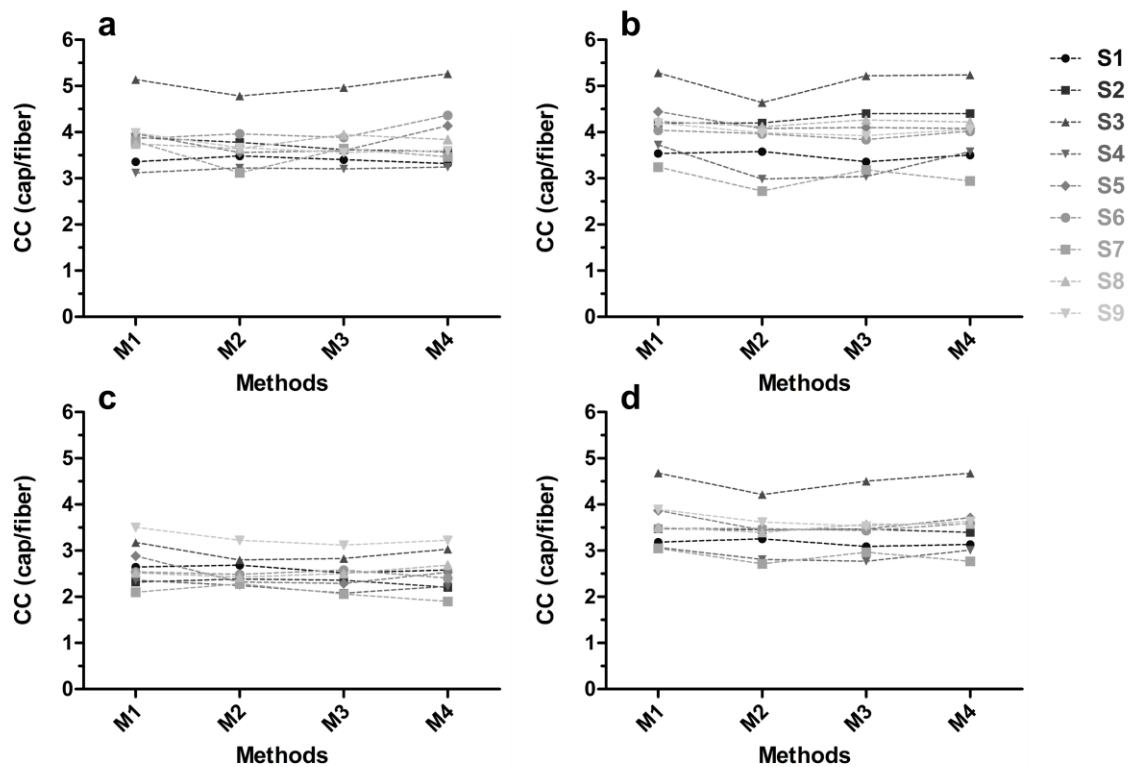
**Tab.3:** Capillary supply assessments between the different methods

Types of fibers	Capillary contacts (CC)				ICC
	M1 (cap/fiber)	M2 (cap/fiber)	M3 (cap/fiber)	M4 (cap/fiber)	
Type I fibers	3.87 ± 0.56	3.69 ± 0.48	3.76 ± 0.50	3.87 ± 0.64	0.89 [0.72;0.96]
Type IIA fibers	4.10 ± 0.59	3.81 ± 0.61	3.92 ± 0.68	4.00 ± 0.64	0.93 [0.81;0.97]
Type IIX fibers	2.67 ± 0.44	2.54 ± 0.32	2.48 ± 0.34	2.53 ± 0.41	0.88 [0.71;0.96]
All types	3.58 ± 0.52	3.37 ± 0.44	3.42 ± 0.49	3.50 ± 0.54	0.95 [0.86;0.98]

cap/fiber: capillaries per muscle fiber, CC: Capillary contacts, ICC: Intra-class correlations, M: Method

Concordance between single and serial cross-section capillary identification also had to be assessed. Differences could have been expected between methods that require a single section analysis (M1 and M3) compared to methods that required a serial cross-section analysis (M2 and M4). As observed in Fig.1, capillaries seem to be less apparent in M1 and M3 because of the colors of surrounding fibers, compared to M2 and M4. Indeed, capillaries seem to be very easy to detect in M2 and M4 for which fiber distribution was observed in a separate slide (M5). This first visual observation tends to suggest that capillarization might be underestimated in M1 and M3. However, capillaries should still be well detected when carefully analyzing large-format images on a large screen, despite colors of the surrounding fibers. Indeed, results showed a high degree of concordance between methods for fibers of type I (ICC: 0.89), type IIA (ICC: 0.93), type IIX (ICC: 0.88), and for all fiber types (ICC: 0.95). In addition, high reliability was also observed in CC values of each individually observed sample (Fig.2),

despite small variations. This small variability could be partly explained by the distance (a few tens or hundreds of micrometers) between muscle sections that were stained by the different methods. Although this is supposed to introduce a minor bias, the fact remains that capillaries can evolve slightly within a few micrometers. This small variability could also be attributed to a random error related to experimenter's reproducibility in the detection of capillaries, which led us to question intra-experimenter reproducibility.



Measurements of capillary contacts for each sample between methods in type I fibers a), type IIA fibers b), type IIX fibers c), and all types of fibers d)  
cap/fiber: capillaries per muscle fiber, CC: Capillary contacts, M: Method, S: Sample

**Fig.2** Individual values between methods by type of fibers

Lin's CCC were calculated between two separate analysis (A1 and A2) of the same images and performed by the same experimenter, in order to test intra-experimenter reproducibility. As displayed in Tab.4, Lin's CCC were systematically higher than 0.96 for each type of fibers and each method. Considering all types of fibers, Lin's CCC [CCC<sub>95%</sub>] of 0.996 [0.990;1.002] was observed for M1, 0.989

[0.974;1.004] for M2, 0.987 [0.970;1.005] for M3, 0.994 [0.986;1.002] for M4, and 0.992 [0.987;0.997] for all methods, thus demonstrating a high degree of intra-experimenter reproducibility.

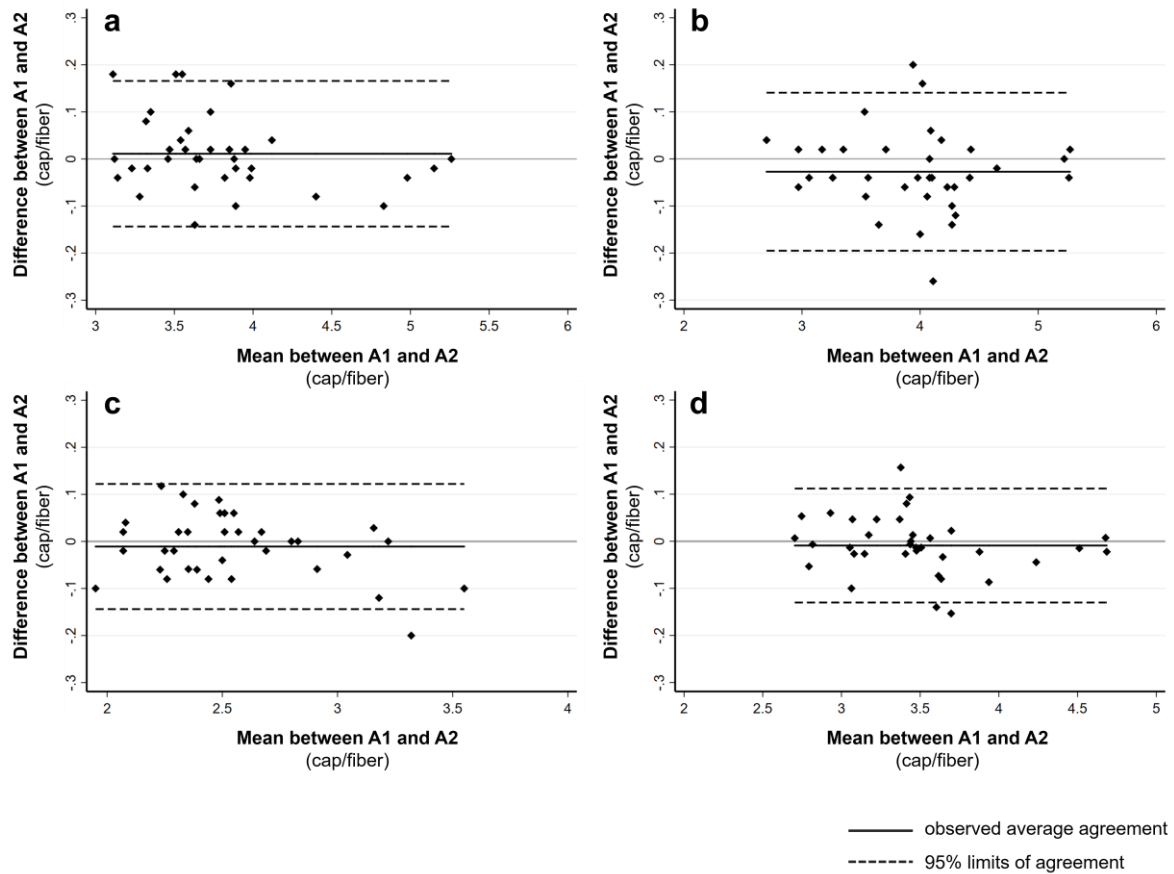
**Tab.4:** Intra-experimenter reproducibility for the different methods

Types of fibers	Capillary contacts (CC)														
	M1			M2			M3			M4			All methods		
	A1	A2	Lin's CCC	A1	A2	Lin's CCC	A1	A2	Lin's CCC	A1	A2	Lin's CCC	A1	A2	Lin's CCC
	(cap/fiber)	(cap/fiber)		(cap/fiber)	(cap/fiber)		(cap/fiber)	(cap/fiber)		(cap/fiber)	(cap/fiber)		(cap/fiber)	(cap/fiber)	
Type I	3.87 ± 0.56	3.88 ± 0.57	0.997 [0.994;1.001]	3.69 ± 0.48	3.68 ± 0.51	0.994 [0.987;1.002]	3.76 ± 0.50	3.68 ± 0.56	0.966 [0.921;1.010]	3.87 ± 0.64	3.90 ± 0.63	0.996 [0.989;1.002]	3.80 ± 0.53	3.78 ± 0.55	0.989 [0.982;0.996]
Type IIA	4.10 ± 0.59	4.12 ± 0.59	0.985 [0.963;1.007]	3.81 ± 0.61	3.81 ± 0.64	0.986 [0.967;1.006]	3.92 ± 0.68	3.94 ± 0.69	0.992 [0.979;1.004]	4.00 ± 0.64	4.07 ± 0.63	0.992 [0.983;1.001]	3.96 ± 0.61	3.99 ± 0.62	0.989 [0.982;0.996]
Type IIX	2.67 ± 0.44	2.67 ± 0.48	0.992 [0.982;1.002]	2.54 ± 0.32	2.55 ± 0.37	0.967 [0.932;1.003]	2.48 ± 0.34	2.48 ± 0.38	0.981 [0.958;1.004]	2.53 ± 0.41	2.56 ± 0.39	0.986 [0.968;1.004]	2.55 ± 0.37	2.57 ± 0.39	0.984 [0.974;0.994]
All types	3.58 ± 0.52	3.58 ± 0.52	0.996 [0.990;1.002]	3.37 ± 0.44	3.38 ± 0.47	0.989 [0.974;1.004]	3.42 ± 0.49	3.40 ± 0.52	0.987 [0.970;1.005]	3.50 ± 0.54	3.54 ± 0.53	0.994 [0.986;1.002]	3.47 ± 0.48	3.48 ± 0.50	0.992 [0.987;0.997]

A: Analysis, cap/fiber: capillaries per muscle fiber, CC: Capillary contacts, CCC: Concordance Correlation Coefficients, M: Method



Bland and Altman's graphical analyses were also performed to assess reproducibility between the first analysis (A1) and the repeated analysis (A2) (Fig.3). Differences between A1 and A2 were plotted against means between A1 and A2, for type I, IIA, IIX fibers, and for all types of fibers (Fig.3), in order to assess global agreement between A1 and A2. First, Fig.3 shows that data were homogeneously dispersed. Then, the average agreement was very close to zero for all fiber types, meaning that A1 and A2 measurements give the same results, without systematic error. Bland and Altman's method also defines the 95% limits of agreement to help in the analysis of random error. Based on CC values already existing in the literature (Proctor et al. 1995; Gavin et al. 2005; Kryger and Andersen 2007; Groen et al. 2014; Verdijk et al. 2016; Galusca et al. 2018; Moro et al. 2019), we had *a priori* defined acceptable intervals of 0.4 capillary per fiber. Fig.3 shows the limits of agreement between 0.1 to 0.2 capillary per fiber. Based on clinical and biological considerations, limits of agreement were therefore acceptable, since 95% of the differences between one measurement to the other were included in a range  $< 0.2$  capillary per fiber. Finally, both Lin's CCC and Bland and Altman's graphics showed a high degree of intra-experimenter reproducibility.



**Comparison between analysis 1 (A1) and analysis 2 (A2) of capillary contacts (CC) for type I fibers a), type IIA fibers b), type IIX fibers c), and all types of fibers d)**  
**A: Analysis, cap/fiber: capillaries per muscle fiber**

**Fig.3** Graphics of Bland and Altman of intra-experimenter reproducibility in capillary contacts analysis

Although we propose here two reliable methods (M1 and M3) to simultaneously identify muscle capillaries, the three types of fiber and basal lamina, some limitations can be pointed out. The disadvantage of M1 using CD31 mouse monoclonal antibody was the requirement to perform the staining in two steps, because of the cross-reactivity with BF-35. The main limitation of M3 using CD31 Ab28364 rabbit polyclonal antibody was a slightly lower sharpness compared with M1, likely due to antibody polyclonality. Therefore, we recommend to an untrained experimenter to use M1 (CD 31 JC70A) for easier detection of capillaries. Yet, high values of ICC were obtained between the different methods, and CD31 Ab28364 was already used in the literature (Snijders et al. 2016, 2017; Nederveen

et al. 2016, 2018; Tan et al. 2018). On the other hand, the single-step M3 procedure was also found reliable and might be relevant for fast staining by trained experimenters. Another limitation might be the small individual variability observed in Fig.2. Nevertheless, this no longer exists by calculating the means of several individuals, which is usually done in clinical studies (Tab.3). Based on the present results, these limitations should be tempered considering the high reliability observed between the different methods.

Our initial objective was to develop a method allowing easy identification of capillaries with simultaneous staining of the three types of fibers and basal lamina. The statistical analyses were therefore conducted on the CC local index – a fiber-type dependent index, and results showed strong reliability in the identification of capillaries. Thus, the two immunohistochemical techniques M1 and M3 can be used for capillaries counting of both global indexes (CD, C/F) and local indexes (CC, CAFA, CFPE). While reliability has been verified for such indexes that only implies capillaries identification, it remains to be confirmed for indexes related to the geometry of capillaries – such as LC/PF or CapTor. We would prefer the M1 procedure to perform morphological analyses of capillaries, since these kinds of analyses require a high level of accuracy. Indeed, from our visual observations, the M1 procedure allows providing sharp images with high quality and almost no artefact.

In perspective, it seems also important to consider the recent literature reporting an increasing number of semi- or fully automatic computational programs that are designed to perform faster and more objective analyses. Most of the time, these kinds of programs allow analyses of muscle fibers distribution and/or cross-sectional areas of fibers (Bergmeister et al. 2016; Wen et al. 2018; Kastenschmidt et al. 2019; Desgeorges et al. 2019; Encarnacion-Rivera et al. 2020), but some free open-source programs (ImageJ, Fiji, CellProfiler...) are even able to perform some capillary analyses (Smith and Barton 2014; Mayeuf-Louchart et al. 2018; Al-Shammari et al. 2019; Sanz et al. 2019). In addition to the staining methods described in the present article, it could be very useful to consider the use of such automated programs to further reduce the time for experimentation and analysis. However,

although these methods undoubtedly represent the future of histomorphometry, today they require, by the users' own admission, high quality and artifact-free biopsies, which is not always the case with the muscle biopsies of patients, for example (Sanz et al. 2019).

In conclusion, we propose here two new reliable, time-efficient, and timesaving immunohistochemical methods (referred to as M1 and M3 in Tab.1 and Tab.2) for capillary supply analysis in human skeletal muscle. These immunohistochemical methods allow simultaneous identification of capillaries, type I, IIA, IIX fibers, and basal lamina. The main advantage of the M1 procedure is its accuracy facilitating the identification of capillaries by an unexperienced experimenter or even potentially by an automated program, and the main advantage of the M3 procedure is its ability to perform fast staining in a single step (< 5 hours). A high degree of similarity in capillaries measurements for the different methods was found, and a high degree of intra-experimenter reproducibility was observed. The major benefit of these single section methods is the suppression of the time-consuming analysis of serial sectioning analyses. With these methods, all the necessary information for a fine fiber typing (3 different types of muscle fibers), assessment of global and local indexes of capillarization, and measurement of muscle fibers morphometry (such as cross-sectional area thanks to the staining of basal lamina) just have to be “read” on the merged picture.

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