

Qualitative comparison of anatomical microdissection, Sihler's staining and computerized reconstruction methods for visualizing intramuscular nerve branches

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Abstract

Stating background This study was designed to examine the entire intramuscular nerve distribution pattern of various human skeletal muscles in fetuses.

Methods In the present study rhomboid major, trapezius, long head of the biceps femoris and masseter muscles were investigated in five 18 weeks old fetal cadavers. Anatomical microdissection was applied to one fetal cadaver. In two fetuses, the extramuscular (main), major and minor nerve branches, and anastomosis were examined using Sihler's staining and labeling. In the remaining two fetuses, consecutive slices with 0.5 mm interval and 5 µm thickness were obtained from each skeletal muscle. These slices were stained with S100 for the demonstration of the nerve fibers and thereafter 3D reconstruction images were constituted using PC software.

Results Anatomical microdissection, Sihler's staining and computerized reconstruction methods were compared to demonstrate the intramuscular nerve distribution pattern. Demonstration of the intramuscular minor nerve branches and anastomosis showed difficulties in anatomical dissected specimens when compared with three-dimensionally reconstructed images and specimens obtained with Sihler's staining technique. Nevertheless, anatomical dissection is a simple method whereas Sihler's technique and computer aided 3D reconstruction are complex methods and take a long time to complete.

Conclusion The obtained information exposed that staining technique and the 3D reconstructions appeared to provide better results than did anatomical dissection.

Keywords Anatomical dissection · Staining method · 3D reconstruction · Intramuscular innervation

Introduction

Comprehensive knowledge of neural distribution within viscera or muscles provides important and useful information about the function of the muscle for anatomists, physiologists and clinicians [11]. Distribution of nerves within the muscles can be demonstrated in 2D with widely used histological methods. However with these methods it becomes difficult to perceive the nerve distribution pattern in skeletal muscle in 3D. There are three ways of visualizing and analyzing the route and distribution of a nerve innervating a muscle. First, anatomical dissection has been the most commonly used method up to now [3, 8, 9, 17]. Second is to render all but neural tissue translucent while staining the neural tissue. This method is known as Sihler's staining and labeling and was found by Sihler [21]. The third method is to acquire serial images of histological or anatomical sections. Images of serial macroscopic or microscopic sections are digitized, transferred to a computer and these images are reconstructed to obtain a 3D appearance of the organ or muscle [13]. Though this method has been available for some time, it gained popularity within the past 10–15 years in conjunction with advances in computer technology [6].

The first 3D reconstruction was performed by Born [2] and His [7] using serial sections. These scientists performed reconstruction by placing paraffin plates on top of each other.

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Prior to the 1970s, graphical reconstruction represented the initial attempt to generate 3D models and consisted of tracing outlines of histological structures on opaque paper. Outlines were superimposed providing a 3D model [13]. The first computer-aided 3D reconstruction was achieved by Glaser and Van der Loos [4]. Considerable effort was spent during the 1980s to develop algorithms capable of modeling surfaces. Two general approaches were developed: surface modeling and solid modeling. Surface modeling reconstructs the object as a shell. Structures are digitized manually or automatically and tissue edges are represented as streams of coordinates or contours. Many reconstruction algorithms and software have been devised for gross or microscopic anatomy. The important point is to use software that fulfills users' requirements, is easy to use and one that is not costly. Nowadays it is possible to obtain good quality and affordable 3D images using reconstruction software [13].

A literature search has shown that there are an insufficient number of studies on traces of nerves within muscles. Studies on this subject were done mainly by anatomical dissections or Sihler's staining. This study is aimed to qualitatively compare three methods (anatomical microdissection, Sihler's staining and labeling and computerized 3D reconstruction) for the demonstration of intramuscular nerve distribution pattern. The advantages, weakness and/or limitations of these techniques were also discussed.

Materials and methods

Five 18-week-old human fetuses were used in this study. Fetuses were aborted therapeutically for spina bifida cystica. Prior to the therapeutic abortion, written consents from families and ethical approval from the Hospital Ethics Board were obtained. After the fetal materials were fixed in 10% formalin solution, skeletal muscles were excised totally, including their origins and insertions. The muscles were excised under $\times 12.5$ – 25 magnification using a dissection microscope (Zeiss Opmi Pico, Oberkochen, Germany) so as not to cause any damage to the neuromuscular compartment or the main nerves or its branches innervating the muscle. Rhomboid major, trapezius, long head of the biceps femoris and masseter muscles were used in this study. Intramuscular nerve ramification and distribution was investigated using anatomical microdissection, Sihler staining and labeling and 3D reconstruction methods. None of the fetuses had distal nerve modifications in their ramification and distribution associated with spina bifida cystica in the investigated skeletal muscles.

We chose fetuses in this study because fetal skeletal muscles are thinner and smaller when compared to adult cadaveric muscles; thus it is much easier to demonstrate the nerve distribution pattern in muscles and reduced time is

spent for Sihler's staining and labeling and 3D reconstruction procedures in fetuses.

In our knowledge, it is the first time that these methods were realized on fetal cadavers.

Anatomical microdissection

Rhomboid major, trapezius, long head of the biceps femoris, and masseter muscles on both sides of one fetus were dissected under the stereoscopic microscope. The dissection in each muscle began from the entry point of the extramuscular (main) nerve branch, followed by the intramuscular major and minor nerve branches until it was no longer possible to follow them.

Sihler's staining procedure

In order to demonstrate the distribution pattern of nerves within the muscles, Sihler's staining and labeling improved by Liem and Van Willigen [10] and Wu and Sanders [22] was carried out in two fetuses step by step in order to:

Maceration and depigmentation

All muscles were macerated in 3% aqueous potassium hydroxide. For the depigmentation, three drops of 3% hydrogen peroxide were added per 100 ml of 3% potassium hydroxide for 21 days.

Decalcification

All the macerated specimens were decalcified in Sihler's solution I (one part glacial acetic acid, one part glycerin and six parts 1% aqueous chloral hydrate) for 14 days. This solution was changed every two days.

Staining

After decalcification, the specimens were stained with Sihler's solution II (one part Erlich's hematoxylin stock solution, one part glycerin and six parts 1% aqueous chloral hydrate) at least for 20 days.

Destaining

For destaining, Sihler's solution I was used. The solution was changed when it became purple. The progress of destaining was checked under a dissecting microscope and was stopped just before the finest branches of the nerves began to fade. Afterwards, the specimens were washed in a weak aqueous solution of lithium carbonate (0.05 g lithium carbonate per 100 ml distilled water) so that the colors of the nerves changed from purple to deep blue.

Clearing

The specimens were cleared with 40% glycerin for 12 h, 50% glycerin for 12 h and 75% glycerin for 24 h, respectively. Finally, they were stored in pure glycerin.

Fascia around the skeletal muscles were carefully removed under a surgical microscope and care was taken not to cause any damage to the nerve running within the muscle after the muscles were stained with Sihler's stain. This technique was carried out on whole muscles and pictures were taken using a photography apparatus (SZ-PT and PM-10K) attached to Olympus SZ-40 stereo microscope.

Computer-aided reconstruction

This method was applied to the remaining two fetuses. First, serial histological sections of skeletal muscles were obtained. For this purpose, 5 μm thick sections with 0.5 mm interval were obtained. Skeletal muscle sections for computer-aided reconstruction did not include the tendons of the muscles both at origin and insertion. Slices from each level of section were stained with a nerve tissue specific immunohistochemical stain (S100) (DAKO Corp., Carpinteria, CA, USA) [1]. The sections were examined and evaluated under microscope (Leica DM 4000B, Germany), transferred to the computer through CCD camera and numbered. Three-dimensional reconstruction software (Surfdriver v3.5, Kailua, Hawaii, USA) was used to reconstruct images. Using the Surfdriver software, the contours were drawn around the nerves on each image semi-automatically. Finally, all individual images were transferred into a single volume in a way that when all images were overlapped, the contours of nerve branches generated the 3D reconstruction of the nerve branches. Computer aided

reconstruction images were produced by using both Surfdriver and Cinema 4D v.8.1 (MAXON Computer, Inc. 566 St Charles Drive, Suite 2 Thousand Oaks, CA 91360) software.

Results

Anatomical microdissection

The main nerve branches entered between the fusion point of one-third proximal and one-third middle part in all the muscles dissected. Afterwards each main nerve branch divided into two to four major branches in all the muscles. The major nerve branches could be followed in their whole length but after they gave off minor nerve branches it was no longer possible to follow them even with $\times 25$ magnification.

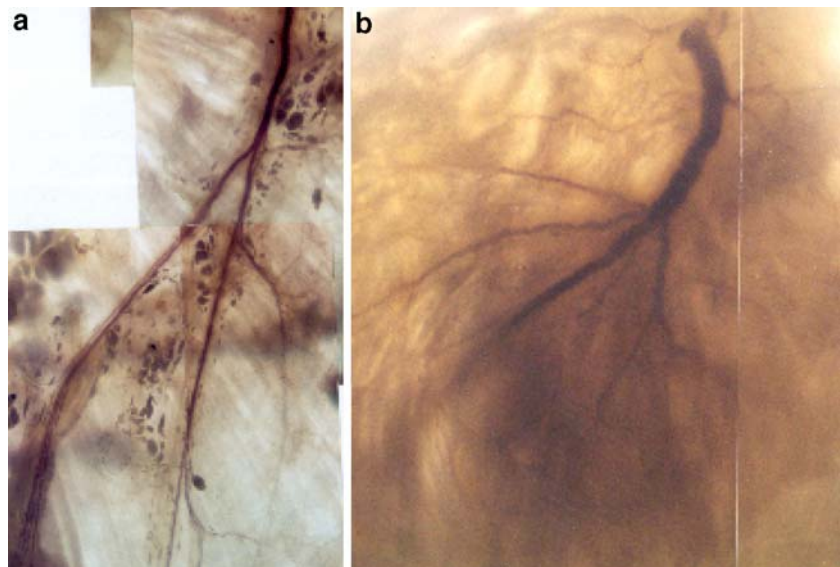
Sihler's staining and labeling

In the context of this study, we manage to demonstrate the route of the nerve running through the muscle disclosed in detail using the Sihler staining and labeling. While the muscle was translucent, the nerve appeared dark blue or purple, creating a good contrast, contributing to the examinations of morphological relations of nerve branches (Fig. 1a, b).

Computer-aided 3D reconstruction of the nerve branches

While there was a marked staining of Schwann cells of the peripheral nerve branches with S100, a non-specific staining was observed in the muscle fibers so that the identification of nerve fibers was easily made (Fig. 2a). Serial

Fig. 1 Nerve distribution pattern of trapezius (a) and masseter (b) muscles by using Sihler's staining method



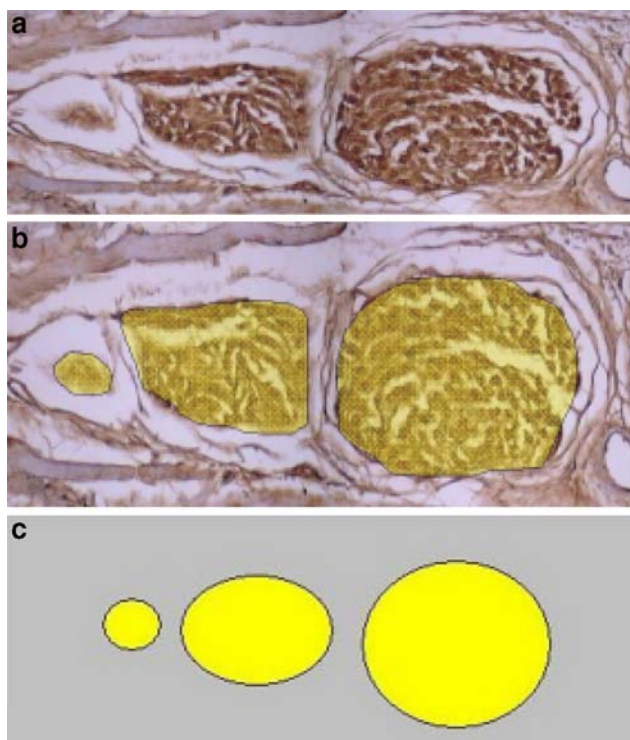


Fig. 2 Histological section stained with S100 (a) and nerve tissue traced with semi-automatic contour-tracing tool (b) (magnification $\times 40$). Schematic drawing of the same section (c)

histological sections were examined under the microscope and transferred to a computer. We made the tracing from the each histological section semi-automatically with the contour-tracing tool provided by Surfdriver 3.5.3 (Fig. 2b). The edges of nerve fibers in original pictures traced semi-automatically were irregular, therefore we used schematic images for 3D reconstruction to provide smooth contours (Fig. 2c). Major and minor branches could easily be seen in 3D reconstructions (Fig. 3). Intramuscular nerve distribution pattern demonstrated in both the 3D reconstruction image and Sihler's specimen was found in harmony (Fig. 4a, b). Spatial relations between these branches could easily be demonstrated by rotating the reconstructed anaglyph image in any plane (Fig. 5). It should be remembered that rotation of the reconstructed images can only be done on a computer. In regions where the diameter of the nerve was large, even anastomosis were visible in the field of reconstruction. It was observed in reconstructions that the diameters of the major nerve branches were larger than those of minor ones and the diameters got smaller as it got closer to the periphery. Reconstruction was of better quality when the intramuscular nerve branches were aligned and in order (Fig. 4b). On the other hand, when the intramuscular nerve branches had a wavy course, the reconstructed images had jagged edges. Similarly, reconstructed images were distorted where the nerves branched (Fig. 6).

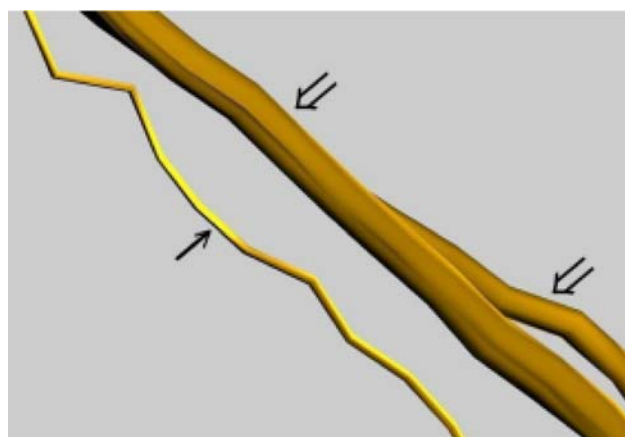


Fig. 3 Three-dimensional reconstruction image of major (open arrows) and minor (small arrow) nerve branches

Discussion

Sihler's staining and labeling is one of the most chosen techniques for the investigation of the nerve distribution pattern within skeletal muscles [5, 10, 12, 14, 15, 16, 18, 19, 20, 22]. In their study, Liem and Van Willigen [10] suggested that serial sections obtained from an organ or skeletal muscle require to be stained for their nervous tissues by silver impregnation techniques or silver proteinate methods whereas nowadays a more specific staining method like S100 is to be of use. During 3D reconstruction, we also used S100 which is specific to myelin sheath in order to stain neural fibers well and to differentiate them from the surrounding tissues.

A literature search revealed very few studies on distribution of nerves reconstructed from serial histological stained sections. This shows that the number of studies on intramuscular nerve distribution is still insufficient. When all studies to date have been scrutinized, it was noted that those related with intramuscular nerve distribution pattern were carried out with anatomical dissections. Anatomical dissections carry the risk of being damaged accidentally as they get thinner from extramuscular nerve branches to intramuscular terminal branches, as the latter are very fine and invisible while they course through the muscle [12]. Moreover, it is not always possible to demonstrate the ends of intramuscular nerve branches, even with a dissection microscope. On the other hand, it is possible to demonstrate the full course and branches of the nerve without inflicting an injury to the nerve by using Sihler's staining and labeling. The muscle is rendered translucent and the nerve itself is stained with this method. This method gives someone the advantage to examine the whole muscle since there are no invasive procedures involved that could injure the muscle

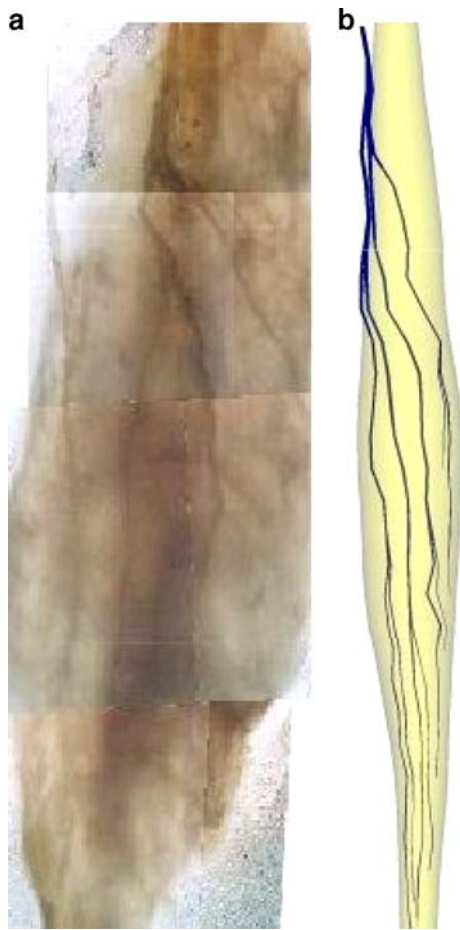


Fig. 4 Demonstration of the intramuscular nerve distribution pattern in the long head of the biceps femoris muscle using the Sihler's staining method (a) and 3D reconstruction image of a different specimen (b)

and the continuity of the nerve branches. This method also has some disadvantages. The success of the method depends on the size of the muscle to be examined. As the size of the muscle increases, the clearing process becomes more difficult, resulting in loss of distribution details and quality. Therefore, the smaller and thinner the muscle, the better the nerve is demonstrated. In the present study, the nerve distribution pattern in rhomboid major and trapezius muscles was clearly demonstrated due to their thinness whereas it was difficult to demonstrate in masseter and long head of the biceps femoris muscles due to their thickness. Another disadvantage is the staining process. The staining process requires several weeks or months to achieve adequate contrast for visualization while computerized reconstruction can be accomplished in a much shorter time. Three-dimensional reconstruction technique also has advantages and disadvantages. When serial sections are obtained from the muscle for reconstruction of the structures, some sections fold onto themselves and become useless. In addition, difficulties are encountered during superposition of images, in other words in the placement of

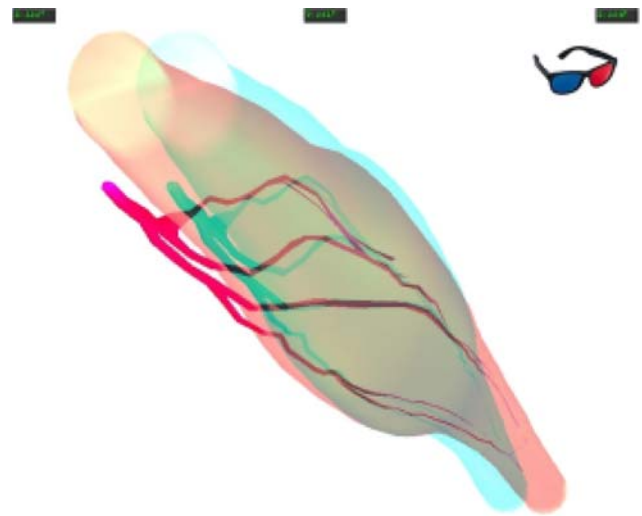


Fig. 5 Anaglyph stereo image of the intramuscular nerve distribution pattern in the long head of the biceps femoris muscle. Special eye-glasses (red-blue) should be used for the perception of depth

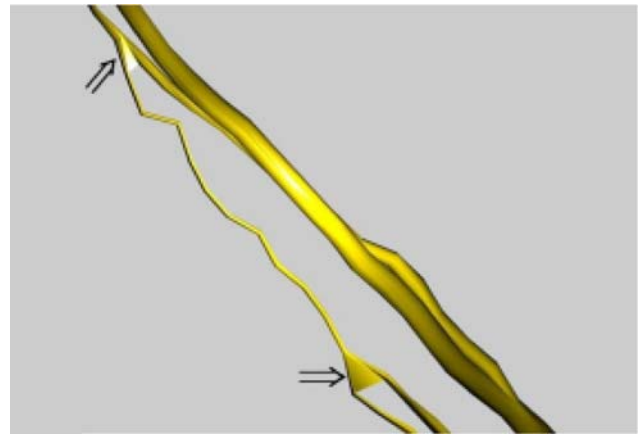


Fig. 6 Three-dimensional reconstruction image of an intramuscular nerve showing a wavy course. Note the distortion (open arrows) on the branching or anastomosing point of the nerve fiber and the jagged edges

contours. Finally this results in poor visualization of the course of the nerve within the muscle in reconstruction images. Three-dimensional reconstruction images can show nerve branches to the full as well as detailed spatial relationships between branches, which is not possible with Sihler's staining and labeling. Three-dimensional reconstructed digital images of nerve branches can be rotated and displayed in any plane (x , y , or z plane). Further, these images can be used to create stereo and anaglyph images for 3D perception. The term "anaglyph" originally referred to a stereoscopic motion or still picture in which the right component of a composite image, usually red in color is superposed on the left component in a contrasting color to produce 3D effect when viewed through correspondingly

colored filters in the form of spectacles. It should be not forgotten that rotation and creation of stereo anaglyph images can only be done on a computer. A disadvantage of the 3D reconstruction method is that the number of sections increases with the size of the muscle. Increase in number and area of serial sections results in increase in number of nerve branches within sections, which makes contour tracing of nerve branches very difficult. In other words, large muscles comprise too many numbers of sections so that reconstruction of the whole muscle could not be done. Due to 3D reconstruction technique, a wavy course of the intramuscular nerve branches demonstrated with Sihler's staining and labeling showed sharp edges on the reconstructed images. Similarly, reconstructed images were distorted where the nerves branched or anastomosed. These factors altogether decrease the success rates of the reconstruction and affect the image quality whereas none of these problems are encountered with the Sihler staining and labeling.

Following recent advances in microsurgery there is an increase in transplantation of flaps. A thorough knowledge of the course of the nerve branches within the flap and its variations are one of the major factors that would increase the success of the surgeon during muscle flap transfer. Based on this assumption, we have tried to look at the intramuscular nerve distribution pattern from a different point of view. Anatomical microdissection requires the least amount of time spent working with the specimen whereas Sihler's technique takes weeks or months. On the other hand, the whole intramuscular nerve distribution pattern can be demonstrated in computer aided 3D reconstruction images but not in anatomical microdissections. Although, we compared the three techniques, the obtained information exposed that staining technique and the 3D reconstructions appeared to provide better results than did anatomical dissection. These techniques complement each other and both of them would be helpful to researchers conducting studies on the course of nerves within muscles and in muscle microsurgery.

References

- Boenish T, Farmilo AJ, Stead RH, Key M, Welcher R, Harvey R, Atwood KN (2001) Handbook immunochemical staining methods, 3rd edn. DakoCytomation, Carpinteria, pp 5–47
- Born G (1883) Die Plattenmodelliermethode. Archiv f Mikroskop Anatomie 22:584–599
- Botha GSM (1957) The anatomy of phrenic nerve termination and the motor innervation of the diaphragm. Thorax 12:50–56
- Glaser EM, Van der Loos H (1965) A semi-automatic computer-microscope for the analysis of neuronal morphology. IEEE Trans Biomed Eng 12:22–31
- Gülekon N, Peker T, Liman F, Anil A, Turgut H.B (2002) Demonstration of the nerve distribution of the extraocular muscles in rabbits (*Oryctolagus cuniculus*). Cur Eye Res 25:179–188
- Heimer L, Robards MJ (1981) Neuroanatomical tract-tracing methods. Plenum, New York, pp 43–53
- His W (1887) Ueber die Methoden der plastischen Rekonstruktion und über deren Bedeutung für Anatomie und Entwicklungsgeschichte. Anat Anz 2:382–392
- Homma T, Sakai T (1991) Ramification pattern of intermetacarpal branches of the deep branch (ramus profundus) of the ulnar nerve in the human hand. Acta Anat 141:139–144
- Homma T, Sakai T (1992) Thenar and hypothenar muscles and their innervation by the ulnar nerve and median nerves in the human hand. Acta Anat 145:44–49
- Liem RSB, VanWilligen JD (1988) In toto staining and preservation of peripheral nervous tissue. Stain Technol 63:113–120
- Liu J, Kumar VP, Lau HK, Pereira BP, Shen Y, Pho RWH (1997) Neuromuscular compartments in the long head of triceps: a morphological study in rabbits. Muscle Nerve 20:897–899
- Liu J, Kumar VP, Shen Y, Lau HK, Pereira BP, Pho RWH (1997) Modified Sihler's technique for studying the distribution of intramuscular nerve branches in mammalian skeletal muscle. Anat Rec 247:137–144
- Moody D, Lozanoff S (1999) Surf driver: rapid and reliable 3D reconstruction, Surf Driver 3.5 Manual
- Mu L, Sanders I (1998) Neuromuscular specializations of the pharyngeal dilator muscles: I. Compartments of the canine geniohyoid muscle. Anat Rec 250:146–153
- Mu L, Sanders I (1999) Neuromuscular organization of the canine tongue. Anat Rec 256:412–424
- Mu L, Sanders I (2000) Sensory nerve supply of the human oro- and laryngopharynx: a preliminary study. Anat Rec 258:406–420
- Parratte B, Tatu L, Vuillier F, Diop M, Monnier G (2002) Intramuscular distribution of nerves in the human triceps surae muscle: anatomical bases for treatment of spastic drop foot with botulinum toxin. Surg Radiol Anat 24:91–96
- Peker T, Turgut HB, Gülekon N, Anil A (2001) Demonstration of the nerve distribution of the masticatory muscles in rabbits (*Oryctolagus cuniculus*). Anat Histol Embryol 30:225–229
- Peker T, Gülekon N, Anil A, Turgut HB, Liman F (2003) Clearing and embedding in polyester resin for demonstrating the nerve distribution pattern of skeletal muscles. Biotech Histochem 78:187–190
- Peker T, Gülekon N, Anil A, Turgut HB, Karaköse M (2006) Observation of the relationship between the shape of skeletal muscle and their nerve distribution pattern: a transparent and microanatomic study. Plast Reconstr Surg 117:165–176
- Sihler C (1895) Ueber Muskelspindeln und intramuskuläre Nervendigungen bei Schlangen und Fröschen. Arch Mikros Anat Entwickl 46:709–723
- Wu BL, Sanders I (1992) A technique for demonstrating the nerve supply of whole larynges. Arc Otolaryngol Head Neck Surg 118:822–827