

# Laboratory methodology for the histological study of skeletal muscle

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## Summary

Skeletal muscle is a malleable and dynamic tissue capable of a high degree of plasticity in regards to its histological configuration. In this sense, microscopic study is an important and essential tool for the analysis of adaptive processes -such as hypertrophy or changes of fiber types- and the regeneration or repair of skeletal muscle after injury, in the fields of sports medicine and traumatology respectively. While light microscopy addresses the study of the different constitutive elements into the skeletal muscle and their relationships with each other that determine the organ histoarchitecture, with electron microscopy an ultrastructural analysis is carried out that allows to relate the structure and function of the individual cells. This article illustrates a pragmatic and practical approach, based on personal experience and a review of the literature, from the conditions in obtaining and sending samples of skeletal muscle to the laboratory to the procedures to prepare them for histological study (sections of cryostat, paraffin sections and electron microscopy). Especially we focus on the description of the processing by freezing and recommendations to follow, as this is the ideal method for this tissue. The aim of this article is to provide useful information on the management of skeletal muscle samples that are processed in the histology laboratory to achieve optimal and reliable results in microscopic analyzes and how to avoid methodological errors that lead to the appearance of artifacts that can get to hinder or invalidate the histological study.

## Key words:

Skeletal muscle. Muscle histology. Muscle ultrastructure. Muscle biopsy. Frozen section. Electron microscopy.

## Metodología de laboratorio para el estudio histológico del músculo esquelético

### Resumen

El músculo esquelético es un tejido maleable y dinámico capaz de un alto grado de plasticidad con respecto a su configuración histológica. En este sentido, el estudio microscópico es una herramienta importante y esencial para el análisis de los procesos adaptativos –como la hipertrofia o los cambios de tipos de fibras– y la regeneración o reparación del músculo esquelético después de la lesión, en las áreas de la medicina deportiva y la traumatología respectivamente. Mientras que con microscopía óptica se aborda el estudio de los diferentes elementos constitutivos del músculo esquelético y sus relaciones entre sí que determinan la histoarquitectura del órgano, con microscopía electrónica se realiza el análisis ultraestructural que permite relacionar estructura y función de las células individuales. Este artículo ilustra un enfoque pragmático y práctico, en base a la experiencia personal y una revisión de la literatura, desde las condiciones en la obtención y envío de las muestras de músculo esquelético al laboratorio a los procedimientos para prepararlas para su estudio histológico (secciones de criostato, secciones de parafina y microscopía electrónica). Especialmente nos centramos en la descripción del procesado por congelación y recomendaciones a seguir, al ser éste el método ideal para este tejido. El objetivo de este artículo es proporcionar información útil sobre el manejo de muestras de músculo esquelético que se procesan en el laboratorio de histología para lograr resultados óptimos y fiables en los análisis microscópicos y cómo evitar los errores metodológicos que conducen a la aparición de artefactos que pueden llegar a dificultar o invalidar el estudio histológico.

## Palabras clave:

Músculo esquelético. Histología muscular. Ultraestructura muscular. Biopsia muscular. Cortes congelados. Microscopía electrónica.

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## Introduction

The microscopic evaluation of skeletal muscle is an essential part of the study of muscle histophysiology in sport and physical activity<sup>1,2</sup>, muscle injuries in sport<sup>3,4</sup> and basic research in myology and experimental myopathology<sup>5-7</sup>. Knowledge of skeletal muscle histology allows us to understand the tissue, cellular and molecular mechanisms involved in the adaptive responses to exercise –hypertrophy, hyperplasia and remodelling of fibre types– and post-injury muscle regeneration<sup>8,9</sup>. It also allows us to know and understand the specific effects that certain types of exercises<sup>10-12</sup>, substances<sup>13</sup>, nutrients<sup>14</sup>, drugs<sup>15</sup>, rehabilitation strategies<sup>16</sup> and regenerative medicine therapies<sup>17</sup> have on the structure and function of muscle fibres, satellite cells, the extracellular matrix, innervation, vascularisation and myoconnective junctions.

The microscopic study of skeletal muscle requires magnification instruments such as *optical* and *electron microscopes*, and *techniques* which reveal the different components of its structure (Figure 1). *Histological* techniques are used to study the structural features of skeletal muscle, *histochemical* techniques are used to observe enzyme and non-enzyme activities which help to characterise the diversity and distribution of fibre types or identify certain substances; and *antibodies* and *immunohistochemical* techniques are used to study and locate specific cellular and extracellular protein components.

While optical microscopy with biopsy or muscle sample sections is used for the *histoarchitectural study or analysis of the whole* of all the elements that make up skeletal muscle as an organ, *electron microscopy* is used for the *ultrastructural study or individualised, detailed analysis* of each element<sup>18</sup>. Another methodology, involving the optical microscopy of individual muscle fibres, allows us to isolate muscle fibres to analyse the behaviour of elements such as myonuclei and satellite cells<sup>19</sup>. Although less used due to the limited information it has to offer, scanning electron microscopy is another very useful option for the three-dimensional examination of muscle fibres and their relationship with the nerve fibres at the level of motor end plates or the connective scaffold of skeletal muscle<sup>20</sup> (Figure 1).

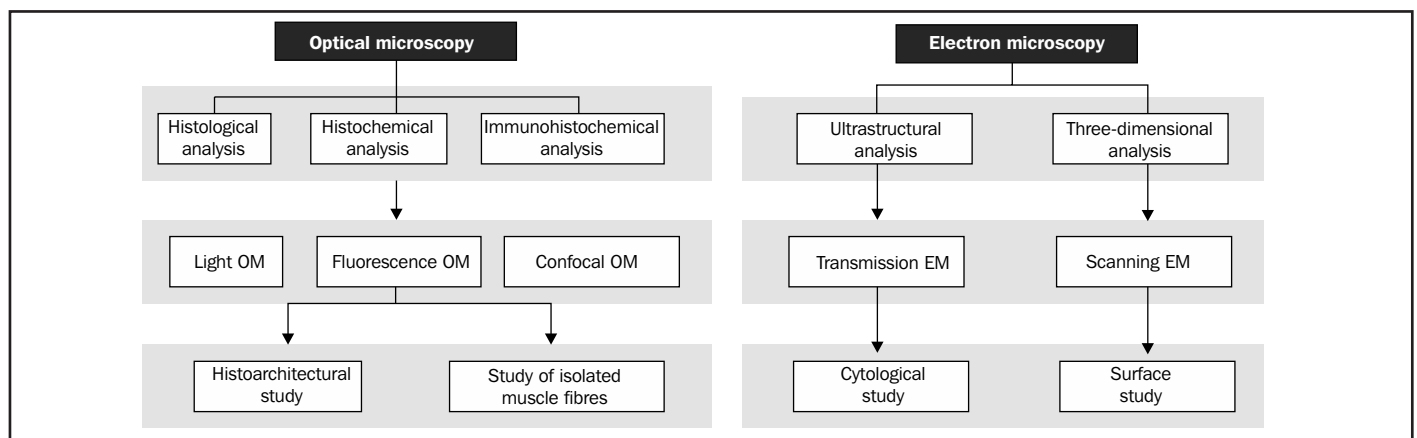
In our opinion, anyone interested in conducting any kind of microscopic study of skeletal muscle must know the procedure or methodology to follow with the samples obtained, primarily because it is a tissue/organ that requires a very specific protocol for laboratory processing which needs to be strictly observed for optimal histological evaluation<sup>21</sup>. This article describes the procedure, the methodology and recommendations to ensure the proper specific preparation of samples, essential for an histological interpretation of skeletal muscle.

## From which muscle, how much and how the sample should be taken

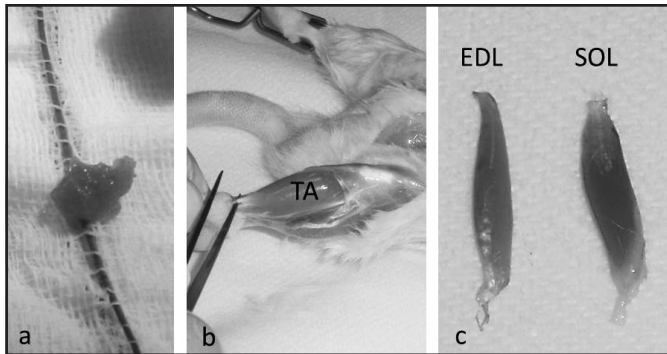
The laboratory procedure to follow is the same for human and experimental animal muscle, except when it comes to taking samples. In both cases, the essential requirements and conditions necessary to obtain the sample and send it correctly to the laboratory are the same and must be followed meticulously to ensure that the sample is not rendered entirely unusable.

There are two standardised procedures for human muscle: open biopsy and needle biopsy<sup>22,23</sup>. While both procedures are used for the diagnosis of neuromuscular diseases<sup>24</sup>, the second is used to study athletes<sup>10,25,26</sup>. *Open biopsy* is carried out in the operating theatre without any special preparation beforehand (see special requirements in specialist literature)<sup>22,27</sup>. After local anaesthesia, a small incision is made to the skin (2-3 cm) over the muscle belly and a block of muscle tissue 0.5 cm in diameter and 1 cm long (equivalent to “a small olive pit”) is removed (Figure 2). *Needle biopsy* requires an instrument -a modified Bergstrom needle- and a skin incision (1 cm) with local anaesthetic, inserting the needle to the muscular layer and extracting the muscle sample (see special requirements in specialist literature)<sup>22,27,28</sup>. There is a risk that the amount of muscle obtained will be insufficient for diagnosis and that the removal procedure will cause artefacts in the sample, in addition to making correct orientation very difficult when mounting. Certain muscles cannot be biopsied using this technique. The specimens obtained must be handled as little as possible, avoiding stretching and crushing. If the sample is bloodied, it should be rinsed rapidly in saline.

Figure 1. Range of microscopic studies for the analysis of skeletal muscle.



**Figure 2. Macroscopic appearance of (a) a fragment of human muscle taken by open biopsy, and (b) complete tibialis anterior muscles in situ (TA), extensor digitorum longus muscle (EDL) and soleus muscle (SOL).**



With both biopsies and corpses, the sample must be taken from the muscle belly. The reasons are: a) this is where the muscle fibres have the largest cross-sectional area, which is essential to assess the sizes of the muscle fibres, and b) the area near the myotendinous region should be avoided (at least 2 cm away) (except when that area is the objective of the study) because, in addition to the muscle fibres decreasing in diameter at their termination, they also have histological features which, while normal, differ significantly from other muscle areas<sup>29</sup>. Muscles with recent trauma or injected with local anaesthetic or electromyography needles should not be biopsied either; in all events, the choice of the muscles for biopsy should be based on medical or scientific criteria depending on the desired objective. In studies with muscles from corpses, the procedures and recommendations are the same. It should be borne in mind both that the sample must be taken within 24 hours after death, because this post-mortem interval does not hamper histochemical analysis of the autopsied tissue<sup>30</sup>, and that examination of autopsied muscle is not recommended with electron microscopy due to the effects of post-mortem autolysis<sup>31</sup>. The study of muscle samples from dead bodies already fixed in formalin is not recommended due to their great tendency to deteriorate, generating artefacts in the muscle fibres such as contraction and cracking, and even artefacts resulting from imperfect fixation<sup>31</sup>.

In research with *experimental animals*, the muscles being studied are normally extracted completely (Figure 2), although again the muscle belly is used for actual analysis. Most of these studies are performed on the soleus and extensor digitorum longus muscles (typically red and white, respectively)<sup>32</sup>, and on the tibialis anterior and gastrocnemius muscle, when a greater muscle volume which is easy to access for handling is needed for analysis<sup>33</sup>.

## How to send the sample to the laboratory

Regardless of the type of study to be conducted, once the sample has been taken, it must be sent to the laboratory immediately because

cellular autolysis starts as soon as it is extracted. The indications for transportation are as follows:

- All efforts should be made to keep the samples cool and moist during transportation by placing them in gauze soaked in saline and then wrung out; under no circumstance should they be sent immersed (or previously immersed) in water, saline or fixation substances, because the excess moisture will subsequently generate artefacts during freezing.
- The time from collection to the beginning of the preparation process in the laboratory must be short. Delays of over 45 minutes before arrival at the laboratory may cause artefacts as a result of hypercontraction or dehydration of the muscle fibres<sup>29</sup>. Although a period of 4 hours should never be exceeded<sup>34</sup>, a delay before freezing of up to 48 hours has no effect on enzyme histochemistry<sup>35</sup>. If the sample cannot be sent to the laboratory immediately, we recommend that it be kept in a refrigerator at 4°C. Another method is to transport skeletal muscle in ACTP (Aedesta™-cell/ tissue preservation media), as it preserves the sample better and results in fewer artefacts than muscle transportation by conventional methods<sup>36</sup>.

## How to process the sample in the laboratory

Once in the laboratory, the sample is divided into three fragments: two for analysis by *optical microscopy* and another for *electron microscopy* (Figure 3). It may also be necessary to take tissue for protein or DNA extraction in biochemical and/or genetic research; the fragment for these studies must be preserved at -70°C<sup>29,37</sup> and then subjected to different procedures to those for microscopic study. Consult these in the specific literature<sup>19,21</sup>.

### Preparation of samples for optical microscopy

The samples must be processed in such a way that it is possible to collect the greatest amount of information from all or most of the elements that constitute the skeletal muscle as an organ (Table 1). To ensure we actually “see” what we are looking for under the microscope, it is necessary to know the microanatomy of the muscle; otherwise, we could prepare the samples inadequately and render the study useless. For example, it is essential to understand the arrangement of the muscle fibres in order to orient the sample cross-sectionally or longitudinally, the motor line area to analyse motor end plates (in conjunction with electrophysiological studies)<sup>29</sup>, the composition and distribution of the fibre types in a given muscle to evaluate their percentage variations correctly or the specific characteristics of the myoconnective junctions for proper assessment when dealing with injuries at this level.

Two fragments can be taken in the sample preparation procedure for optical microscopy, one for *fixation in 10% formalin and embedment in paraffin* and another for *freezing in isopentane cooled in liquid nitrogen*. However, the first type of fragment (the kind usually used for other sorts

Figure 3. Schematic overview of the three paths for processing skeletal muscle tissue.

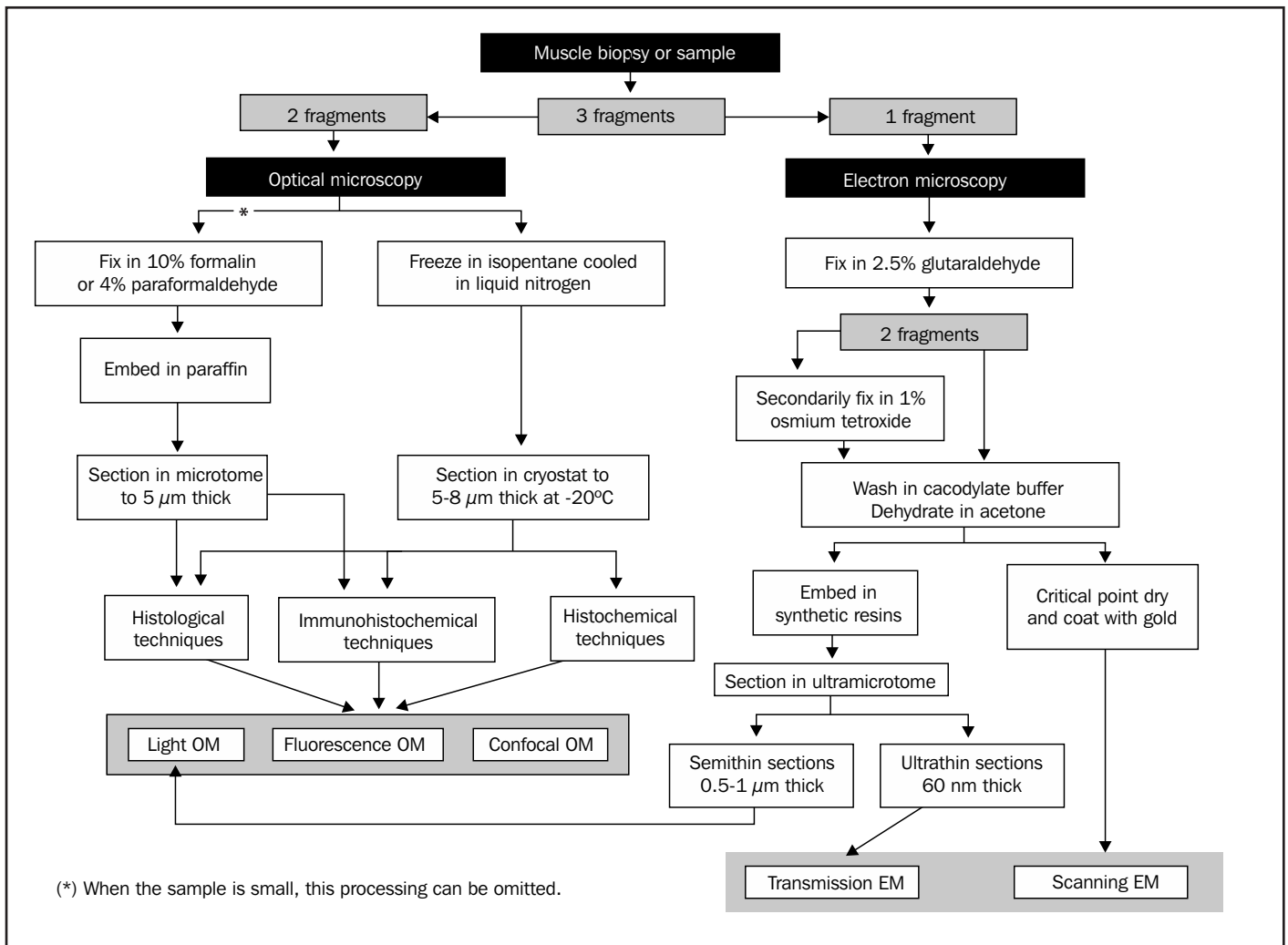


Table 1. Objectives of analysis of a histoarchitectural study.

Optical Microscopy: histoarchitectural study
<b>General histological features of muscle fibres:</b>
- Shape and size.
- Location of the myonuclei.
- Fibre types: percentages and intramuscular distribution.
<b>Satellite cells.</b>
<b>Organisation of and relationships between sheaths of connective tissue:</b>
- Endomysium.
- Perimysium.
- Epimysium.
<b>Vascular elements and their distribution.</b>
<b>Nerve elements:</b>
- Nerves.
- Neuromuscular junctions.
- Neuromuscular spindles.
<b>Myoconnective junctions:</b>
- Myotendinous.
- Myofascial.

of tissue/organ) is of very limited value in the case of skeletal muscle. The reasons are: a) fixation in formalin and embedding in paraffin produces artefactual changes in the muscle structure which hinder correct microscopic interpretation<sup>38</sup>, and b) certain cellular components (enzymes, lipids, etc.) are not preserved, thereby considerably limiting the classification of fibre types and impeding recognition of certain types of changes which cannot be seen in samples processed using the general histological technique<sup>39</sup>. However, we recommend that, whenever possible, a fragment be kept for the usual processing procedure.

### Processing samples by freezing

This is the preferred method for the microscopic study of skeletal muscle because it preserves not only the microanatomy but also the antigenic structure and enzyme content of the tissue intact, as well as detaining autolysis and tissue putrefaction. The key to the correct fixation of tissue by freezing lies in doing it instantly, because slow cooling leads

to the formation of intracellular ice microcrystals which can aggregate with the passage of time and cause the tissue to break, hindering or completely impeding microscopic analysis. Problems of this kind are known as “freezing artefacts” and can be avoided by following certain recommendations: i. the sample size to freeze must be small (1.0 x 1.5 cm)<sup>40</sup>, and ii. freeze using a mediator such as isopentane cooled in liquid nitrogen, because this speeds up freezing and prevents the formation of intracellular crystals which break the muscle fibres when the freezing process is slow or only liquid nitrogen is used<sup>39</sup>. The entire procedure is as follows and it should be stressed that extreme care must be taken when handling the sample so as not to generate artefacts:

#### 1. Shaping and mounting (Figures 4a-f):

- The muscle fragment or specimen should be approximately 4 mm in diameter and 8 to 10 mm long (Figure 4a). If it is too thick, the inside will not freeze optimally. For shaping, we recommend a razor blade split in half lengthways; while one half is used to hold the sample against the surface, the other is used to cut (Figure 4b).
- A microscopic magnifying glass can be used to correctly orient the sample during shaping; this is necessary when the samples have been obtained by needle biopsy (Figure 4c).
- The trimmed fragment is mounted on a small sheet of cork (on the base of which the sample is identified) using OCT Compound Tissue®, applied only to the cork mounting (Figure 4d) and being extremely careful not to smear or cover the muscle with OCT®, which would result in very severe freezing artefacts by acting as an insulator and preventing quick freezing<sup>27,38</sup>.
- During mounting, the correct orientation of the sample is fundamental to obtain cross sections (type of section which should be used to assess a muscle biopsy), which involves orienting the muscle fibres perpendicular with respect to the mounting surface (cork) and cutting surface (cryostat blade). Use fine tweezers and histological needles to orient the specimen, being careful not to damage the tissue (Figure 4e).
- If the specimen is too long, it could curl, resulting in the muscle fibres skewing and losing their transverse orientation<sup>41</sup>. To avoid this in our laboratory, we stick a needle in the cork to act as a support, preventing the sample from falling in the moments prior to freezing (Figure 4f). When freezing has been completed, we remove the needle.

#### 2. Freezing (Figures 4 g-l):

The freezing of skeletal muscle requires the use of isopentane (2 methylbutane) because it cannot be directly immersed in liquid nitrogen. This is due to the relative warmth of the tissue, which would cause the vaporisation of the liquid nitrogen next to it, acting as insulation against freezing and generating significant artefacts<sup>39</sup>. The solution is to freeze the specimen in isopentane cooled in liquid nitrogen, which does not penetrate the tissue or prevent or alter later staining processes.

The freezing procedure is as follows:

- Pour 80 cc of isopentane into a 100 ml beaker (Figure 4g).
- Suspend the beaker and immerse it in liquid nitrogen (-160°C)

(without it overflowing from the vessel), ensuring that the nitrogen does not enter the isopentane (Figure 4h). The first time the beaker enters, there is still a large temperature difference between the two substances, causing the liquid nitrogen to vaporise a great deal and hinder vision of the vessel (Figure 4i). This phenomenon usually dies down in 2-4 minutes.

- When the isopentane freezes, a solid white coating starts to appear on the walls and bottom of the beaker, indicating that it has reached the temperature of the liquid nitrogen (Figure 4j). In our laboratory, we remove the beaker when this has happened and carefully scrape the base and walls until this coating disappears. We then repeat the process. After this, the beaker is ready to receive the sample.
- Re-immerses the beaker; the walls and bottom will solidify very quickly. Then use long tweezers to insert the sample into the isopentane on its cork base (Figure 4k).
- Keep the specimen in the isopentane for 20 seconds (Figure 4l) and then remove it (Figure 4m), transferring it immediately to the cryostat in order to section it.

The freezing time varies according to different authors (8 to 40 seconds), in some cases because they use a mediator other than isopentane, such as acetone and dry ice or Freon 22.

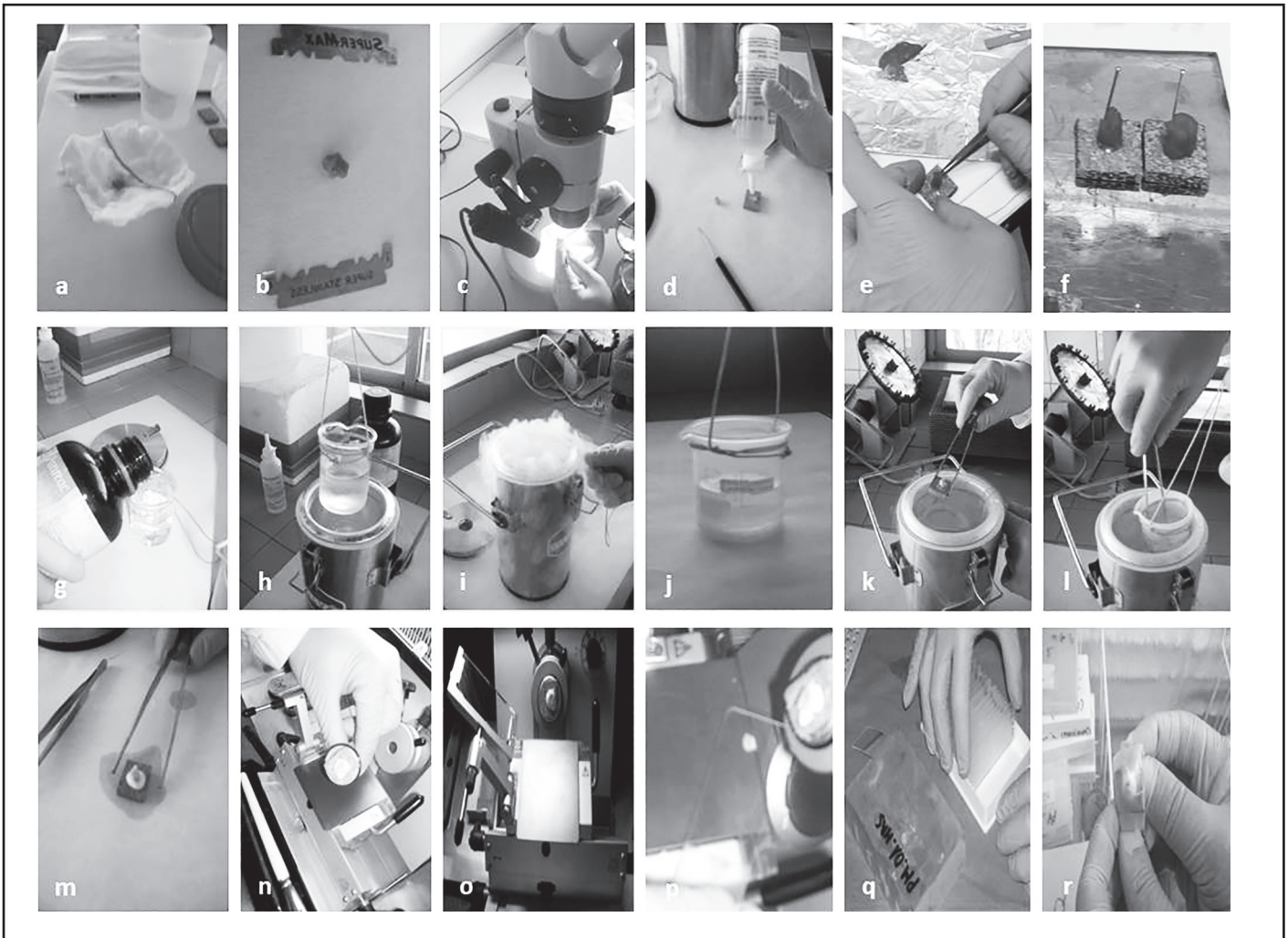
#### 3. Sectioning in cryostat (Figures 4 n-p):

- The ideal temperature for slicing skeletal muscle is -20°C, although -22°C is better when the sample contains abundant adipose or fibrous tissue<sup>40</sup>.
- Place the cork mounting with the sample on it on the metal supports of the cryostat with OCT® and wait for it to solidify and stabilise (Figure 4n). After this, fit it on the specimen holder on the arm and orient it with respect to the blade (Figure 4o).
- Then proceed to trim the surface being cut until it is smooth and uniform, and slice the muscle; the recommended thicknesses are 8-10 µm for histochemistry and 4-6 µm<sup>41</sup> or 5-7 µm<sup>22</sup> for immunohistochemistry. Sections of 2 µm are recommended for cytochemical studies of small structures, such as neuromuscular plates<sup>35</sup>. If the objective is to study the innervation of skeletal muscle with argentic impregnation techniques, the thickness of the sections should range between 50 and 100 µm<sup>42</sup>.
- The sections are collected from the surface of the blade by placing the slide, to which they adhere due to temperature difference, on top (Figure 4p). The slides are kept outside the cryostat and only enter it to collect the section, taking only those slices which are not wrinkled.
- While obtaining sections, the slide boxes can be kept outside the cryostat to help the tissue on the slide dry and reduce the appearance of artefacts due to detachment when applying the techniques (especially histochemical techniques).

#### 4. Storage of specimens and sections (Figures 4 q-r):

The slides are stored in boxes (containing 25 sections, a number sufficient for the different staining techniques) (Figure 4q) in a freezer (-20°C) prior to histological, histochemical or immunohistochemical

**Figure 4. (a-f) Sequence of the procedure for mounting specimens for freezing. (b-l) Sequence of the procedure for freezing in isopentane cooled in liquid nitrogen. (m-r) Sequence for sectioning in cryostat and storage of slides and specimens.**



staining. The slides should be dried at room temperature for about 30 minutes before staining the sections.

The frozen specimens on their cork mountings can be stored at  $-70^{\circ}\text{C}$  for an unlimited period of time and can be sectioned again (for which they need to be acclimatised to the cryostat temperature,  $-20^{\circ}\text{C}$ , for at least 20-30 minutes), retaining their aptness for staining with histological, histochemical and immunohistochemical techniques, and giving satisfactory results even decades after initial freezing<sup>43</sup>. In our laboratory, we store the specimens (both those kept at  $-20^{\circ}\text{C}$  and those kept at  $-70^{\circ}\text{C}$ ) wrapped in Parafilm® to prevent them from drying out and allowing them to be sectioned in the cryostat at any time (Figure 4a).

### What to do if you do not have the equipment and resources for processing by freezing?

In this case, the procedure to follow should be the general histological technique, as used for most histological studies. This basically

consists of fixing the specimen by immersing it in a flask containing 10% formalin for 24 hours, embedding it paraffin and sectioning it (thickness  $5\mu\text{m}$ ) with a microtome. The sections, collected on glass slides, can be stored indefinitely before staining, for which they first need to be deparaffined and hydrated.

Although this procedure generates important artefacts, it does not render the muscle sections entirely useless for histological analysis. While it does not permit enzyme histochemical techniques, it is possible to identify fibre types by means of immunohistochemical techniques using monoclonal antibodies against fast and slow myosins<sup>44</sup>.

### Preparation of samples for electron microscopy

The main drawbacks of examination using electron microscopy are related to the great amount of work involved in preparing the samples and the high cost involved. These two reasons are given for

not using it and replacing the information it can provide with techniques through which to mark the different elements which make up muscle fibre and other elements. However, in our opinion, while the information obtained through ultrastructural study is no substitute for this information, it does complement and expand on it. This type of microscopy is used when it is necessary to analyse the subcellular or ultrastructural characteristics of the elements which make up skeletal muscle, especially in research which requires very precise views of the structures (Table 2). The procedure is as follows:

### Shaping and mounting (Figures 5 a-f)

When the sample is received, a small fragment (no more than 5 mm thick) should be kept for processing using transmission electron microscopy. This fragment is inserted in a well with saline (or phosphate buffer) for 2-5 minutes to prevent artefacts in the myofibrils due to hypercontraction.

The fragment is cut into small cubes measuring 2 mm long by 1 mm wide (Figure 5a). These cubes are then transferred to a small tube in which they are covered with 2.5% glutaraldehyde (Figure 5b). The samples are so small in order to ensure that the fixative penetrates the tissue sufficiently. The samples must be fixed for at least 48 hours (maximum 2 weeks) and should be kept at 4°C meanwhile. After this time, the fixative should be replaced with phosphate buffer (Figure 5c)

**Table 2. Objectives of analysis of an ultrastructural study.**

Electron microscopy: ultrastructural study
<p><b>General cytological features of muscle fibres:</b></p> <ul style="list-style-type: none"> <li>- Basal lamina and plasma membrane.</li> <li>- Myonuclei.</li> <li>- Myofibrils.</li> <li>- Cytoskeleton.</li> <li>- Mitochondria.</li> <li>- Sarcoplasmic reticulum and T system.</li> <li>- Inclusions: glycogen, lipid droplets.</li> <li>- Specific areas:                             <ul style="list-style-type: none"> <li>• Motor end plate.</li> <li>• Myotendinous junction.</li> </ul> </li> </ul> <p><b>Satellite cells.</b></p> <p><b>Interstitial:</b></p> <ul style="list-style-type: none"> <li>- Capillaries.</li> <li>- Pericytes.</li> <li>- Interstitial cells: histiocytes, fibroblasts.</li> <li>- Nerve fibres.</li> </ul>

and stored in a refrigerator (4°C, because such a low temperature slows down the cellular autolysis processes and anoxic changes which usually occur in the deeper parts of the sample before fixation<sup>27</sup>). The sample is then secondarily fixed with osmium tetroxide. The best way to send the samples is in Eppendorf tubes with hermetic lids.

### Embedment in synthetic resins, preparation of blocks (Figure 5d)

Standardised procedures<sup>22,38,45</sup> are followed to embed the samples in synthetic resins such as araldite/epon. Following polymerisation and demoulding of the embedment capsules (Figure 5d), the specimens are trimmed prior to slicing.

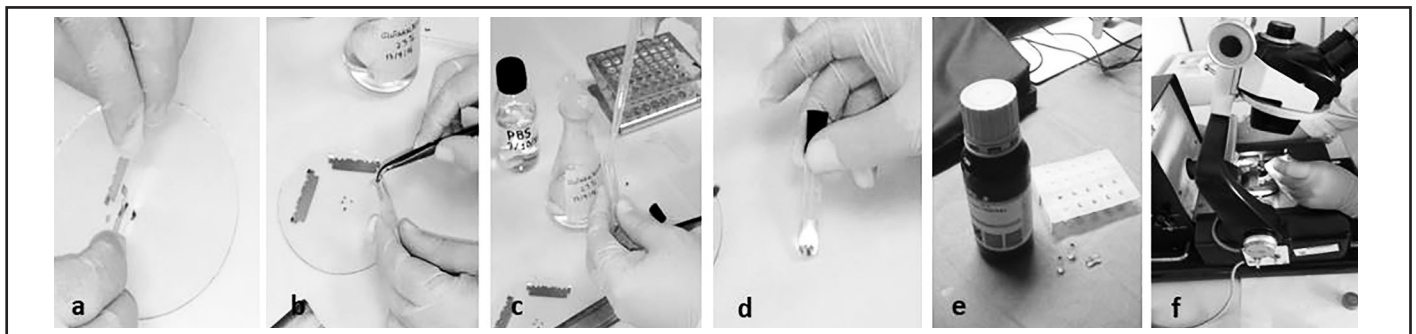
### Semithin and ultrathin sectioning (Figure 5f)

An ultramicrotome is used to obtain two types of section: semithin (0.5-1 micron) and ultrathin (50-60 nanometres). The former are collected on glass slides and stained with toluidine blue or p-phenylenediamine for prior evaluation under a light microscope; sections of this type provide a view similar to an electron microscope at very low magnification, with the advantage that they offer a much larger area of study than in transmission electron microscopy. This also allows us to select the sections and areas which interest us for later ultrastructural analysis<sup>27</sup>. These sections are collected on copper grids for contrasting (or “staining”) with uranyl acetate and lead citrate. The ultrathin sections are then analysed under a transmission electron microscope; longitudinal sections are recommended for ultrastructural study.

### Artefacts

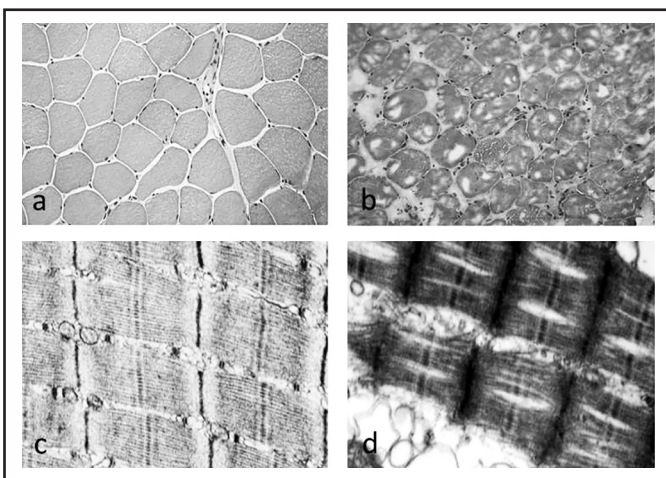
Anyone setting out to perform a microscopic examination should be aware not only of the steps involved in handling and processing histological samples of any type of tissue or organ, but also of what we call artefacts (Figure 6). Artefacts are “errors” or “flaws” that appear in histological preparations as the result of an inappropriate methodology or misuse of the equipment or apparatus. Their appearance is quite common in histology and should be avoided because not only can they render a study unviable, they can also be misinterpreted as lesions or could conceal underlying pathological changes.

**Figure 5. Sequence of the procedure for sample preparation for transmission electron microscopy.**



**Table 3.** List of recommendations to prevent the appearance of the most common artefacts due to errors in the method used to prepare skeletal muscle samples.

Point in the procedure	Don'ts	Consequences
Sample taking	Don't take samples from areas near to tendon	Overestimation of fibrosis and the percentage of nuclear internalisation
Sample taking	Don't take samples from areas previously: - Biopsied - Infiltrated with local anaesthetic - Used for EMG	Tissue necrosis, regeneration and repair may be observed as a result of previous damage
Send samples to the laboratory	Don't send samples in formalin	Histochemical techniques not possible Morphometric analysis not assessable
Processing by freezing	Don't embed the tissue in OCT	"Hole"-type artefacts appear in the more peripheral muscle fibres of the sample
Processing by freezing	Don't freeze the muscle sample directly in liquid nitrogen	Formation of ice crystals which break the muscle fibres
Storage	Don't store the samples in the freezer without protecting them with Parafilm®	The samples dry out and cannot be cut
Fixation for electron microscopy	Don't keep the muscle fragments in saline prior to fixation in glutaraldehyde	Hypercontraction of myofibrils
Fixation for electron microscopy	Inadequate fixation in glutaraldehyde: too much or too little	Mitochondrial breakage or swelling

**Figure 6.** Cross sections of human muscle stained with hematoxylin and eosin, showing (a) correct freezing and (b) freezing artefacts. Longitudinal sections of the inside of a muscle fibre under a transmission electron microscope, with (c) relaxed myofibrils showing their different bands and (d) myofibrils artefacted by hypercontraction.

In the case of skeletal muscle, artefacts are produced throughout the process, especially during freezing, possibly raising doubts about the reliability of the data obtained or even making study impossible. If this occurs, biopsies may need to be repeated or experimental animals may need to be used for study and both these solutions are ethically

questionable. Knowledge of artefacts, how they are produced and how to solve them, therefore, is essential in order to obtain the best possible microscopic preparations and guarantee analysis of the highest quality (Table 3).

## Conclusions

Knowledge of and adherence to the methodology for processing skeletal muscle samples for microscopic analysis ensures quality material and facilitates reliable results for trustworthy, precise evaluation. This sample processing by freezing methodology allows us to obtain samples suitable for a wide range of histological, histochemical and immunohistochemical techniques which, with the use of different types of microscopes, offer a more complete vision of muscle histology and, therefore, provide us with an essential aid to find out more about the responses of muscles in the field of medicine and sports traumatology.

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