Chapter

Current Approaches in Immunoassay Methods Focus on Skeletal Muscle Proteins

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Abstract

The skeletal muscle is a complex tissue that represents most of the muscle tissue in mammals and plays a key role in health and in the body's function. It is a heterogeneous tissue whose contractile and metabolic functions depend on type, size, and quality of a large number of proteins. The multitude of proteins, the relationships that exist between them, and functional changes that occur in different muscle pathologies make their investigation to be challenged. In this chapter, current approaches in proteomic studies, its application, specific technical advice, and recent progress of the most important techniques based on antigen-antibody interactions used for the analysis of muscle proteins involved in different muscle diseases are presented.

Keywords: antigen, antibody, immunofluorescence, western bolt, antisense oligonucleotides

1. Introduction

Proteins, key players responsible for good muscle function, are the main structural and functional constituents of the muscle [1]. In the last three decades, a considerable amount of skeletal muscle proteins that present a vast variation in size, shape, abundance, and expression have been identified. Based on their localization, muscle proteins are grouped as muscle membrane (dystrophin, sarcoglycans, dysferlin, and caveolin-3), the extracellular matrix (a2-laminin and collagen VI) and from the sarcomere (telethonin, myotilin, titin, and nebulin), the muscle cytosol (calpain 3, myotubularin, TRIM32), the nucleus (emerin, lamin A/C, SMN) [2]. Mutation in the gene that encodes for specific muscle proteins is responsible for the changes in expression of most of them and lead to different forms of neuromuscular diseases.

Evaluation of the localization of a protein on tissue, its expression, cellular level, and understanding of the interactions with different other proteins can elucidate the molecular basis of muscle pathology.

The differences in terms of size (from titin with a molecular mass around 1200 kDa, nebulin 700 kDa, to caveolin 3–25 kDa) and location of each of them make their investigation difficult [3].

However, the examination of the muscle protein expression by immunostaining reactions, as well as investigation of the abundance and size using SDS gel electrophoresis by methods based on antigen-antibody interaction method, has been

developed and improved over time [4]. The immunoassay methods represent a powerful tool that offers comprehensive details about the molecular alteration of different muscle pathology. Different strategies have improved over time in terms of quality and sensitivity, but each of them has the specific requirement as well as limitations.

Applications of immunoassay techniques contribute to both diagnostic and research purposes for biomarkers identification and therapeutic drug monitoring.

This chapter presents the most used techniques based on the interaction between antibodies (Ab) with a specific antigen (Ag) for muscle protein analysis, providing necessary knowledge for obtaining a good result, and provides some suggestions for the inherent problems that may be encountered in different stages.

2. Current immunoassay technologies and approaches

Immunoassay methods, technologies based on the properties of the antigen-antibody interaction, allow both qualitative and quantitative analysis of interest protein. Most important and used ***qualitative*** methods for the investigation of muscle proteins are immunohistochemistry (IHC) and fluorescent immunohistochemistry "gold standard" in muscle biopsy analysis. Their use for the evaluation of muscle proteins regarding localization, loss, accumulation, or maldistribution of different proteins involved in different myopathies has increased since the 1960s [5]. Advances in basic muscle research and development of new antibodies have increased the broad diversity of the proteins that can be analyzed. Also, technological advances in image capture, as well as improved image analysis software, now allow better interpretation and quantification of the protein expression levels both for research and for clinical pathological diagnosis. Because the successful outcome of IHC depends strictly on choosing a specific and sensitive primary antibody, and to avoid possible false-positive results, the need for an alternative method to confirm results was imperative [6]. However, for the detection and quantification of proteins in complex biological mixtures as well as for confirmation of immunohistochemistry results, western blot represents a good alternative. Western blot, also known as immunoblot, is an important quantitative method for qualitative and semiquantitative sample analysis and differential diagnosis [7].

Both immunoassay methods have various applications and have been widely used in the identification of biomarkers, diagnosis of diseases, therapeutic drug monitoring, and drug discovery, as well as characterization of protein expression and function by use of antibodies (**Table 1**).

2.1 Antigens and antibodies

2.1.1 Antigens

Antigens, also called immunogens, are any macromolecule of natural or synthetic origin such as proteins, polypeptides, polysaccharides, nucleic acids, and infectious agents that stimulate the immune system to induce an immune response (e.g., to produce a specific antibody). An antigen (Ag) molecule consists of two components: a carrier that is the largest part of the molecule and specific antigenic determinants or epitopes that are immunologic active regions of the antigens recognized by an antibody. An epitope is usually a protein segment by 5 to 8 six amino acids in length, specific for each antigen. The fixation of the antigen at the combining site of the immunoglobulin occurs based on the complementarity between epitope and paratope by multiple noncovalent bonds, van der Waals forces, hydrogen, and hydrophobic-type interactions. The rate formation of the antigen-antibody

Abbreviation	Method	Relevance to muscle proteomic studies
IHC	Immunohistochemistry	Localization of a protein Accumulation of a protein in a tissue Qualitative pattern: presence, absence, or reduction Secondary reduction
IF	Immunofluorescence	Localization of a protein Accumulation of a protein in a tissue Qualitative pattern: presence, absence, or reduction Secondary reduction
ICW	In-cell Western	Detect and quantify target proteins localized in-cell Quantitative analysis of cellular signaling pathways
WB	Western blot	Evaluation of molecular mass and abundance Differential diagnosis Level of expression
MWB	Multiplex Western blot	Localization of a protein Accumulation of a protein in a tissue Qualitative pattern: presence, absence, or reduction Secondary reduction

Table 1.
Methods and their relevance to proteomic studies.

complex depends not only on the preservation and accessibility of epitopes to the antibodies [8] as well as the concentration of the reactants but also on the mutual affinity. The alteration or destruction of epitopes by different treatment determines the reduced or abolished antigen-antibody interaction.

Because proteins are the most powerful antigens, the immunohistochemistry reaction is primarily concerned on their identification and localization as well as glycoprotein and lipoprotein compounds. The successful rate of immunohistochemistry reaction-detection of the antigen-antibody complex-depends on several factors of which the most important are: (i) the reactivity and quality of the reagents; (ii) antigen concentration in the analyzed tissue; (iii) antigen retrieval methods; and (iv) labeling conditions and methods used [9].

2.1.2 Antibodies

Antibodies also called immunoglobulins (Ig) are large Y-shaped animal glycoproteins made up of four polypeptide chains: two identical heavy chains (H) and two identical light chains (L) linked together by disulfide bonds.

While there are two main types of light chains *kappa* (κ) and *lambda* (λ) that decide the *type of immunoglobulin* molecule, for the heavy chains, 5 isotypes are found in the molecule in mammals such as humans and mice and determine the *class of immunoglobulins:* IgG, IgM, IgA, IgD, and IgE, each playing a specific role [10]. The main immunoglobulin (Ig) found in human serum that makes up about 75% of the total immunoglobulins is IgG, which is further divided into four subclasses: IgG1, IgG2, IgG3, and IgG4, each with its own biologic properties [10]. IgA can be split into two subclasses: IgA1 and IgA2 [11].

Ig is also a bifunctional molecule presenting two antigen-binding sites called Fab fragment and one complement-binding site called Fc fragment. For both heavy and light chains, the C terminal parts contain constant regions of the antibody, while the N-terminal contains variable domain through the antibody that binds to the antigen named *binding site* or *paratope*. The specificity, affinity, and antibody diversity are determined by the structure of this region [12].

The use of antibodies for the identification of protein antigen in human tissue was a revolutionary step for methods like western blotting, immunofluorescence, and immunohistochemical studies. The two most used types of antibodies in these studies are monoclonal and polyclonal antibodies.

For a successful immunohistochemistry experiment, a few things related to antibodies must be known such as whether the antibody will react with the antigen under conditions of fixation and processing system used, the immunoglobulin class to which the antibody belongs, the species in which the antibody was generated, which epitope to be targeted, type of antibody used (monoclonal or polyclonal), and analysis methods.

2.1.3 Monoclonal versus polyclonal antibodies

Besides the crucial and active role, which it plays in producing the mammal's normal immune response following the action of pathogens, antibodies are powerful tools for research and diagnostic purposes. Due to high exquisite specificity and selectivity, antibodies are an excellent tool utilized in a wide variety of therapeutic applications including detection and quantification of molecules of interest [13].

Antibodies used for research and diagnostic applications are produced by repeatedly injecting a laboratory animal (e.g., mice, rats, rabbits, and goats) with a specific antigen until it confirms the occurrence of antigen-specific antibodies in the blood serum. Generally, based on production and purification methods, antibodies can be classified into two groups: polyclonal or monoclonal.

Monoclonal antibodies (mAbs) refer to a specific antibody for a single antigenic epitope secreted from a single B-cell plasma clone. Subsequent, B-cells are fused with a myeloma cell line and grown in culture.

Polyclonal antibodies (pAbs) are produced by injecting a laboratory animal (e.g., mice, rats, rabbits, and goats) with a specific antigen and are a heterogeneous mixture of immunoglobulin molecules that are usually produced by different B-cell clone species. They can recognize and bind to several antigenic epitopes of a single antigen.

For the identification of the same antigen, both monoclonal and polyclonal antibodies are available. Choosing a primary monoclonal or polyclonal antibody for a specific target in immunoassay methods depends on the purpose of the experiment.

In the immunostaining methods based on antigen-antibody interaction, higher specificity and lower affinity of the mAbs as well as lower specificity but higher affinity of pAbs must be taken into account. To assess changes in molecular conformation, protein-protein interactions, as well as to identify the members of protein families, mAbs are more useful because of their monospecificity [14]. Polyclonal antibodies prove their usefulness in the studies they are aiming to detect variants of a particular protein of interest [15] by being able to amplify the signal of interest protein with low expression level (**Table 2**).

2.1.4 The characteristics of antibody-antigen interaction

Antigen-antibody complex (immune complex) is a bimolecular association that occurs when an antigen combines with an antibody based on the affinity of the antibody for the antigen. The reaction of the antibody-antigen is highly specific. The antibodies recognize the epitope region of the antigens and interaction between them is maintained due to exclusively noncovalent bonds such as hydrogen bonds, ionic bonds, hydrophobic interactions, and van der Waals forces [16]. The bonds are irreversible, but the antigen-antibody interaction can be affected during washing steps leading to dissociation of the complex. A lot of factors can influence the Ag-Ac complex such us temperature of incubation, pH buffer, the concentration of

	Monoclonal antibodies	**Polyclonal antibodies**
Advantages	Higher specificity to a single epitope Higher reproducibility Moderate sensitivity Reduced cross-reactivity Provide better results	Rapid generation and less expense costs Higher affinity–recognize and bind to many different epitopes of a single antigen Less technical skill required for production Easy to store Quicker binding to the target antigen Stable to pH and buffer conditions
Disadvantages	More time – 4-6 months to produce and develop Expensive costs for production Less tolerance to pH and buffer condition changes	Cross-reactivity due to a recognition of multiple epitopes High variability between different lots produced in different animals at different times

"Sensitivity" refers to the limit of detection of an assay for a specific target in a sample.
"Specificity" refers to the ability of a method to measure a specific target in a sample.

Table 2.
Characteristics of monoclonal and polyclonal antibodies.

antigen/antibody, and incubation time as well as the number of antigen sites per cell (zygosity) [16, 17].

2.1.5 Detection systems

The Ag-Ab complexes are not visible with standard microscopy and must be labeled. A wide range of fluorochromes is commercially available. An important property of a fluorochrome is the absorption spectrum. Fluorochromes absorb light at one wavelength [18] and emit light at a different wavelength.

The most commonly used markers for labeling are:

a. Fluorochromes: mostly used are fluorescein (absorbs blue light and emits yellow-green) and rhodamine (absorbs yellow-green light and emits deep red)

b. Enzymes for histochemical techniques (e.g., peroxidase, alkaline phosphatase, and glucose oxidase)

c. Metals for use in electron microscopy (ferritin and colloidal gold)

Most often, the sensitivity of an immunolabeling reaction depends on the selection of the detection system, which makes the choice of detection to be done carefully. The permanent attempt to obtain better results and significant advances in the biology field has led to the development of numerous methods for visualizing the antibody-antigen complex. Detection systems are classified as direct or indirect methods depending upon whether the fluorochrome is attached to the primary, secondary, or tertiary antibody. Regardless of the method chosen for labeling, both direct and indirect assay make possible distribution and precise localization of a specific protein or specific cellular components within a tissue or cell as well as the study of protein expression and function (**Table 3**).

2.1.5.1 Direct labeling

The direct labeling method is used for the detection of the point of interest in a specimen using a single primary Ab directly coupled with a reporter molecule (**Figure 1**). The method is usually shorter, involves one incubation step reaction,

	Direct	Indirect
Advantages	Single labeling step Short time procedure No cross-species reactivity	Great sensitivity-high amplification of the signal Production of the secondary antibodies is inexpensive
Disadvantages	Less sensitivity Lower signal Higher costs Restricted availability of direct conjugate antibodies	Double labeling steps Long procedure Extra incubation and wash steps are required High background due to endogenous activity

Table 3.
Advantage and disadvantage of immunofluorescence methods.

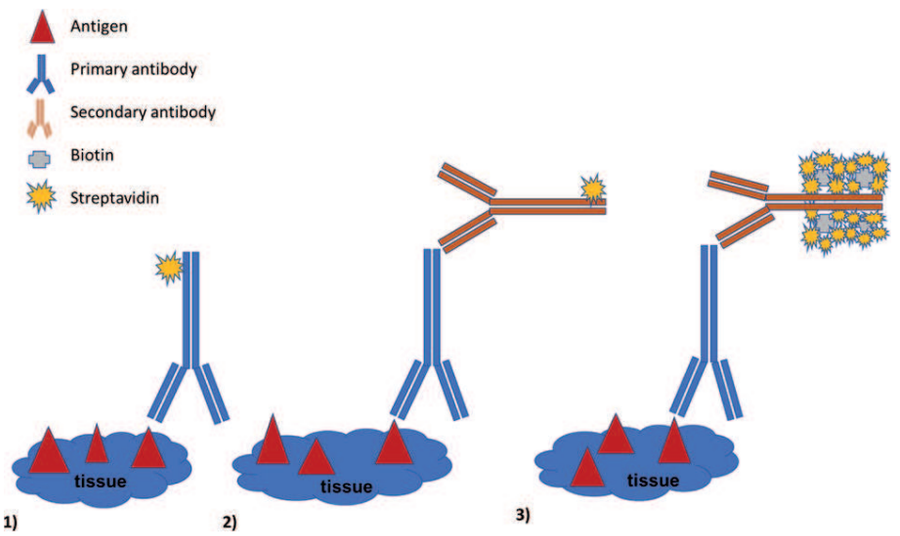

Figure 1.
Schematic representation of immunolabeling mechanisms. (1) Direct labeling; (2) Indirect labeling with secondary antibody conjugate with fluorophore; (3) indirect labeling with biotinylated secondary antibody.

and is widely used for detecting highly expressed antigens in a tissue. The advantage of the direct labeling method is the avoidance of antibody cross-reactivity or nonspecificity.

Nevertheless, direct labeling is not routinely employed in clinical and research applications due to insufficient sensitivity of the method in detecting of antigens in a tissue, the weak intensity of obtained signal, as well as optimal given preparation process of attaching fluorochrome to the antibody, which can affect antibody affinity. To date, for muscle protein analysis is used indirect labeling due to the higher sensitivity of the method.

2.1.5.2 Indirect labeling

The need to improve the sensitivity of detection methods of antigens with low expression led to the development of labeling methods in several stages, which intensifies the signal. *Coons et al*. developed in 1941 a detection method that involved two distinct steps: i) an unconjugated primary antibody incubated with tissue section that binds to the target molecule and forms an invisible antigen-antibody complex and ii) secondary antibody, which carries the fluorophores, enzyme, and biotin, recognizes, and binds to the primary antibody facilitating

detection by producing a fluorescent signal or a collared substance. This method was a progress for labeling method because it allowed the amplification of the signal by increasing the number of fluorophore molecules that target the antigen. There are several advantages of the method that make it so popular: different primary antibodies can be used with the same secondary antibody, the possibility of use of secondary antibodies conjugated with different dye fluorophores, and the ability of the unconjugated primary antibody to bind to up to five secondary antibodies [19].

Thus, the indirect method is no longer used in its original form but continues to develop both the identification and production of a large number of fluorescent molecules, as well as the improvement of specific methodologies for coupling fluorochromes with antibodies.

2.1.6 Important parameters for immunomarkers

Therefore, even the immunostaining methods may seem simple in concept, there are many critical steps in performing it. To ensure success and to obtain good results, several parameters should be considered as follows:

1. ***Sample collection and tissue handling*** immediately after surgery play an important role in immunostaining. For example, [20] showed that a long time after harvesting increases degradation of calpain 3 in muscle due to *autolysis* that can affect the detection of this protein involved in a type of muscular dystrophy by immunostaining methods.

2. ***Specimen fixation*** is another important step in immunostaining. The alteration, damage, or maskings of the epitope of some antibodies against sarcolemmal membrane-associated proteins by fixation in formalin-fixed and paraffin-embedded skeletal muscle tissue have been reported [21].

3. ***Slide storage conditions***: Following freezing and until sectioning, tissue must be stored at ultra-low temperature (−80°C). The tissue architecture undergoes damage due to freeze-thaw [22]. Also, longer storage tissue sections lead to cracking as well as decrease in intensity signal levels. Furthermore, protecting the tissue slide from oxidation must be considered.

4. ***Probe size***: Depending on the purpose, the tissue section for immunostaining can vary. Previous reports have shown that the intensity of staining is indeed dependent on tissue thickness [23]. Generally, for muscle protein analysis, the recommended thickness of tissue slides is 7um for tissue cryosection [...] and 3–5 μm for FFPE sections [21].

5. ***Antibody selection (monoclonal vs polyclonal):*** This step is critical for immunostaining methods. Knowing the differences between monoclonal antibodies (that recognize a single epitope in an antigen) and polyclonal antibodies (that bind to multiple different epitopes from an antigen) for the interest target must be selected the antibodies with higher specificity. In addition, antibody concentration, working temperature, and duration of incubation affects the immunostaining reaction results and must be optimized.

6. ***Controls*****:** Due to insufficient specificity of antibodies or procedures used, sometimes unexpected binding and labeling can occur. To avoid confusing or inconsistent obtained results as well as to demonstrate that the detected signal

is specific for the target protein, a set of controls is required to demonstrate the reliability of labeling. Therefore, while planning immunolabeling experiment, more types of controls are essential [24] and should be included to validate the staining reaction and to show that the protocol works properly:

Tissue (antigen) controls

- *Positive control*: a tissue section, which is known to express the target protein; successful staining reactions of this control demonstrate the protocol and parameters used to detect the target protein.
- *Negative control*: a tissue section known not expressed the target protein and no response is expected. It is useful for the identification of positive errors.
- *Tissue background control*: used to avoid interpretation of autofluorescence mainly comes from mitochondria, lysosomes, for example, as positive results.

Reagent specificity controls

- *Primary antibody controls*: useful to demonstrate specific binding of the primary antibody to the antigen
- *Secondary antibody controls*: useful to show specific binding of the secondary antibody to the primary antibody

The use of antibodies in immunohistochemical microscopy-based experiments is a helpful tool for identification, localization, and expression patterns of muscle proteins. This technique is still routinely used in research to study the role of interest protein both in healthy and in pathological muscle as well as for diagnostic purposes or optimized treatment regimes and therapeutic drug monitoring. However, the use of one method only for the complete characterization of a muscle protein is not enough. The advances in the proteomic field led to the improvement of the analytical method for identification and quantification of proteins avoiding thus inconsistent and confusing immunocytochemical results.

The most used method for confirmation of immunohistochemical results as well as for the size of the protein is western blot. There are many different types and methods for Western blotting.

2.2 Western blot

The Western blot (WB) method also known as protein immunoblotting is an important analytical and quantitative technique used to identify, to separate a specific protein from a given complex biological mixture of proteins from a tissue/cellular homogenate, and to determine the amount of antigens (proteins) in reaction with a specific antibody. Initially, the proteins are electrophoretically separated into a polyacrylamide (PAA) gel electrophoresis. The most widely used technique for large scale protein from a mixture of proteins is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) due to the possibility of separation of proteins based on their molecular weight under the action of electric current after linearization of the proteins. Special gradient gels are used to separate proteins with a wide variety of molecular mass. After the separation, the proteins are electrophoretically transferred on a solid substrate such as nitrocellulose, PVDF, or cationic nylon membrane. To suppress nonspecific adsorption of the antibodies,

the unreacted nonreactive binding sites on the membrane are blocked, which causes immobilized proteins to react specifically with monoclonal or polyclonal antibodies. Primary antibodies can be applied single when we target the expression of a single protein or in a cocktail when a simultaneous analysis of several proteins is necessary. Commonly used conjugates for secondary antibody are color, radioactivity, enzyme, as well as biotin. Antigen-antibody complexes are radiographically, chromogenically, or chemiluminescently visualized.

With this technique, the size (molecular mass) and abundance of the interest protein are evaluated by comparing with the control. The need for the analyses of multiple different target proteins simultaneously led to great improvement of sample separation resolution.

2.2.1 Multiplex Western blot

The multiplex detection methods have improved over the past few decades and have led from the analysis of a single specific protein to the detection of simultaneously multiple target proteins with a different molecular weight in a complex of cellular homogenates [25, 26]. For the simultaneous analysis of muscle proteins, a biphasic polyacrylamide gel system with different concentrations is used. With this system, the separation of muscle proteins is done based on their molecular weight (proteins with molecular weight more than 200 kDa are separate in the top part of the gel, e.g., dystrophin, while the smaller proteins under 150 kDa, e.g., calpain 3 in the bottom). Highlighting of proteins is achieved with a cocktail of specific primary antibodies.

Simultaneous analysis of multiple proteins involved in different muscle pathologies revolutionized the medical diagnosis, reduced cost and time for analysis, and improved differential diagnosis in muscle pathology.

2.2.2 In-cell western (ICW) assay

ICW also known as in-cell ELISA (ICE) is quite a novel quantitative immunofluorescence-based technique suitable for the detection of protein levels and signaling events performed in cell culture grown on microplate format [27]. It is an extremely sensitive method and accurately quantifies, which can detect two targets normally labeled with specific primary antibody followed by incubation with secondary antibody fluorescent conjugated with spectrally distinct dyes and quantification of the signals from fluorophores conjugated at different wavelengths on two detection channels. The technique allowed the quantification of proteins directly in cell culture [28]. The accuracy of quantification is increased by normalization due to adjustments of the cell number in wells. This technique is also useful in the study of the drug effect on multiple points.

2.3 Data acquisition and image analysis

For the evaluation of antigen expression by ***immunohistochemistry*** (IHC) and fluorescent IHC, a comprehensive microscopic evaluation of the signal beside specimen preparation and staining protocol depends also on the type of microscope used for acquisition of imagines.

2.3.1 Light microscopy

In bright-field microscopic IHC, antigen expression in muscle is visualized by a combination of a secondary antibody with an enzyme and utilizing a colorimetric

substrate that produces a colored reaction product detected by light microscopy. The most widely used enzymes in chromogenic detection are the horseradish peroxidase (HRP) and alkaline phosphatase (AP), which convert 3,3′ diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC) into brown and red end color, respectively. The reaction product is stable for a long time and visualization can be done any time. Multiple labeling is also possible since different substrates are now available for the same enzyme, but low contrast and resolution for samples is a limitation of bright-field microscopy.

2.3.2 Fluorescent microscopy

Although a lot of muscle proteins can be detected by IHC with peroxidase and visualized in light microscope field, the labeling of antigen with an antibody coupled with a fluorochrome permitted a superior viewing of many muscle proteins and offered significant advantages in accurately identifying certain protein localizations and distinguishing subtle difference in protein expression patterns. The major advantage of fluorescence microscopy is given with the possibility of uses of different dyes that span the entire visible spectrum to track different target proteins. Also, the identification of the interaction between proteins as well as their specific localization is better observed by colocalization when the color of two or more different fluorescent dyes used appears changed as a result of the overlap. One limitation of fluorescence microscopy remains the loss of the fluorescent capacity *of* fluorophores (photobleaching) due to many cycles of excitation and emission [29]. Therefore, it is recommended to keep the samples marked in dark when not in use and use a mounting medium with an antifade agent to fix this problem.

In combination with laser confocal scanning microscope, fluorescence is preferable to evaluate the degree of colocalization and relative quantitation of proteins by specialized software [30].

2.3.3 Confocal microscopy

Confocal microscopy is a powerful laser scanning method used for the imaging analysis of fluorescently labeled specimens.

The technique provides high-resolution detailed information about the 3D structure of the tissue sections and cells allowing detailed analyses and measurements of double, triple, and even quadruple stained sections [31, 32]. This is possible due to labeling of secondary antibodies with different fluorophores, which emitted on a different wavelength.

The z-stack mode permitted three-dimensional reconstructions of optical sectioning useful for the study of the relationship between stained structures.

2.4 Qualitative and quantitative analysis of proteins

Muscle pathology usually involves changes in level and protein expression. For muscle protein diagnosis, a good knowledge is required of the expected cellular location: at sarcolemmal *level (dystrophin, sarcoglycans, dysferlin, and caveolin-3),* in the cytoplasm (*calpain 3 and TRIM32),* and nuclear (*emerin and lamin A/C)* level. The initial choice of a qualitative or quantitative method for protein analysis depends on the availability of the methods and the specificity of the antibodies. It should be mentioned that the results obtained by one method have to be confirmed by another; see **Table 4**.

Current Approaches in Immunoassay Methods Focus on Skeletal Muscle Proteins
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Disease	Gene	Primary protein defect	Secondary changes	Localization level	Clue to diagnosis	References
					IF/WB	
DMD	DMD	Dystrophin	Utrophin upregulated Sarcoglycans reduced/absent Dystroglycan reduced/absent nNOS absent	Subsarcolemmal	Absent, reduced/absent	[2, 43]
BMD	DMD	Dystrophin	Utrophin upregulated Sarcoglycans reduced/absent Dystroglycan reduced/absent nNOS reduced/absent	Subsarcolemmal	Reduced/Reduced in size/amount Absence of at least one antibody	[2, 43]
DMD/ BMD carriers	DMD	Dystrophin	Utrophin upregulated Sarcoglycans reduced/absent Dystroglycan reduced/absent nNOS may be absent	Subsarcolemmal	Mosaic pattern/ Reduced in size	[2, 43]
LGMD2C-F	SGCG SGCA SGCB SGCD	Sarcoglycans	Reduction of other sarcoglycan B-Dystroglycan reported Possible reduction Dystrophin Loss of nNOS reported	Sarcolemmal	Variably reduction/absent	[44]
LGMD2B	DYSF	Dysferlin	Caveolin-3 reduced	Sarcolemmal	Absent/reduced	[45]
LGMD1C	CAV3	Caveolin-3	Dysferlin reduced	Sarcolemmal	Absent/reduced	[45]
CMD	LAMA2	Laminin α2	Deficiencies of laminin β2, α-dystroglycan and integrin α7	Extracellular matrix	Completely or partially absent	[46]
Bethlem or Ullrich myopathy		Collagen VI	Deficiency of laminin β1 chain	Extracellular matrix	Reduced/absent	[47]
LGMD2G	TCAP	Telethonin	—	Sarcomere	Reduced/absent	[44, 48]
LGMD2J	TTN	Titin	Calpain 3 reduction	Sarcomere	Absence of calpain 3/loss of C-terminal fragments of titin results in the reduction of higher molecular weight	[48, 49]

Disease	Gene	Primary protein defect	Secondary changes	Localization level	Clue to diagnosis	References
					IF/WB	
LGMD1A	MYOT	Myotilin	Secondary laminin γ reduction	Sarcomere	Protein aggregates	[45, 48]
LGMD 1E	DES	Desmin	Myotilin, αB-crystallin, VCP cytoplasmic aggregates	Exosarcomeric	Desmin cytoplasmic aggregates	[50]
LGMD2A	CAPN3	Calpain 3	Dysferlin reduced	Cytosol	Labeling may be absent or reduced on sections/ Calpain 3 bands may be variably reduced	[44, 50]
LGMD 2H	TRIM32	Trim 32	—	Cytosol	Reduced expression/reduced level	[51]
Emery-Dreifuss MD	EMD	Emerin	—	Nucleus	Absent /reduced level	[52]
LGMD1B	LMNA	Lamin A/C	Laminin β1 reduction	Nucleus	Lamin A/C normally expressed	[44]

Table 4.
The skeletal muscle-specific proteins.

2.4.1 Analysis of immunohistochemical expression

Immunohistochemical staining is useful for the identification of the interest markers and provides valuable information about their distribution, localization, and expression in a tissue or cell. The obtained results should be interpreted by comparing a given protein pattern in normal and affected tissue in the presence of the controls of the reaction mentioned above. The evaluation of muscle protein expression is generally made in a qualitative manner based on their presence, absence, or variable reduction. Depending on the manifestation of the protein identified by immunohistochemical analysis, the progression of different neuromuscular disorders can be evaluated.

The usefulness of the method lies in the identification of the primary protein abnormalities in recessive diseases [33] and secondary reduction of interconnected proteins. For example, in the primary reduction of dystrophin, protein involved in Duchenne/Becker muscular dystrophy and secondary reduction of sarcoglycans [22] and of cytosolic calpain 3 [25] was reported. The reduction of dysferlin also is accompanied by the reduction of calpain 3.

Because of the currently limited access to next-generation sequencing that permitted analysis of simultaneous gene involved in a different type of muscular dystrophies, analysis of muscle biopsy by the immunoassay method is still a helpful method in many laboratories. Different computational methods developed in the last decade for quantitative immunohistochemical (IHC) image analysis of proteins have begun to be increasingly used.

How much protein is needed for proper muscle function has always been a problem that researchers have been trying to solve. Immunohistochemistry quantification of dystrophin proteins involved in the most severe type of muscular dystrophy has come to the attention of researchers with the improvement of image analysis software. Thus, L. E. Taylor [34] and Antony K [35] developed a method of image analysis that allows immunofluorescent quantification of dystrophin expression in sections that proved to be robust and reliable method of biomarker detection. These methodologies contribute to and improve the final diagnosis and especially are used in the analysis of a protein after a drug and specific treatment.

However, increasing the contradictory results obtained by this method and reported in the literature, a final and accurate diagnosis requires the confirmation of the results by another quantitative method, such as Western blot.

2.4.2 Interpretation of Western blot results

Western blot methods have become very popular in diagnostic laboratories due to the ability to analyze several proteins simultaneously avoiding thus the preservation of large portions from an affected muscle [36, 37].

The rapid evolution of the quantitative methods has improved over time from the analysis of one protein to simultaneous analysis by SDS gel electrophoresis of several proteins with different molecular mass. Multiplex Western blot technique developed by Andreson [25] represented a significant improvement in muscle protein analysis regarding efficiency and cost. By this method, can be evaluated the molecular mass (normal or reduced size) and abundance of the proteins by comparison with controls and the use of quantitative software analysis.

Western blot has greater importance in muscle protein analysis especially in the differential diagnosis of muscular dystrophies (distinguish between DMD and BMD patients); for analysis of calpain 3 in it, there are no antibodies available for immunoreactions on the sections.

2.4.2.1 Applications, utility, and importance of immunoassay methods

The immunohistochemical reactions are widely used both in the investigation and in the pathological diagnosis evaluations of diseases based on the antigen-antibody reactions. The identification and localization of an antigen in a tissue or cells can provide valuable information, which otherwise could not be obtained by other methods. Because most of the antigens are usually proteins, identifications of abnormal expression patterns of proteins in diseased tissue are also helpful in differential diagnosis and detecting primary protein defect involved in the pathology. Besides the identification of a primary defect and protein expression chances, immunohistochemistry is also helpful in the pharmaceutical analysis area for drug discovery and therapeutic drug monitoring.

2.4.3 Therapeutic drug monitoring

With knowledge advance in disease pathogenesis and the development of novel therapeutic strategies, there is a need to generate new treatments for specific neurodegenerative disorders. In the last few years, the use of antisense oligonucleotides (AOs) has increased interest in the treatment of the different types of muscular dystrophy such as Duchenne muscular dystrophy.

Antisense oligonucleotides, a new class of the synthetic single-stranded molecules of nucleic acids as RNA or DNA, have been reported that modulate the gene expression and splicing process interacting with specific gene transcripts through a variety of mechanisms and restore the expression of functional protein [38, 39].

The quantification of protein levels after treatment will be monitored by the immunoassay method after administration of a specific dose of specific AOs. Positive results obtained in clinical trials with AOs for different diseases increase the interest in the clinical application of antisense strategies. However, more clinical trials and more data are necessary to make this strategy clinically available.

Difficulties, limitations, and disadvantages of immunostaining methods and troubleshooting are discussed further.

a. Difficulties

Although it is a simple technique, many parameters must be considered and optimized before. The quality of the results depends on these critical factors that begin from handling the specimen to tissue fixation procedure, detection system, staining protocol, antibody selection, and sensitivity. Also, the ability, experience, the rigor of execution of the researcher in performing the reaction, and good knowledge and understanding of the methodology and morphological changes in a specific pathology are necessary for an accurate overview of protein expression and interpretation of the results to avoid misinterpretation [40, 41].

b. Limitations

One of the major limitations of the immunoassays is antibody specificity. The nonspecific binding occurs when an antibody attaches to a cell without a specific epitope for that antibody. Several reasons are responsible for nonspecific binding such as higher concentrations of the antibody and binding by *Fc* segment contained by most of the antibodies to *Fc* receptors of immune cells (e.g., neutrophils,

monocytes, and macrophages). The decrease of antibody concentration and the use of an Fc blocking reagent will minimize the nonspecific binding [42].

The nonspecific binding of the antibody can cause errors and confusion in the interpretation of the obtained results. To eliminate the interpretational error, antibody specificity controls must be introduced into the reaction to obtain reliable staining [43].

A limited number of available antibodies for muscle protein represent another limitation of this method as well as the choice of an insufficiently specific and sensitive antibody for the desired target. Often, the resolution microscope is a limitation in determining the proper intensity of the signal of the target antigen as well as subtle differences in protein expression level. This problem can be solved by confocal laser scanning microscopes, which offer a possibility to overcome this problem by providing images in the highest resolutions and can predict accurately variation in staining intensity. It must be considered that the image quality degrades over time.

c. Disadvantages

Both direct and indirect immunofluorescence methods have some advantages and disadvantages, which are presented in **Table 3**. Besides, these immunostaining methods have some disadvantages among which we mention the lack of worldwide standardized protocol that determines the introduction of a level of subjectivity both in the working procedure and in the interpretation of the obtained results. Once again, the experience of the personnel who work this technique is crucial.

3. Conclusion

Immunoassay methods are a powerful tool for characterization of the proteins regarding localization, understanding the quantitative and qualitative characteristics, as well as interactions of proteins at the cellular level. The progress of the development of the specific antibodies, as well as improvement in image analysis software, leads to more sensitive and specific immunoassay methods used for characterization of the proteins. However, a combination of qualitative and quantitative methodologies offers great value for the results. Further advances in immunoassay methods will lead to a better understanding of the functional role of proteins.

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Conflict of interest

The author declares that there are no competing interests.

Acronyms and Abbreviations

Ag	antigen
AOs	antisense oligonucleotides
BMD	Becker muscular dystrophy
DMD	Duchenne muscular dystrophy
CMD	congenital muscular dystrophy
ICW	in-cell Western
IF	immunofluorescence
Ig	immunoglobulins
IHC	immunohistochemistry
LGMD	limb-girdle muscular dystrophy
MAbs	monoclonal antibodies
MEB	muscle-eye-brain disease
PAbs	polyclonal antibodies
WB	Western blot

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