Muscle Biopsy: A Diagnostic Tool in Muscle Diseases

Lee Nowak and Patricio F. Reyes
Barrow Neurological Institute, St. Joseph’s Hospital and Medical Center, Phoenix, AZ

Abstract
Muscle biopsy is an important and essential tool in the diagnosis and treatment of muscle disease. For several decades, histological staining of paraffin-embedded tissue was used in muscle pathology. In the past few decades, fresh-frozen enzyme histochemical techniques have become the standard for assessing muscle disease. They have been complemented by the use of electron microscopy to characterize ultrastructural changes. More recently, the discovery of certain muscle proteins and the development of antibodies made possible the combination of enzyme histochemical and immunohistochemical methods to better assess muscle changes. In this review, we discuss the roles of histochemistry, enzyme histochemistry, immunohistochemistry, and electron microscopy in the diagnosis of skeletal muscle pathology (The J Histotechnol 31:101, 2008).

Submitted March 3, 2008; accepted with revisions May 24, 2008

Key words: actin, ATP, histochemistry, immunohistochemistry, myoglobin, myosin, NADH, skeletal muscle, tropomyosin

Introduction
A muscle biopsy involves obtaining a tissue sample that will be processed and examined under a light microscope for pathological alterations in muscle, connective tissue, and blood vessels. Although muscle biopsies have been performed since the 19th century, they did not become a standard diagnostic tool until the mid-20th century (1).

Earlier biopsy procedures illustrate the difficulties in obtaining appropriate skeletal muscle specimens for morphologic examination. Because contraction artifacts can distort the morphology of muscle fibers, particularly skeletal muscle, sutures were inserted into each end of the muscle in situ and attached to a wooden applicator to “splint” the tissue sample obtained for microscopic evaluation (2). This procedure has been replaced by more-sophisticated and less-invasive methods.

At present, muscle biopsies (3–8) normally are performed with the patient under local anesthetic. He or she may experience a burning sensation from the anesthetic, “tugging” during the procedure, and soreness in the area for a week after the procedure (9). Current methods of muscle biopsy include needle biopsy, in which a hollow needle is inserted through the skin and the appropriate tissue is aspirated (10–14). In many cases, open muscle biopsy is used to obtain fresh and frozen tissue specimens to be used in histochemical, immunohistochemical, and electron microscopy protocols (15).

Muscle Types
There are three muscle types: skeletal, smooth (visceral), and cardiac. Although the three types all use the movement of actin against myosin to create a contraction, each mechanism of action is different. Understanding the mechanisms of action of different muscle types can indicate which methods of staining would be most appropriate.

Skeletal muscle fibers are innervated by a motor axon. Its action begins at the motor end plate or neuromuscular junction, where a motor axon terminal releases acetylcholine, which binds to receptors localized in the muscle membrane at the motor end plate, resulting in local depolarization and triggering an action potential (16).

Smooth muscle differs from skeletal muscle in that electrical activation is passed from cell to cell by gap junctions instead of discrete, well-defined neuromuscular junctions. Contraction in the smooth muscle of the gut is independent from direct neural control, whereas the iris of the eye is under more direct, precise neural control (17).

The action potential in cardiac muscle is generated within the heart itself, passed from fiber to fiber through gap junctions, causing the muscles to contract in a synchronous wave that sweeps from the atria through the ventricles, pumping blood out of the heart (18).

Skeletal Muscle Fiber Types
Skeletal muscle is made up of muscle fibers, polygonal and uniform in size. They bundle into groups called fascicles. In 1678, Stefano Lorenzini described muscles simply
as “red” and “white,” differentiated by their hue and anatomical distribution. These descriptions have now been related to the metabolic and physiological properties of muscles (19). Red refers to Type I skeletal myofibers, also called “slow twitch,” which are responsible for long-duration, low-intensity activity. They receive their energy from mitochondria and lipids. White Type II fibers, also called “fast twitch,” have less myoglobin and are responsible for short-duration, high-intensity activity and quick bursts of energy. Their main source of energy is glycogen (20). Type II fibers can be further subclassified as Type IIa (glycolytic), muscles less easily fatigued; Type IIb (glycolytic/oxidative), muscles easily fatigued; and Type IIc, believed to be fiber-changing types as the result of disease or injury (21,22).

Muscle types and subtypes can be determined by incubating frozen muscle sections at various pH of adenosine triphosphatase (ATPase), an enzyme critical for adenosine triphosphate (ATP) production. Muscle contraction is powered by the breakdown of ATP produced in the mitochondria (23). Staining for ATPase will result in differentiation of muscle types by color depending on the pH of preincubation solutions. The procedure is performed specifically to compare the number, size, location, and distribution of Type I and Type II fibers. At pH 9.4, Type II fibers will be dark, Type I fibers light, and Type IIc fibers intermediate. At pH 4.6, Type I fibers are the darkest, Type IIb and c fibers intermediate, and Type IIa the lightest (Figure 1a). At a pH of 4.3, Type I fibers are the darkest, Type IIc fibers intermediate, and Type IIa and b the lightest (24).

Normally, Type I and Type II fibers are distributed randomly throughout a muscle fascicle in a mosaic pattern. If the normal mosaic pattern is disturbed, it may result in “fiber-type grouping,” a phenomenon usually associated with chronic denervation and reinnervation, and easily distinguished by such a staining procedure (Figure 1b) (20).

Structural skeletal muscle changes may occur in systemic disorders. Muscle atrophy of Type II fibers may be seen in patients with cachexia, a condition frequently seen in the elderly, possibly due to muscle disuse or malnutrition, end-stage cancer, chronic obstructive pulmonary disease, and some autoimmune diseases (25–27).

**Enzyme Histochemistry**

Oxidative enzymes, such as the dehydrogenases, the adenosine triphosphatases (ATPases, which are important in the functioning of the contractile mechanism), and enzymes associated with the glycolytic pathway, such as phosphor-
Lases, are enzymes considered relevant when one assesses muscle fiber integrity. Hydrolytic enzymes such as galactosidase and phosphorylase, which are found in mammalian muscle fibers, are useful in the diagnosis of glycogen-storage disorders. Phosphorylase activity is reciprocally distributed to oxidative enzymes. Small fibers with concentrated oxidative enzymes show weak or negative phosphorylase reaction. Conversely, large fibers containing low oxidative enzyme activity show intense phosphorylase activity. Medium fibers show intermediate activity (28). Among the hydrolytic enzymes, esterase may be used to identify degenerating denervated fibers (29).

The subcellular distribution of most enzyme histochemical reactions falls into one of two patterns: myofibrillar pattern (ATPase reaction) and intermyofibrillar network pattern in the oxidative enzyme reactions (mitochondria, sarcoplasmic reticulum, transverse tubular system, aqueous sarcoplasm) (30).

Nicotinamide adenine dinucleotide (NADH) (Figure 2) is an oxidative enzyme staining procedure that reveals myofibrillar architecture, mitochondria, and target fibers. It may also differentiate between Type I and Type II fibers. Its staining is based on the theory that flavoprotein enzymes transfer hydrogen from NADH to various dyes, usually tetrazolium compounds, which accept the hydrogen, staining the tissue (23). A purple precipitate forms at site of mitochondria in sarcoplasmic network. Type I fibers are darker than those of Type II (because of greater enzyme activity in Type I fibers and increased numbers of mitochondria). Architectural changes in the muscle, like central cores, whorled, lobulated, and moth-eaten fibers, usually are demonstrated by NADH. These changes are believed to occur as the result of displacement of the intermyofibrillar network and mitochondria (20).

Succinate dehydrogenase, which also is an oxidative enzyme, will distinguish relatively more oxidative from less oxidative fibers. The more succinate dehydrogenase a fiber possesses, the more intense the stain. Oxidative fibers will be a deeper purple with a densely speckled appearance, whereas nonoxidative fibers will have a more sparsely speckled, purple appearance (31). Glucose-6-phosphate dehydrogenase can be used to detect degenerating or regenerating muscle fibers without increased staining in denervated or atrophic fibers (32).

**Histochemistry**

Routine stains used in muscle biopsies include hematoxylin and eosin (Figure 3), which stains for nuclei and other basophilic structures (blue) and cytoplasm and acidophilic structures (light to dark red) (28). Gomori’s trichrome stain (Figure 4a) shows subsarcolemmal nuclei and normal muscle myofibrils (33). It may also demonstrate increased endomysial, perimysial and epimysial connective tissue in muscular dystrophies, abnormal accumulations of mitochondria (seen as ragged red fibers) (Figure 4b) (34) in mitochondrial diseases, and nemaline rods, usually found

![Figure 2](image-url). NADH showing Type I (dark) and Type II (light) myofibers. (a) Normal myofibrillar architecture (total magnification, ×400). (b) Target fibers with vacuoles and loss of myofibrillar architecture, indicating a neurogenic process (total magnification, ×200). Photo courtesy of Dr. Stephen Coons.
in nemaline rod myopathy, a congenital and hereditary neuromuscular disorder (35). Nemaline bodies can be differentiated from ragged red fibers because they lack enzyme activity (36).

Other stains can be used as warranted. Oil Red O is a fat-soluble dye used to demonstrate triglycerides and lipids in frozen sections (deep orange red) in Type I muscle fibers (Figure 5) (37,38). It may also stain some lipids and lipoproteins in paraffin-embedded tissue (39). It is useful in showing abnormal deposits of fat in lipid disorder diseases, in other tissues after long bone fractures, and in tumors arising from and containing fat cells (40). Lesions associated with vacuoles such as target fibers and artifacts can mimic fat globules, but fat stains are negative.

Periodic acid Schiff (PAS) is used to demonstrate muscle glycogen, mucin and some basement membranes (red to purple), fungi (red to purple), and nuclei (blue) (41). PAS, for glycogen, will be more distinct in Type II fibers. PAS stain is important when confirming and distinguishing glycogen-storage diseases (20). Van Gieson’s stain also may be used to differentiate collagen from connective tissue. Increased collagen may be seen in muscle disorders accompanied by excessive collagen deposition. Cytoplasm, muscle, fibrin, and red blood cells appear yellow, whereas collagen is bright red (42).

Immunohistochemical Staining

In the past, histochemical techniques were the only methods used in diagnostic muscle biopsies. With the discovery of certain muscle proteins and other cellular and subcellular components came the development of antibodies and new immunohistochemical protocols that have been used to verify the presence, absence, abnormal accumulation, or maldistribution of certain proteins in neuromuscular disorders.

Furthermore, antigen-retrieval techniques and antibodies to specific muscle proteins made possible the development of immunohistochemical methods to demonstrate fiber types, denervation, and the characterization of dystrophic...
myopathies (1). Paraffin-embedded methods are used primarily to look for organisms such as parasites and bacteria and muscle involvement in systemic disease such as infection, malignancy, and immunologic disorders.

Immunohistochemistry (IHC) uses antigen–antibody interaction, visualized by fluorescent, enzymatic, or another marker, to localize specific antigens in tissue sections. This method of staining is more specific than special or enzymatic staining procedures that only identify a limited number of proteins, enzymes, and tissue structures. Immunohistochemical methods are more sensitive and can detect even small amounts of proteins (31,43). IHC also can be used to help identify key proteins found in muscle fibers, neuromuscular junctions, and supporting elements, including desmin, which forms the Z line in striated muscle, and actin, which works with myosin in muscle contraction, and troponin and tropomyosin, which regulate the interaction of actin and myosin. In the skeletal muscle, nebulin is an actin-binding protein localized to the I-band. Mutations in nebulin cause nemaline myopathy in which nemaline rods cluster in subsarcolemmal and perinuclear locations. Nemaline rods also may show immunoreactivity for other proteins, including telethonin and myotilin (1). Titin (connectin), the largest known protein, attaches the Z line to the M line in striated muscles (44). Telethonin is a protein involved in stabilizing the Z-disk by anchoring titin within Z lines. It is absent in limb-girdle muscular dystrophy (LGMD) 2G. Myosin loss is associated with both acute and chronic muscle-wasting disorders. IHC for myosin demonstrates fiber type differentiation and for desmin is the standard immunostain for myofibrillary myopathies (1).

In biopsies, histochemical and immunohistochemical methods are used for the diagnosis of inflammatory myopathies, certain muscular dystrophies, mitochondrial cytopathies, and metabolic myopathies (45). Antibodies against dystrophin can be used to diagnose Duchenne muscular dystrophy and Becker-type muscular dystrophy, because gene abnormalities will result in the loss of the dystrophin protein from the myofibers’ plasma membrane complex. Antibodies against merosin can be used to diagnose congenital muscular dystrophy. Merosin helps to anchor myofibers to the extracellular matrix. Mutations result in merosin deficiency, which accounts for approximately half of the cases of congenital muscular dystrophy. Lamin A/C, dysferlin, and sarcoglycans can be used in the diagnosis of various forms of LGMD. Caveolin deficiency results in LGMD 1C, also known as rippling muscle disease. Antibodies against tropomyosin, myotubulin, and desmin can be used to identify nemaline myopathy, centronuclear myopathy, and desmin storage myopathy, respectively. Alpha-dystroglycan is also reduced in congenital muscular dystrophies with brain abnormalities, such as congenital muscular dystrophy with severe mental retardation and abnormal glycosylation, muscle-eye-brain disease and Walker-Warburg syndrome.

![Figure 5](image-url) Oil Red O. (a) Normal lipid staining (total magnification, ×200). (b) Increased lipid staining (total magnification, ×400). Photo courtesy of Dr. Stephen Coons.
and congenital muscular dystrophy with respiratory failure and muscle hypertrophy (1,25,46). In inflammatory myopathies, the antibodies most frequently used are CD3, CD4, CD8, CD20, and CD68 to determine lymphocytes from macrophages in necrotic muscle fibers (1).

**Electron Microscopy**

Electron microscopy (EM) is generally used to determine the ultrastructural changes in subcellular components that may not be identified by histochemistry or immunohistochemistry. It can improve diagnostic accuracy in muscle disorders. It is recognized that a definitive diagnosis cannot be made by EM alone; however, it remains useful in diseases in which antigen–antibody and histochemical methods are not helpful (47). The major roles of EM in muscle biopsy are to distinguish normal from abnormal, to clarify features observed in the light microscope, and to identify features not visible with the light microscope (48). Sampling error in EM can be a concern because some muscle tissue may appear “normal” in earlier stages of disease. Proper sampling and identifying precisely every phase of the EM of skeletal muscle are critical to achieve a reliable result (49). The introduction of immunohistochemical methods has led to a more limited application of EM in many neuromuscular disorders.

In diseases associated with defects of protein components of the sarcolemma, extracellular matrix and nuclear membrane, EM is a valuable tool to confirm diagnoses suggested by immunohistochemical and molecular analysis. In mitochondrial disorders, EM can show changes in size, number, distribution, or structure of the cristae (Figure 6) (48). Moreover, ultrastructural studies are most valuable in showing sarcoplasmic anomalies, including vacuoles, inclusion bodies, and other changes not revealed by light microscopy. Finally, EM is helpful in understanding pathological mechanisms of muscular diseases as well as correlating subcellular abnormalities with genetic and molecular investigations (50).

**Conclusion**

Histochemistry, enzyme histochemistry, IHC, and EM play important roles in the diagnosis and treatment of neuromuscular disorders. IHC is a relatively new frontier in diagnostic medicine. As more cellular and subcellular proteins are identified, the diagnostic value of IHC will increase. Future advances in immunopathology and molecular pathology will lead to the development of more sophisticated diagnostic techniques and a better understanding of muscle diseases.

**References**

5. Rohe M, Appell HJ, Schneider PG: Muscle biopsy—still a

---

**Figure 6A-B.** EM images of abnormal mitochondria showing (a) numerous mitochondria creating an irregular “ragged” profile; (b) electron-dense inclusions. Photos courtesy of Dr. John Beggs.