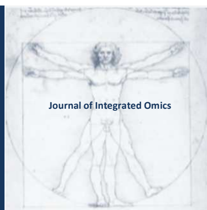

JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

REVIEW ARTICLE | DOI: 10.5584/jiomics.v2i2.111

Organelle proteomics in skeletal muscle biology

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Received: 18 September 2012 **Accepted:** 17 October 2012 **Available Online**: 04 November 2012

ABSTRACT

The cell biological profile of skeletal muscle tissues is highly complex and variable due to the molecular heterogeneity and cellular plasticity of contractile fibres and their supportive structures. Mass spectrometry-based proteomics has been used to study global changes in muscle during maturation, differentiation and physiological adaptations, as well as following pathological insults. However, due to the dynamic protein expression range of contractile cells, the findings from large-scale biochemical surveys of crude tissue extracts were limited to mostly soluble and abundant protein species. To overcome this technical problem, organelle proteomics was applied to study distinct subcellular fractions from skeletal muscle preparations. Tissue pre-fractionation procedures significantly reduce sample complexity and thus allow a more comprehensive cataloging of highly complex protein mixtures. This article reviews the impact of recent subproteomic studies of skeletal muscle and discusses findings from changes in the proteome of mitochondria, surface membranes, sarcoplasmic reticulum, cytosol and the contractile apparatus in normal, transforming and pathological muscle.

Keywords: Contractile apparatus; Mitochondria; Muscle proteomics; Sarcolemma; Sarcoplasmic reticulum.

1. Introduction

Comparative proteomics presents one of the most powerful analytical tools to determine global changes in distinct protein constellations, including the establishment of alterations in protein abundance, isoform expression patterns, protein interactions and post-translational modifications [1]. This makes proteomic datasets a fundamental part of modern systems biology and network analysis of biological processes [2], as well as the discovery of disease biomarkers [3]. Mass spectrometry is now the method of choice for swift and reliable protein identification in almost all high-throughput biochemical surveys of biological or pathological processes [4-6]. However, the routine proteomic investigation of crude tissue extracts can be complicated by sample complexity. Physicochemical properties of individual protein isoforms can differ considerably within a given proteome [7]. Thus, the identification and characterization of thousands of proteins by standard separation techniques, such as liquid chromatography [8] or gel electrophoresis [9], often only achieves the coverage of the near-to-complete tissue proteome. Differences in size, charge, hydrophobicity and density seriously hamper the comprehensive identification of all protein species in a dynamic cellular system.

Organelle proteomics attempts to overcome these technical limitations by reducing sample complexity [10] using sophisticated pre-fractionation steps prior to proteomic analysis [11-13]. The analysis of membrane-associated proteins is especially challenging in large-scale proteomic studies [14-16] and is mostly due to the limited solubility, dy-

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namic properties and relative low abundance of integral proteins in most biological systems [17-19]. Skeletal muscles contain a diverse range of protein constituents making the application of organelle proteomics an essential part of basic and applied myology. Voluntary contractile fibres exhibit a large dynamic range of proteins, a unique set of extremely high-molecular-mass proteins, numerous supramolecular membrane complexes and an extremely diverse isoform expression pattern of proteins involved in excitation-contraction coupling, contractile functions, muscle relaxation, ion homeostasis and metabolic pathways [20]. This review discusses the impact of recent subproteomic studies of normal, adapting and pathological skeletal muscle.

2. Defining the skeletal muscle proteome

Extensive mass spectrometry-based cataloging of the skeletal muscle proteome has resulted in the identification of thousands of fibre-associated protein isoforms, as well as the degree of crucial post-translational modifications, such as glycosylation, phosphorylation, tyrosine nitration and carbonylation [21]. Liquid chromatography, one-dimensional gels or two-dimensional gels using isoelectric focusing in the first dimension and slab gel electrophoresis in the second dimension are routinely employed to separate muscle proteins. The majority of fibre-associated proteins identified by proteomics belong to the class of contractile proteins of the thick and thin filaments (myosin light chains, myosin heavy chains, actins), regulatory proteins of the contractile apparatus (troponins and tropomyosins), enzymes of anaerobic metabolism (glycolytic enzymes), enzymes of oxidative metabolism (mitochondrial proteins), metabolic transportation (fatty acid binding proteins, albumin, myoglobin), as well as proteins responsible for detoxification and the cellular stress response (heat shock proteins). These studies included various muscles from humans [22] and animals that play a key role in biomedical research [23], as well as muscle samples crucial for the meat industry [24]. The proteomic comparison of fast versus slow muscles has revealed clear differences in protein expression patterns, showing altered levels and isoform distribution of key regulatory, functional and structural proteins belonging to the contractile apparatus, excitation-contraction coupling, ion homeostasis, cell signaling, stress response and muscle metabolism [25-27].

The proteomic characterization of human *vastus lateralis* muscle revealed that mitochondrial proteins accounted for 22% of the accessible skeletal muscle proteome [28]. This emphasizes the importance of mitochondria for muscle metabolism and shows that global proteomic studies can make an important contribution to our overall understanding of muscle physiology and bioenergetics. It also implies that organelle proteomics will play a key role in future studies of the skeletal muscle proteome during physiological adaptations or pathological insults. Thus, proteomic cataloguing studies are crucial for the establishment of comprehensive protein databanks for skeletal muscles in health and disease. Besides the establishment of extensive proteomic maps of skeletal muscle based on gel electrophoretic studies, the application of shotgun proteomics to the biochemical characterization of human skeletal muscle has identified a large number of muscle-associated proteins. The proteomic analysis of biopsy specimens from 31 individuals has identified more than 2,000 protein species [29]. These databanks can now be employed as references for large-scale comparative studies of skeletal muscle samples. Whole tissue proteomics has investigated the effects of physical exercise, weight loss, muscular atrophy, muscle growth and fibre transitions, as well as the pathological impact of nerve damage, diabetes, sepsis, hypoxia, muscular dystrophy, inclusion body myositis, myotonia, age-related muscle wasting and motor neuron disease, as critically examined in several recent reviews [21, 22, 30-32].

3. Subproteomics of skeletal muscle tissues

Since all current large-scale biochemical separation methods have technical limitations with respect to properly separating all components within complex mixtures of heterogeneous proteins, routine proteomic studies of crude extracts do not usually cover the entire protein constellation of a given biological sample [33]. Although body liquids, such as saliva, urine or plasma with their almost exclusively soluble components, present exceptions and have been cataloged in their entirety by standard proteomics [34-36], the mass spectrometric identification of all proteins present in biological tissues is technically more challenging [37]. The extensive dynamic expression range of proteins in complex tissues and the diversity in charge, size and post-translational modifications of soluble proteins versus membrane proteins makes a reduction in sample complexity a prerequisite for inclusive proteomic studies. The subproteomes of distinct fractions from skeletal muscle have been described for nuclei [38], mitochondria [39-43], the contractile apparatus [44], sarcolemma [45], sarcoplasmic reticulum [46] and the cytosol [38, 47, 48], as described in detail in the below subsections.

3.1 *Subcellular fractionation of skeletal muscle tissues*

Muscle organelle proteomics attempts the cataloging and characterization of discrete subcellular fractions or supramolecular protein assemblies from contractile tissues, employing pre-fractionation steps prior to final protein separation. This may include micro-dissection approaches, differential centrifugation schemes, density gradient centrifugation, affinity isolation methods based on ligand or antibody technology, the usage of narrow pH ranges during the isoelectric focusing of low-abundance proteins, offgel electrophoresis for investigating proteins with extreme p*I*-values, detergent phase extraction or differential detergent fractionation techniques for studying integral proteins, and immobilization and blotting methods using on-membrane digestion for identifying extremely high-molecular-mass or very hydro-

phobic proteins [7, 11-13, 37, 49]. Emerging separation techniques are free-flow electrophoresis and fluorescent-assisted organelle sorting [50], which have not yet been widely applied to skeletal muscle biology. The flowchart of Figure 1 summarizes routine pre-fractionating steps for the isolation of major organelles, membrane systems and functional units from skeletal muscle homogenates. This includes the preparation of the contractile apparatus, nuclei, mitochondria, sarcoplasmic reticulum, transverse tubules, sarcolemma, extracellular matrix, cytoskeleton and the cytosolic fraction.

Standardized protocols for the subcellular fractionation of skeletal muscle homogenates consist of repeated centrifugation steps at progressively higher speeds and longer centrifugation periods. Distinct fractions enriched in nuclei, the contractile apparatus, mitochondria and microsomes are routinely achieved by centrifugation for 10 minutes at 1,000*g*, 10 minutes at 10,000*g*, 20 minutes at 20,000*g* and 1 hour at 100,000*g*, respectively. After high-speed centrifugation, the final supernatant contains almost exclusively components from the cytosolic fraction. Since myosins constitute one of the most abundant classes of proteins in muscle homogenates, myosin heavy and light chains often cross-contaminate subcellular fractions following differential centrifugation. However, trapped or adsorbed myosin molecules can be easily removed from membrane preparations by mild salt washes [51]. The heterogeneous content of the microsomal fraction can be further separated by density gradient centrifugation. If a sufficiently high *g*-force is employed, surface membranes can be separated from the highly abundant sarcoplasmic reticulum, which usually divides into fractions enriched in longitudinal tubules, terminal cisternae and triad junctions [51]. The sarcolemma and transverse tubules can be further separated by affinity purification methods, such as differential lectin agglutination [52].

Subcellular marker proteins for distinct fractions from skeletal muscle tissue are represented by laminin for the basal lamina, the Na^+/K^+-ATPase for the sarcolemma, dystrophin for the sub-sarcolemmal membrane cytoskeleton, vimentin for the cytoskeleton, the dihydropyridine receptor for transverse tubules, the ryanodine receptor Ca^{2+}-release channel for triad junctions, SERCA-type Ca^{2+}-ATPases for longitudinal tubules of the sarcoplasmic reticulum, calsequestrin for the terminal cisterna region, sarcalumenin for the lumen of the sarcoplasmic reticulum, galactosyl transfer-

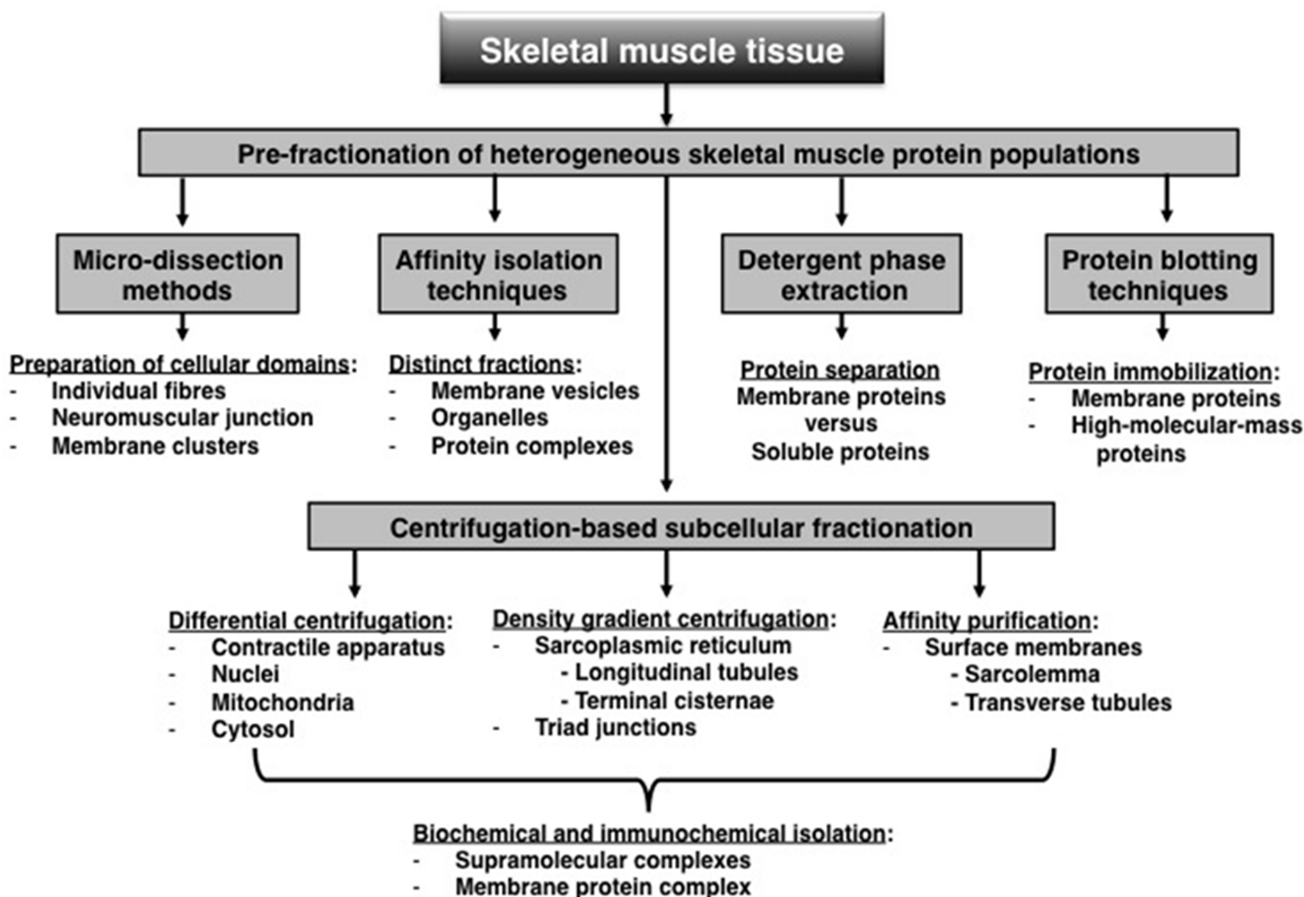

Figure 1. Overview of routine pre-fractionating techniques used for the isolation of major organelles, membrane systems and functional units from skeletal muscle. The flowchart summarizes the various experimental approaches employed to prepare fractions highly enriched in distinct organelles or large cellular structures, including micro-dissection methods, affinity isolation techniques, detergent extraction, protein blotting techniques and centrifugation-based subcellular fractionation protocols.

ase for the Golgi apparatus, histones for nuclei, ribosomal proteins for ribosomes, succinate dehydrogenase for mitochondria, acid phosphatase for lysosomes, catalase for peroxisomes, myosin heavy chains for the contractile apparatus and lactate dehydrogenase for the cytosol. For the subfractionation of intact organelles, the application of consecutive swelling, shrinking and sonication steps yields usually distinct membrane species. For example, mitochondrial subfractions can be identified by the enrichment of marker enzymes, such as succinate dehydrogenase of the inner membrane, glutathione transferase of contact sites and monoamino oxidase of the outer membrane.

3.2 *Contractile apparatus*

The distribution of myosin heavy chains, myosin light chains and fibre type-specific enzymes is indicative of the main fibre types I, IIa, IIx/d and IIb of skeletal muscles [53]. Since contractile proteins are extremely abundant in muscle fibres, comparative proteomic studies using crude tissue extracts routinely identify myosins, actins, troponins and tropomyosins by mass spectrometry [22]. Alterations in the abundance of specific contractile protein isoforms have been shown to occur during muscle adaptations, muscular atrophy and the natural aging process, as well as in a variety of neuromuscular diseases [21]. Previous biochemical studies have demonstrated that chronic low-frequency stimulation causes myosin light and heavy chains, as well as regulatory troponin subunits, to undergo a stepwise replacement from fast to slow isoforms [54]. Electrostimulation-induced muscle transformation has been confirmed by mass spectrometry -based proteomics and shown rapid fast-to-slow transitions due to enhanced neuromuscular activity [55, 56]. However, the detailed study of changed protein expression levels and/or isoform switching usually requires enriched preparations of the contractile apparatus.

Fractions enriched in myofibrillar proteins can be easily isolated from tissue homogenates by differential centrifugation [57, 58]. The proteomic characterization of isolated myofibrils from young adult versus senescent skeletal muscle has established a drastic increase in the abundance of slow myosin light chain MLC-2 during aging [44]. Phosphoproteomics could furthermore show that this contractile protein exhibits the highest change in phosphorylation during aging [44, 59]. The MLC-2 isoform of myosin light chain is an excellent indicator of fibre transformation in skeletal muscle [54] and its abundance changes and post-translational modifications establish this protein as an excellent candidate for being a suitable biomarker of sarcopenia of old age [60]. Comparative immunoblot analysis and immunofluorescence microscopy were used to verify proteomic data and confirmed a switch between fast and slow myosin heavy chains in aged muscle fibres [44]. Hence, these proteomic findings support the pathophysiological concept of an age-related shift to a slower-twitching fibre population that expresses predominantly slow isoforms within myosin hexamers [61]. In the long-term, the proteomic identification of altered concentration levels of individual muscle protein species may be useful in the biochemical evaluation of novel pharmacological or nutritional treatment options to counter-act age-related muscle wasting, as well as improved exercise regimes to prevent sarcopenia and frailty in the elderly.

3.3 *Skeletal muscle mitochondria*

Global changes in mitochondrial proteins are intensively studied [62, 63]. A recent review by Silvestri et al. [64] has described in detail the importance of various analytical approaches, including two-dimensional gel electrophoresis, blue native polyacrylamide gel electrophoresis, fluorescence difference in-gel electrophoresis, shotgun proteomics and stable isotope labeling analysis, mitochondrial protein arrays and mitochondrial protein databanks. The mammalian mitochondrial proteome consists of over 1,000 different proteins [65-67], which are distributed throughout the inner matrix region, a heavily folded inner membrane structure, a smooth outer membrane and contact sites between the two membranes, as well as an inter-membrane space that is continuous with the cytosol [68, 69]. While a small number of mitochondrial proteins are encoded by a unique mitochondrial genome [70], a large population of proteins is imported into mitochondria via sophisticated transport mechanisms [71, 72]. Mitochondria represent one of the most diverse class of organelles in the body with respect to multiple metabolic functions and manifold involvements in regulatory processes. Having originated as endosymbionts of a primordial eukaryote, highly evolved mitochondria play a key metabolic role as the primary site for energy generation via oxidative phosphorylation [68]. They are furthermore involved in the integration of intermediary metabolism, protein transport, the production of heme and iron-sulfur clusters, cell cycle progression, calcium ion signaling and the regulation of apoptosis [69]. The proteome of mitochondria exhibits considerable tissue heterogeneity [39, 40] and the protein constellation of skeletal muscle mitochondria can be distinguished by their subsarcolemmal versus intermyofibrillar location [73]. Interestingly, proteomics has shown that the mitochondrial oxidative phosphorylation system is associated with sarcolemmal lipid rafts during myogenesis [74] and that muscle mitochondria are closely coupled to lipid droplets probably promoting metabolic channeling [75].

Proteomic profiling of human *vastus lateralis* muscle has identified 823 distinct mitochondrial proteins [41], making mitochondria one of the most extensively catalogued organelle from voluntary contractile tissue [29]. Post-translational modifications have also been extensively studied in isolated muscle mitochondria by mass spectrometry [76, 77]. Since altered numbers of mitochondria, functional alterations within this crucial organelle and/or changed expression levels within the mitochondrial proteome play a central role in various disorders [67, 78] and especially during the natural aging process of skeletal muscles [79-81],

numerous subproteomic studies have targeted mitochondria and their involvement in senescence of the neuromuscular system [82]. In agreement with comparative studies of total tissue extracts from adult versus aged muscle [21, 22], organelle proteomics identified a large number of changed mitochondrial proteins during aging [42, 43]. Proteomic profiling in combination with Blue Native PAGE analysis, which has been widely applied to defining the mitochondrial protein population [83], revealed interesting age-related changes in the abundance of mitochondrial complexes involved in oxidative phosphorylation [43]. The mitochondria-enriched fraction from senescent rat muscle was shown to exhibit drastically increased levels of NADH dehydrogenase, succinate dehydrogenase, creatine kinase and ubiquinol cytochrome-c reductase [42]. In addition, the offgel electrophoretic analysis of basic proteins could also show increased levels of mitochondrial creatine kinase and ubiquinol cytochrome-c reductase in aged muscle [84]. These findings agree with the pathobiochemical concept of a drastic metabolic shift during fibre aging [61]. Skeletal muscle tissues appear to perform a higher degree of aerobic-oxidative metabolism in a slower-twitching fibre population during age-dependent fibre degeneration [60]. However, this process is probably not an adaptive mechanism during aging, but rather a pathological consequence of the selective denervation and preferential loss of type II fibres in senescent muscles [85]. The progressive loss of muscle mass and function in the elderly has now been termed sarcopenia and is believed to play a major pathophysiological role in the overall functional decline of the senescent organism [86-88].

Regular physical activity and a healthy lifestyle play a crucial role in preventative medicine, including the avoidance of an early onset of certain forms of diabetes, obesity or sarcopenia of old age. Thus, studying the effects of endurance exercise versus disuse atrophy by proteomics can give crucial molecular and cellular insights into global mechanisms of muscle adaptation [31]. Changed functional demands have profound effects on protein expression levels and proteomic studies have shown that on the one hand chronic neuromuscular activity triggers fast-to-slow transitions [56, 89] and on the other hand muscular atrophy is clearly associated with slow-to-fast transformation processes [90-92]. The fact that endurance training triggers extensive modifications of the mitochondrial protein constellation was recently confirmed by organelle proteomics. Egan et al. [93] have shown that a large number of mitochondrial markers are increased in human *vastus lateralis* muscle following endurance exercise, including creatine kinase, ATP synthase and malate dehydrogenase. These proteomic findings support the idea of skeletal muscle tissue being highly plastic in its physiological response and that the neuromuscular system can quickly adapt to new physical challenges.

In contrast to physical training, muscular disuse and obesity has been linked to a higher susceptibility to type 2 diabetes. Diabetes mellitus does not only affect the heart in a major way, but also triggers contractile weakness and metabolic disturbances in skeletal muscles [94]. Muscle proteomics has shown major changes in the protein profile of patients with insulin resistance [95], whereby an oxidative-to-glycolytic shift appears to occur in diabetic fibres [96]. The proteomic profiling of isolated mitochondria from diabetic skeletal muscle has shown a generally perturbed protein expression pattern with a reduction in NADH dehydrogenase, cytochrome b-c1 complex and isocitrate dehydrogenase. Isoforms of ATP synthase and pyruvate dehydrogenase showed differential changes in their expression levels [97]. Mitochondrial abnormalities probably cause a diabetes-related impairment of oxidative phosphorylation and may thus be crucial for metabolic disturbances and the development of insulin resistance [32].

3.4 *Surface membranes*

The highly complex arrangement of the sarcolemma and its membranous invaginations, the transverse tubular system, is involved in a plethora of cellular functions in skeletal muscle fibres. This includes synaptic transmission, propagation of action potentials, excitation-contraction coupling, the essential stabilization of the fibre periphery during contraction-relaxation cycles, indispensable nutrient uptake, metabolite transportation, the maintenance of cell signaling systems, the provision of natural repair mechanisms following membrane rupturing, the regulation of ion homeostasis and general structural support for the preservation of fibre integrity [98]. Since the sarcolemma and transverse tubules are relatively low in abundance as compared to the intricate membrane system of the sarcoplasmic reticulum, the biochemical isolation of distinct surface membrane vesicles from skeletal muscle homogenates without cross-contaminations is a difficult task. However, an important prerequisite of meaningful muscle organelle proteomics is the biochemical purity of the starting material for protein analysis [7, 37]. Hence impurities due to non-specific protein adsorption and/or cross-contaminations due to membrane entrapments during isolation procedure have to be kept to a minimum. Ideally suited for such applications are affinity isolation methods that exploit distinct cellular, immunological or physicochemical properties of subcellular fractions and their constituents.

Affinity isolation of plasmalemma vesicles in combination with one-dimensional gradient gel electrophoresis and on-membrane digestion has been used to determine the protein composition of highly purified sarcolemma vesicles from rabbit skeletal muscle [45]. This novel approach has overcome several technical limitations of standard gel electrophoresis-based proteomics that routinely employs two-dimensional gel electrophoresis and subsequent in-gel digestion for mass spectrometric studies. Two-dimensional gel electrophoresis is an excellent method for the large-scale separation of urea-soluble and abundant proteins that fall into a molecular mass range of approximately 10 to 200 kDa. However, proteins with a low copy number, very high mo-

lecular mass, mostly hydrophobic properties, extensive post-translational modifications and/or extreme p*I*-values are poorly represented by this electrophoretic technique. Thus, for studying integral proteins of high molecular mass it is advantageous to pre-fractionate tissue samples to reduce sample complexity and then use large one-dimensional gradient gels for protein separation. In addition, since high-throughput proteomic surveys using in-gel digestion procedures are sometimes complicated by an inefficient trypsination of certain protein species, on-membrane digestion with superior protein sequence coverage can be used as an alternative [99-101].

When studying highly enriched sarcolemma vesicles from skeletal muscle, the combination of lectin affinity purification, one-dimensional gradient gel electrophoresis and on-membrane digestion has resulted in the comprehensive cataloguing of major protein bands representing the plasmalemma [45]. Mass spectrometric screening of sarcolemma-associated proteins identified a number of very large muscle components, including the membrane cytoskeletal element dystrophin of 427 kDa. The Dp427 isoform, the absence of which causes x-linked muscular dystrophy, had not previously been identified in numerous proteomic studies of normal versus dystrophic muscles due to its very high molecular mass [30]. Hence, affinity organelle isolation and on-membrane digestion were shown to be highly appropriate for the proteomic identification of high-molecular-mass proteins that would otherwise not be properly recognized by standard two-dimensional gel electrophoresis.

3.5 *Sarcoplasmic reticulum*

The specialized endoplasmic reticulum of skeletal muscles, the sarcoplasmic reticulum, is a highly abundant organellar structure and acts as a crucial physiological controller of Ca^{2+}-cycling throughout the muscle interior [102]. The sarcoplasmic reticulum thus plays a key role in the regulation of the excitation-contraction-relaxation cycle of muscle fibres [103]. Since the contractile status of muscles is determined by cytosolic Ca^{2+}-levels, the spatiotemporal organization of Ca^{2+}-release versus energy-dependent Ca^{2+}-uptake by the sarcoplasmic reticulum regulates excitation-contraction coupling and fibre relaxation. A large number of Ca^{2+}-channels, Ca^{2+}-ATPases, Ca^{2+}-exchangers and Ca^{2+}-binding proteins are involved in the highly complex maintenance of Ca^{2+}-homeostasis in skeletal muscle tissues [104]. Transient opening of the nicotinic acetylcholine receptor by neurotransmitter binding triggers the massive influx of Na^{+}-ions into muscle fibres at the neuromuscular junction, which in turn causes the propagation of an action potential along the sarcolemma via voltage-dependent Na^{+}-channel activation. Within the transverse tubular membrane, the voltage-sensing α_{1S}-subunit of the dihydropyridine receptor interacts with the ryanodine receptor complex by direct physical means through its II-III loop domain and triggers the opening of the Ca^{2+}-release channel at the triad junction [105]. Passive efflux of Ca^{2+}-ions along a steep gradient raises the level of this second messenger in the cytosol and initiates muscle contraction by binding to the troponin TnC subunit. Fibre relaxation is caused by the active re-uptake of Ca^{2+}-ions into the lumen of the sarcoplasmic reticulum by slow SERCA2 and fast SERCA1 Ca^{2+}-ATPase complexes. Ion shuttling within the organelle is provided by sarcalumenin and ion storage and channeling to the ryanodine receptor is mediated by the high-capacity Ca^{2+}-binding protein calsequestrin [106].

As already outlined in above section on sarcolemma proteomics, standard gel electrophoresis-based proteomics does not cover all protein species present in crude extracts or subcellular fractions. This is especially problematic in the case of very large integral proteins of which the sarcoplasmic reticulum contains many examples, such as the ryanodine receptor Ca^{2+}-release channel monomer of 565 kDa [107]. In order to be able to identify this hydrophobic protein of the sarcoplasmic reticulum by mass spectrometry, a combination of subcellular fractionation, gradient gel electrophoresis, protein blotting and on-membrane digestion had to be employed [46]. The proteomic survey of the sarcoplasmic reticulum revealed the presence of 31 distinct protein species, including all major Ca^{2+}-regulatory proteins involved in the excitation-contraction-relaxation cycle, such as Ca^{2+}-ATPase, calsequestrin, sarcalumenin and the Ca^{2+}-release channel. Previous ultrastructural studies had localized glycolytic enzymes on sarcoplasmic reticulum vesicles [108]. The biochemical concept of a close physical coupling between the energy-dependent sarcoplasmic reticulum and the ATP-producing glycolytic pathway was confirmed by proteomics. Mass spectrometry clearly identified the presence of the glycolytic enzymes phosphofructokinase and aldolase in the purified sarcoplasmic reticulum [46]. Thus, organelle proteomics using on-membrane digestion methodology is an excellent way for studying high-molecular-mass proteins and hydrophobic proteins.

The cationic carbocyanine dye 'Stains-all' labels most gel-bound proteins with a light pinkish colour, but stains Ca^{2+}-binding proteins with a characteristic dark purple colour whereby dye-protein complexes absorb maximally at 615 nm [109]. This property of 'Stains-all' dye, in combination with mass spectrometry and immunoblotting, was exploited in a subproteomic study of the fate of Ca^{2+}-binding proteins in dystrophic muscle [110]. Duchenne muscular dystrophy is an x-linked inherited muscle wasting disease and due to primary abnormalities in the membrane cytoskeletal protein dystrophin [111]. Micro-rupturing of the dystrophin-deficient sarcolemma and inefficient repair mechanisms are believed to cause irregular ion handling, which is probably a key pathophysiological factor that renders skeletal muscle fibres more susceptible to necrosis [112]. Comparative subproteomics demonstrated that the reduced Ca^{2+}-buffering capacity of the sarcoplasmic reticulum from dystrophic fibres is caused by drastically decreased levels of the main luminal Ca^{2+}-reservoir protein calsequestrin [110]. Proteomic

analyses have also established that the luminal Ca^{2+}-shuttle protein sarcalumenin is reduced in dystrophin-deficient muscle [113]. Hence, a reduction in essential luminal Ca^{2+}-binding proteins probably plays a key role in the molecular pathogenesis of x-linked muscular dystrophy. With respect to potential therapeutic implications, it is important to mention that the proteomic evaluation of experimental exon-skipping treatment has shown a reversal of calsequestrin reduction in treated fibres [114]. There appears to be a direct connection between the re-establishment of the sub-sarcolemmal membrane cytoskeleton and the Ca^{2+}-handling apparatus of the sarcoplasmic reticulum in muscle following reversal of the primary defect in dystrophinopathy [30].

3.6 *Muscle cytosol*

The sarcoplasm provides the aqueous environment within a muscle fibre and supports and surrounds organelles with its semi-fluid material containing water, anions, cations, organic molecules, nutrients and numerous metabolic and signaling enzymes. The cytoplasm functions as a polar solvent for soluble cellular constituents and facilitates the transportation of essential metabolites and messenger molecules within the contractile fibre. A key anaerobic pathway for energy production and metabolic integration is glycolysis, which occurs almost exclusively in the cytosol of muscle fibres. The enzyme hexokinase is the only exception and its activity levels are regulated by binding to the mitochondrial outer membrane for metabolic channeling purposes. Subproteomic studies have confirmed the high abundance and central metabolic position of glycolytic enzymes in skeletal muscle tissues. Proteomic analysis has clearly established that the most abundant protein species present in the diffusible fraction of the skeletal muscle proteome are the 10 glycolytic enzymes [47]. The high density and solubility of the enzymes that mediate the core glycolytic pathway makes them ideal biomarker candidates to be evaluated by standard biochemical separation methods and mass spectrometry, which has been covered in an extensive review [115].

A subproteomic study by Vitorino et al. [38] of the nuclear, mitochondrial and cytosolic fractions from fast versus slow muscles has established a considerable number of cytosol-associated proteins. Although the application of conventional subcellular fractionation methods results in a considerable degree of cross-contaminations between individual fractions, these kinds of proteomic maps may become useful in future comparative studies for evaluating the effects of physiological adaptations or pathological insults on the muscle cytosol. Previously, cytosolic protein changes were studied in the mdx animal model of Duchenne muscular dystrophy [116, 117] and atrophying mouse skeletal muscle [48] by subproteomics. The proteomic comparison of 3-month old dystrophic mdx muscles versus age-matched normal controls revealed the differential expression of approximately 40 proteins from the cytosolic fraction [116, 117]. Abnormal levels of adenylate kinase isoform AK1 and creatine kinase indicate disturbed regulation of nucleotide ratios and energy metabolism in dystrophin-deficient muscle tissue [116] and these altered protein levels were also shown to exist in the severely dystrophic mdx diaphragm muscle [118, 119].

3.7 *Skeletal muscle secretome*

The proteins secreted by cells and tissues have a great potential to be exploited as disease- and stage-specific biomarkers of a variety of pathological conditions [120]. The entirety of molecules secreted from living muscle fibres are referred to as the muscle secretome, whereby released proteins are involved in myogenesis, cellular signaling, cell-cell communication, proliferation and cell migration [121]. This makes the cataloguing of the fibre secretome an important aspect of studying autocrine and paracrine signaling mechanisms of the neuromuscular system. For proteomic studies of the muscle secretome, the collection of the released protein fraction under controlled conditions is challenging and often hampered by cross-contamination and/or interference by experimentally uncontainable cellular events other than true protein secretion. Besides these technical problems, initial proteomic studies focusing on the muscle secretome have identified interesting groups of extracellular factors that may be involved in the triggering and regulation of intracellular events involved in development, muscle repair and fibre adaptations [122-126]. Secretome proteomics has been especially applied to understanding the role of external factors during myogenesis, including myoblast proliferation, myoblast differentiation and myotube formation [121].

The proteomic survey of cultured C2C12 muscle cells that were grown in a serum-free medium resulted in the identification of a large number of secretory proteins involved in extracellular matrix remodeling, cellular proliferation, migration, and cellular signaling [122]. Quantitative proteomic analysis of the dynamic muscle secretome at different time points of myoblast differentiation identified over 600 released proteins, including cytokines, growth factors and metallo-peptidases [123]. The proteomic identification of changes in the human myotube secretome due to exposure to insulin or tumor necrosis factor TNF-α revealed several new mediator candidates that are being secreted at lower or higher levels following insulin stimulation or during insulin resistance [125, 126]. This indicates that skeletal muscle is a prominent secretory organ that produces a considerable amount of extracellular factors that mediate signaling to the highly complex contractile system and surrounding tissues.

3.8 *Proteomics of select protein populations and protein complexes*

Comparative biochemical studies are ideally performed with crude total tissue extracts in order to avoid potential artifacts due to extensive subcellular fractionation procedures. Extensive tissue manipulation and numerous preparative steps in the enrichment of a particular organelle or sub-

cellular fraction may cause uncontrolled protein release, protein adsorption, protein entrapment or non-specific protein loss. Preparation of tissue homogenates and the subsequent isolation of membrane fractions often result in the production of mixed vesicle populations, including membrane sheets, leaky vesicles, inside-out vesicles, right-side-out vesicles and smaller vesicles entrapped in larger membrane assemblies. Thus, in comparative organelle studies these technical complications have to be taken into account and may seriously limit the proper evaluation of protein levels in physiologically challenged or pathologically damaged muscle preparations. In order to reach a high degree of validity, subproteomic studies have to use highly purified samples with a minimum of impurities from other cellular compartments. An alternative to organelle proteomics is the biochemical isolation of distinct protein populations or the preparation of the entire protein complement from a given tissue sample.

Filter-aided sample preparation is a method that exploits a combination of detergent solubilization of the entire protein constellation of a cell, retention and concentration of the sample on an ultra-filtration device, detergent removal, exchange with urea buffer, chemical modifications and finally controlled protein digestion for mass spectrometric analysis [127]. This procedure covers theoretically the entire proteome of a given sample. If a proteomic analysis focuses on soluble versus hydrophobic proteins, detergent phase extraction would be the method of choice. This separation technique exploits the principle of temperature-dependent phase extraction with the detergent Triton X-114. Phase separation occurs at temperatures above 22°C using Triton X-114. A detergent phase with predominantly hydrophobic proteins and an aqueous phase enriched in hydrophilic proteins occurs reproducible at an experimental temperature setting of 37°C [128]. As can be seen with most separation methods, detergent phase transition approaches also cause a certain degree of cross-contamination between integral and soluble protein species. The fluorescence difference in-gel electrophoretic analysis of aging skeletal muscle proteins using nonionic detergent phase extraction revealed alterations in a large number of muscle-associated proteins involved in energy metabolism, metabolic transportation, regulatory processes, the cellular stress response, detoxification mechanisms and fibre contraction [129].

Skeletal muscle tissues contain several extremely large membrane-associated protein complexes, such as the nicotinic acetylcholine receptor of the post-synaptic membrane folds, the ryanodine receptor Ca^{2+}-release channel of the triad junctions and the dystrophin-glycoprotein complex that encompasses the membrane cytoskeleton, the sarcolemma and the extracellular matrix. Proteomics has been used to characterize the supramolecular dystrophin-associated complex and confirmed the composition of this protein assembly by mass spectrometry [45, 130]. Immunoprecipitation was used to isolate sarcolemmal β-dystroglycan and its tightly associated members of the dystrophin-glycoprotein complex from detergent-solubilized skeletal muscle [130]. Another proteomic approach used biochemically purified dystrophin and its associated glycoproteins and identified individual components by mass spectrometry following gradient gel electrophoresis and on-membrane digestion [45]. Proteins from digitonin-solubilized muscle membranes were separated by ion exchange chromatography, lectin binding and sucrose gradient centrifugation. The mass spectrometric analysis clearly established a tight linkage between dystrophin of 427 kDa and integral glycoproteins of the sarcolemma [45]. This demonstrates that mass spectrometry-based proteomics can be successfully applied to the identification and characterization of relatively low-abundance and membrane-associated muscle protein complexes. These new isolation and detection methods can be extremely useful for detailed future studies into the pathobiochemical role of the dystrophin complex in muscular dystrophy.

3.9 *Proteomic markers of muscle organelles*

Skeletal muscle proteomics has both confirmed the usefulness of established subcellular markers in high-throughput studies and identified novel candidates for the characterization of organelles and membrane systems in large-scale biochemical surveys. The diagram of Figure 2 gives an overview of select subcellular markers of skeletal muscle fibres. As listed in Table 1, this includes proteins associated with the contractile myosin-containing filaments (myosin heavy chains, myosin light chains), contractile actin-containing filaments (various actin isoforms), regulatory complexes of the contractile apparatus (tropomyosins TM, troponin subunits TnT, TnI, TnC), the sarcolemma membrane (dysferlin, Na^{+}/K^{+}-ATPase, β-dystroglycan), the sub-sarcolemmal membrane cytoskeleton (full-length dystrophin isoform Dp427), the cytoskeleton (vimentin, desmin), the extracellular matrix (laminin, collagen), the transverse tubular membrane system (voltage-sensing dihydropyridine receptor complex), the extracellular space (albumin), the longitudinal tubules membranes of the sarcoplasmic reticulum (SERCA-type Ca^{2+}-ATPases), the terminal cisternae region of the sarcoplasmic reticulum (Ca^{2+}-binding protein calsequestrin), the lumen of the sarcoplasmic reticulum (Ca^{2+}-shuttle protein sarcalumenin), the triad junction membrane (ryanodine receptor RyR1 Ca^{2+}-release channel complex), the outer membrane of mitochondria (VDAC porin), the inner membrane of mitochondria (Complex I, NADH dehydrogenase; Complex II, succinate dehydrogenase; Complex III, cytochrome b-c complex; Complex IV, cytochrome c oxidase; and Complex V, ATP synthase), and the mitochondrial matrix (isocitrate dehydrogenase), as well as cytosolic compartments that provide metabolite transportation (myoglobin, fatty acid binding protein), the cellular stress response (molecular chaperones) and metabolic pathways (glycolytic enzymes).

Table 1. List of marker proteins representative of distinct subcelluar fractions and pathways that are routinely identified by muscle proteomics

Subcelluar muscle fraction	Muscle-associated marker proteins
Contractile apparatus Myosin filament	Myosin heavy chains: MHC I, IIa, IIdx, IIb Myosin light chains: MLC 1f, 2f, 3f, 1s, 2s
Contractile apparatus Actin filament	Actins: various a isoforms
Contractile apparatus Tropomyosin filament	Tropomyosins: TM as, af, b
Contractile apparatus Troponin complex	Troponin subunits: TnT (1f-4f), TnI (f, s), TnC (f, s)
Sarcolemma membrane	Dysferlin, Na^+/K^+-ATPase, β-dystroglycan
Sub-sarcolemmal membrane cytoskeleton	Dystrophin (full-length isoform Dp427)
Cytoskeleton	Vimentin, desmin
Transverse tubular membrane	Dihydropyridine receptor complex
Extracellular space	Albumin
Extracellular matrix	Collagen, laminin
Sarcoplasmic reticulum longitudinal tubules membrane	SERCA-type Ca^{2+}-ATPases (SERCA1, SERCA2)
Sarcoplasmic reticulum terminal cisternae region	Calsequestrin Ca^{2+}-binding protein (CSQf, CSQs)
Sarcoplasmic reticulum lumen	Sarcalumenin Ca^{2+}-binding protein (SAR)
Triad junction membrane	Ryanodine receptor Ca^{2+}-release channel complex (Muscle isoform RyR1)
Mitochondria - outer membrane	VDAC porin (VDAC1 isoform)
Mitochondria - inner membrane Complex I	NADH dehydrogenase
Mitochondria - inner membrane Complex II	Succinate dehydrogenase
Mitochondria - inner membrane Complex III	Cytochrome b-c complex
Mitochondria - inner membrane Complex IV	Cytochrome c oxidase
Mitochondria - inner membrane Complex V	ATP synthase
Mitochondria - matrix	Isocitrate dehydrogenase
Cytosol – metabolite transportation	Myoglobin, fatty acid binding protein FABP3
Cytosol - molecular chaperones	Small heat shock proteins (αB-crystallin, cvHsp)
Cytosol - glycolytic particle	Glycolytic enzymes

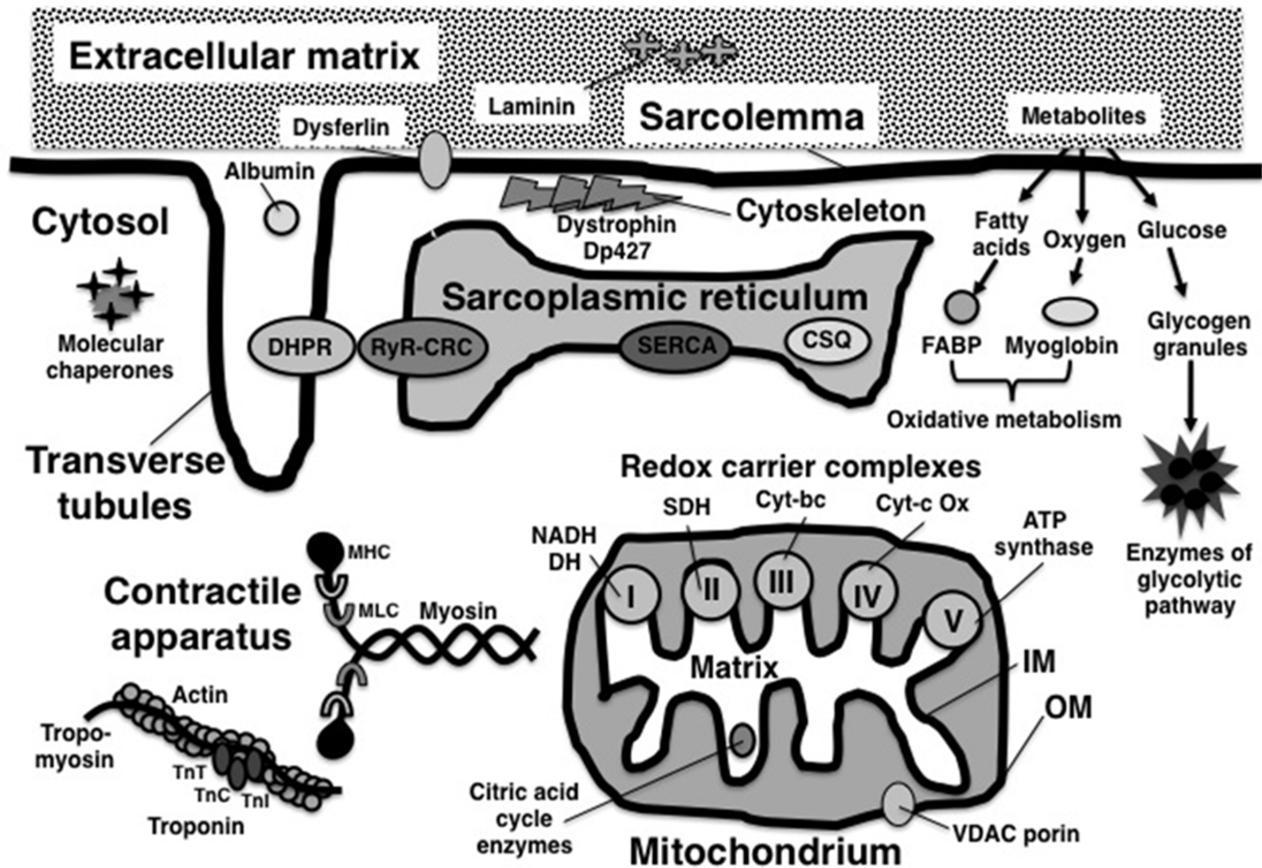

Figure 2. Overview of proteomic markers representative of distinct subcelluar fractions from skeletal muscle. The diagram summarizes the subcellular position of established and frequently used marker proteins of the extracellular matrix, extracellular space, sarcolemma, transverse tubules, cytoskeleton, sarcoplasmic reticulum, contractile apparatus, mitochondria and cytosol.

4. Future perspectives

The determination of global cellular mechanisms and proteomic biomarker identification are now at the forefront of many large-scale biochemical surveys of normal, adapting and pathological skeletal muscles. However, the dynamic expression range of muscle proteins and the extremely diverse biochemical properties of individual proteins with respect to size, charge, hydrophobicity and extent of post-translational modifications seriously complicate the comparative proteomic analysis of whole tissues. Thus, in the foreseeable future the usage of subproteomics for studying isolated organelles and distinct cellular fractions will be a necessity for covering the majority of constituents in a tissue proteome. In order to improve the analytical impact of organelle proteomics, it will be essential to utilize more precise mass spectrometric approaches even for the identification of proteins of very low abundance, but also develop superior subcellular fractionation techniques that exhibit a minimum degree of cross-contamination and employ wide-ranging separation schemes that coalesce the technical advantages of gel electrophoresis, liquid chromatography and optimum protein digestion for identifying a maximum number of proteins. The miniaturization of isolation methods, especially in the preparation of individual muscle fibres with an intact morphology and the microscopical dissection of cellular domains, promises to have a considerable impact on organelle proteomics. Once more comprehensive databanks of muscle organelle proteomes have been established, it will be crucial to use sophisticated bioinformatics to better integrate findings from genomics, transcriptomics, proteomics, metabolomics and cytomics for the systems biological evaluation of fundamental aspects of muscle biology and neuromuscular pathology.

Acknowledgements

The author would like to thank the Irish Health Research Board, the Irish Higher Education Authority (PRTLI5 Bio-AT programme), Science Foundation Ireland and Muscular Dystrophy Ireland for continued funding of our proteomics research projects focusing on skeletal muscle tissues in health and disease.

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