Patients with severe sarcopenia lose mobility and experience imbalance and falls, indicative of a disability that requires intensive medical care. The etiology of sarcopenia is believed to be multifactorial, including reduced physical activity, muscle motor-unit remodeling, oxidative stress, hormonal imbalance, malnutrition, and increased inflammatory cytokines (26, 54). The aging population (>60 years) is anticipated to increase from a current 600 million to 1.9 billion by 2050, highlighting the need to develop safe and effective therapies to treat sarcopenia (53). Characterization of muscle mass, performance, and structure in preclinical models could yield the necessary parameters to evaluate efficacy of novel therapies for sarcopenia. The aim of this study was to develop, improve, and validate efficient technologies for functional muscle testing, muscle mass analysis, and histological characterization of muscle architecture, including fiber-type and fiber-size distributions.

Preclinical functional muscle testing is frequently conducted with in situ muscle contraction assay systems in rodents. These systems require surgical procedures to expose the hindlimb plantarflexor muscle groups and the Achilles tendon, which are connected to a force transducer. These assay systems attempt to minimize disturbance to muscular architectures, innervation systems, and blood circulation and allow an accurate measurement of muscle strength (3, 14, 22).

Longitudinal monitoring of changes in muscle size and mass during aging is important to diagnose sarcopenia and to evaluate potential novel therapies. Noninvasive imaging tools, such as micro X-ray computed tomography (micro-CT), have been used and enable the repetitive monitoring of muscle mass changes during drug treatment (37, 52). However, micro-CT images often require manual analysis, which limits its application for routine animal studies and consequently, its use in drug discovery. Hence, an automated data analysis program would greatly facilitate the general application of this assay system.

Skeletal muscle consists of several subtypes of myofibers (predominantly types I, IIa, IIb, and IIx), which exhibit different contractile properties and use different energy sources (9). Fast-twitch type IIb fibers provide a fast burst of strong force but fatigue within ~1 min. Fast-twitch type IIx fibers fatigue within ~5 min (9, 13). In addition, there are slow-twitch type IIa fatigue-resistant fibers, which fatigue after ~30 min. Slow-twitch type I fibers contract for hours without fatiguing but provide lower output force.

Assaying muscle fatigue over the course of strenuous exercise provides significant insight into strength and type of myofiber, which contribute to performance (5). When skeletal muscle is tested for maximum strength repeatedly over time, it
fatigues in two major steps—early fatigue and late fatigue. The underlying mechanisms involved in early and late fatigue have been described in previous studies (11, 12, 15, 16, 18, 29). Fatigue envelope curves can be fitted to double-sigmoidal functions to generate multiple descriptive parameters, such as maximum force (F_{max}), intermediate force (F_o), and minimum force (F_{min}) (13). F_{max} represents the combined force of all available muscle fibers in a given muscle, whereas F_{min} represents the fatigue-resistant fibers, e.g., types I and IIa. The early fatigue force (F_{max} - F_o) is mostly contributed by the slow fatigue myofibers (type I), whereas the late fatigue force (F_o - F_{min}) is mostly contributed by the fast fatigue myofibers (type IIb). The slopes of early fatigue and late fatigue are represented by slope (S_1) and slope (S_2), respectively (8, 13, 29). Plantarflexor muscle groups [i.e., the combination of gastrocnemius, soleus, and plantaris (P)] contain a mixture of fast- and slow-twitch fibers and exhibit the typical double-sigmoidal fatigue envelope (8, 9).

Sarcopenia is frequently associated with a significant loss of type II fibers (1, 30, 31), structural changes that affect “muscle quality”, including motor unit remodeling (39), altered muscular architecture (21, 42), type I fiber grouping (35), reduced type II fiber size (34, 36), changes of major histocompatibility complex expression pattern (28, 49), and changes in extracellular matrix composition. To characterize myofiber remodeling, ATPase activity-based muscle histology enables differential staining of subtypes of myofibers (23, 48).

In this study, we characterized muscle aging through integrated approaches, including noninvasive muscle imaging, muscle force measurement, and histological analysis. To improve assay throughput, two automated, in vivo evaluation methods were developed. An automated micro-CT imaging analysis algorithm was used to efficiently support longitudinal muscle-size analysis. An automated in situ muscle-contraction assay system, which includes optimized animal surgical procedures, enhanced force measurement precision, and automated data acquisition (DAQ) and data analysis, was evaluated. Muscle fatigue envelope curve fitting, force parameter analysis, and ATPase staining of rat muscle cross-sections were performed. Compared with traditional in situ systems, the workflow was simplified significantly and is applicable of routine preclinical study. Furthermore, we performed the integrated data correlation analysis among data of muscle cross-sectional area (CSA), muscle mass, muscle force, myofiber size, and fiber-type distribution. The analyzed data indicated that although muscle mass correlated well with muscle function between aged and adult rats, other factors, such as myofiber structural changes and the distribution and interaction between subtypes of myofibers, also affect aging muscle function.

MATERIALS AND METHODS

Animals. Naive, 9- to 11- and 18- to 21-month-old male breeder mice (C57BL/6, Taconic, New York) were used for this study. A subpopulation of the 18-month-old mice was aged in-house until 26 mo. Naive, 17-month-old male rats (Sprague Dawley, Charles River, Wilmington, MA) were maintained on a calorie-restricted diet, between 3 and 17 mo of age, and were then transferred to ad libitum diet until the end of life (~33 mo). These rats were subjected to CT imaging at different time points before the endpoint in situ muscle contraction assay. Naive, 9- to 11-month-old retired breeder rats (Sprague Dawley, Taconic) and mice were maintained on a normal diet and were housed in a 12:12-h day and night cycle. These animals were subjected to either micro-CT imaging or in situ assay or both. All animal procedures were approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories (West Point, PA) and in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Body composition. Animal body composition, e.g., fat mass and lean body mass, was monitored using quantitative NMR (qNMR; EchoMRI, Houston, TX).

Micro-CT imaging and data analysis. Muscles were imaged using the LaTheta micro-CT (LaTheta LCT-100A, Aloka Instruments, Japan). This system was selected, due to its use of a complementary metal oxide semiconductor (CMOS) chip instead of a charge-coupled device. The CMOS chip requires lower X-ray energy for imaging and hence, reduces noise and enables a clear separation of fat and muscle tissue in the scanned images. In the imaging system, a stack of 15 slices was selected between the knee and fibula tibia junction. After scanning, each slice’s image was exported to the naive raw data as a matrix of Hounsfield units (HU). The HU were proportionally converted into gray-scale pixel values and assembled into two-dimensional tagged image file format (TIFF) images using a custom MATLAB (MathWorks, Natick, MA) script.

A custom algorithm was developed in the image analysis software Definiens Developer XD (Definiens, Parsippany, NJ) to facilitate the automated analysis of muscle cross-sections in the TIFF images. The algorithm first segments regions of similar brightness via a multiresolution algorithm and identifies the location of the animal in the image. The segmented regions were then classified as bone, muscle, and other tissue types, based on their original HU. The fibula was identified as the region of bone inside a region of muscle, which was farthest from the black background. The muscle region that determines the CSA of whole muscle in the lower leg was then grown around the central fibulae bone as an elliptical fit, until the shape reaches the fat tissue between the biceps muscles (Fig. 1, A and B). The CSA of the segmented muscle region was then exported into an Excel file (Microsoft, Redmond, WA), and the maximal CSA of all 15 slices was used for further analysis. The automated process could analyze 600–900 CT images (40–60 animals) in 4–7 h and requires minimal operator intervention (<30 min).

In situ assay overview. In situ assay systems were developed for both rat and mouse, which were controlled by a custom software application to generate sciatic nerve stimulation, re-establish constant tension before each stimulation event via a feedback loop, measure the force of the plantarflexor muscle group during involuntary exercise, and obtain a fatigue envelope curve (19, 22, 24). Hardware integration, software programming, and assay protocols were designed to maximize the accuracy of force measurements and to enhance assay efficiency to achieve the necessary throughput for routine animal studies. The surgical preparation procedure preserved blood circulation in the tested muscles and minimized tissue damage and surgical steps.

In situ assay procedure. The left femur bone of an anesthetized animal (Isoflurane, Webster Veterinary, Devils, MA) was surgically accessed through the quadriceps (cautery unit; Fine Science Tools, Foster City, CA) and secured to a vertebrate clamp (David Kopf Instruments, Tujunga, CA). The animal was arranged on the heated support in a prone position, the clamp was secured to the frame, and the left foot was cable tied to an integrated foot rest (Fig. 2A). Kevlar thread was knotted to the prepared Achilles tendon, above a remaining piece of the posterior calcaneus bone, and then was connected to the lever of the servomotor. Femur clamp and foot rest were adjusted on the frame until femur and tibia were fully stretched in a straight line with the thread (24). The thread was then tensioned mechanically to a suitable baseline force, which was found empirically and set to 0.1 N.
for mice and 1 N for rats. To compensate for muscle elongation during the exercise, baseline tension was re-established before stimulation by an automated feedback-loop algorithm, which made adjustments to the lever position (43). Next, the sciatic nerve was surgically exposed below the hip and connected to a modified bipolar-stimulating electrode (Harvard Apparatus, Holliston, MA). All exposed tissues were coated with paraffin oil to reduce evaporation. Stimulation and force measurement were conducted as described below. After

![Diagram of the in situ assay system.](image)

**Fig. 1.** Computed tomography (CT) imaging of the rodent tibial muscles cross area and illustration of the automated muscle-size analysis. **A:** typical hindlimb tibial CT image from a 13-mo-old rat with the legs raised above the abdomen. The fibular bone (F) is located near the center of the leg muscles, and the larger tibia bone (T) is near the top. **B:** tibial muscle region identified by the Definiens (Parsippany, NJ) algorithm. The algorithm first identifies the fibular bone based on tissue density [Hounsfield unit (HU) values] differences. The algorithm then elliptically expands to identify the tibial muscle region based on both the HU values and the geometric distribution until it reaches its boundary. Red and green lines, cross-sectional area (CSA) boundaries for left and right leg, respectively; thinner blue lines, body outline and fat-to-lean boundary.

![Diagram and workflow illustrations of the rodent in situ assay system.](image)

**Fig. 2.** Diagram and workflow illustrations of the rodent in situ assay system. **A:** diagram of the in situ assay system. The rodent femur bone is exposed and clamped, and the foot was tightly fixed to a foot holder. The hind paw is fastened on a foot rest, and the hindlimb is aligned with a muscle lever. The sciatic nerve and plantarflexor muscle group are exposed surgically. The muscle tendon is tightened by a Kevlar thread, tied to a muscle lever, and adjusted to an appropriate tension. The exposed sciatic nerve is connected to an electrode. **B:** workflow of the in situ assay system.
In situ assay hardware. The assay hardware integrated a custom-built assay frame and commercial hardware components, including a force-sensing servomotor (Dual-Mode Muscle Lever Systems 305C-LR and 310C-LR for mouse and rat, respectively; Aurora Scientific, Ontario, Canada), a DS3 Isolated Constant Current Stimulator (Digitimer, Hertfordshire, UK), USB-6221 DAQ/control device (National Instruments, Austin, TX), and an Intel-based personal computer. The assay frame was designed to stably hold the animal on a heated support, while under anesthesia, and to connect and properly align the plantarflexor muscle group via its Achilles tendon to the lever arm of the servomotor. An analog voltage signal (~10 to +10 V direct current) was initiated by a custom software application (I-S MUSCON) and then generated through the DAQ device and used as a position command for the servo motor and lever arm. The Aurora Scientific subsystem handled low-level motion control of the lever arm and force-transducer measurements. Stimulation pulse timing and generation were implemented in the DAQ device hardware by two, 32-bit counter/timers, which were programmable through the I-S MUSCON interface. The low-level digital pulses from the DAQ device trigger the Digitimer (DS3), where the amplitude of the constant current was set, and isolated square wave stimulation signals were delivered to the electrode (Fig. 2B).

In situ assay software. The architecture of the instrumentation system electronics was based on a custom software application (I-S MUSCON), developed in LabVIEW (National Instruments). The software facilitated and synchronized stimulation, muscle force measurement/recording, closed-loop muscle force baseline control, and data display, based on configuration parameters supplied by the operator. Individual assays could be automated with a high degree of customization, repetition, and throughput (Fig. 2B).

The I-S MUSCON software was developed in-house as an executable application using LabVIEW, version 8.6, Professional Development System (National Instruments). The software architecture used two task structures: a low-priority structure, which runs an event-driven user interface mainly for system configuration, and a high-priority structure, which handled control algorithm execution, DAQ, storage, and display.

The force envelope filter, contained in the high-priority structure, generates real-time force values, which were displayed on the monitor and saved to a disk file. The assay operation, configuration, and programming component of the low-priority task structure enabled automation of the assay. The operator had access to configuration of the assay timer, pulse generator, data storage, and force control algorithm parameters.

The close-loop muscle force baseline control algorithm ensured that each stimulation began with the muscle at the initially configured baseline force (tension), so as to minimize external disturbance. A “Run” control on the user interface began all assay functions (stimulation, timing, control, recording, etc.), which continued until the end of the assay and was signaled by the configured timer or the end of the assay program.

In situ assay stimulation protocol. The stimulation protocol was evaluated and optimized by stimulating the plantarflexor muscle groups with trains of 0.1 ms square pulses at different pulse frequencies, train intervals, and current amplitudes. For mice, a 50-ms train of 2.5 mA pulses at 100 Hz (five pulses/train) produced intense and forceful muscle contractions. To obtain complete fatigue envelope curves, trains were repeated at a frequency of 0.8 Hz (data not shown) for 300 s, including 10 s of lead time without stimulation, to a total of 232 contractions. The above-mentioned stimulation protocol parameters were identical in mouse and rat studies, but to ensure complete muscle stimulation, a supramaximal stimulation current of 4 mA was used in mice vs. 12 mA in rats.

In situ assay data analysis. The fatigue envelope curves of plantarflexor muscle contractions, which were generated in our studies, can be modeled accurately with double-sigmoid curves (13). To this end, a custom MATLAB (MathWorks) script was written to analyze the fatigue envelopes. Several parameters representing the fatigue envelopes were extracted, analyzed, and plotted, including $F_{\text{max}}$, $F_o$, $F_{\text{min}}$, $F_{\text{max}} - F_o$, $F_o - F_{\text{min}}$, maximal $S_1$ and $S_o$, and time constants of early and late fatigue forces. Characteristics of each force parameter are described in INTRODUCTION.

Histology. After performing the in situ assay, animals were necropsied, and several tissues were collected, weighed, and snap frozen in liquid nitrogen. The frozen plantarflexor muscle groups (gastrocnemius and soleus) were immersed in prechilled Tissue-Tek O.C.T. Compound (Miles Diagnostic Division, Kankakee, IL) and frozen in a slurry of ethanol and dry ice for mounting. Thick cryostat sections (12 μm) were transferred onto glass slides (ProbeOn+, Fisher Scientific, Pittsburgh, PA) and stored at −80°C.

ATPase activity staining was performed following a standard protocol described previously (23, 41). Briefly, the staining protocol was as follows: slides were preincubated at room temperature at pH 4.55 (0.037 M barbitral acetate in 0.053 M HCl) for 5 min, rinsed, and incubated in ATP solution for 25 min or preincubated at pH 10.2 (0.02 M sodium barbital and 0.036 M calcium chloride) for 15 min, rinsed, and incubated in ATP solution (60 mg ATP powder in 6 ml 0.1 M sodium barbital, 21 ml deionized water, and 3.0 ml 0.18 M calcium chloride, adjusted to pH 9.4) for 15 min. Slides were stained by three washes of 1% calcium chloride solution for 10 min each, incubating in 2% cobalt chloride for 10 min, five changes of 5 mM sodium barbital solution, five washes with water, incubating in 2% v/v ammonium sulfide for 20–30 s, and followed by rinsing with five exchanges of water. The slides were dehydrated by a series of ethanol solution incubations (50%, 70%, 80%, 2× 95%, and 2× 100%) and two changes of xylene. The slides were imaged on an Ariol SL50 microscope system using 5× objective.

Data analysis and statistics. The fatigue envelope parameters, analyzed by the double-sigmoidal curve fittings from the in situ assay, CSA measurements computed from micro-CT image analysis, and the body composition data acquired by qNMR were stored in a database (Labmatrix, BioFortis, Columbia, MD) for data retrieval and data mining. The correlation coefficients ($R^2$) were analyzed in Excel (Microsoft) for two-parameter analysis and JMP (SAS, Cary, NC) for multiple parameter matrix analysis. Statistical analysis for significance was done using a one-way ANOVA statistical test. Error bars represent the SE.

RESULTS

CSA and muscle mass. The lower-hindlimb muscles from 9- to 11-mo-old rats were imaged upon arrival and at 14–16 mo. Similarly, the aging rats were imaged longitudinally at 19, 22, 24, 26, and 30 mo (Fig. 3A). The micro-CT images were analyzed using custom MATLAB and Definiens Developer XD software algorithms, where the maximal CSA of whole muscle in the lower legs was extracted from 15 slices (Fig. 1, A and B). The data show that at 19 mo, the CSA values started to decline, progressing to a severe muscle loss by 30 mo. Similar results from the CSA obtained by micro-CT, in correlation with muscle mass, were also observed in the mouse model (data not shown).

To correlate CSA with muscle weight, 10 rats each from three different age groups (13, 18, and 30 mo old) were analyzed using micro-CT. All muscles of the lower hindlimb were excised during necropsy and weighed. CSA and muscle mass were observed to decline significantly with age, and the rats’ CSA values were correlated highly ($R^2 = 0.94$) with the mass of their lower-hindlimb muscles (Fig. 3B). The results indicated that male rats reach their maximum hindlimb muscle mass...
mass at ~13 mo. After 24 mo, the rats exhibited significantly reduced muscle mass and increased mortality. Based on these observations, we chose ~13- and 26-mo-old rats as adult and aged models for functional characterizations. Similar patterns of CSA and muscle mass changes were also observed in aging mice (data not shown).

In situ assay performance. The force recording of a typical rodent fatigue assay (mouse) showed the before-mentioned phases of early and late fatiguing (Fig. 4A). A short potentiating phase before reaching maximum force at the beginning of the assay was observed. Early fatiguing (~10 s) represents a mixture of initial fatigue phase of both fast- and slow-twitch muscles. Late fatiguing (~100–120 s) indicates a phase of fast-twitch (type IIb) muscle fatiguing before reaching the long-lasting lower force, contributed by slow-twitch muscles (types I and Ia).

The most significant muscle lengthening occurred early in the assay, as indicated by a loss of baseline tension after contraction (Fig. 4B). Activating the baseline control algorithm, 600 ms after stimulation onset for 500 ms, provided enough time for the muscles to relax and to reposition the lever arm several times as needed to re-establish the set baseline tension for rats (1 N) and for mice (0.1 N) before the next stimulation occurred at 1,250 ms (Fig. 4B).

The algorithm also compensated for any overshooting corrections (Fig. 4B), thus keeping the muscle tension tightly regulated within a band of ±50 mN. Regulation was then suspended for the last 150 ms, so as not to interfere with the following stimulation. The throughput of the current design was approximately six mice or three rats/h.

Functional parameters and muscle weight. To obtain fatigue envelope curves, 13- and 26-mo-old rats (550–800 g body wt) and 11- and 26-mo-old mice (33–45 g) were tested using their respective in situ assay systems with identical fatigue assay settings, except for a species-specific, supramaximal-stimulating current (Fig. 5, A and B). Since plantarflexor contains a mixture of all subtypes of myofibers, the fatigue envelope is expected to be best fitted with the double-sigmoidal fitting. The averaged R² from fitting the fatigue envelopes with the double-sigmoidal equation was >0.998, indicating an appropriate fit. Table 1 lists the averaged values of the functional parameters, plantarflexor mass, and specific forces of adult and aged rodents (i.e., rats and mice). Compared with their younger counterparts, 24- to 26-mo-old rats and 26-mo-old mice showed significantly decreased plantarflexor muscle mass, i.e., 3.59 ± 0.10 g vs. 2.28 ± 0.12 g for rats and 0.163 ± 0.003 g vs. 0.129 ± 0.003 g for mice. The aged rats showed more profound muscle mass loss than mice, i.e., 37% vs. 21% mass reduction. The aged rats and mice also showed significantly decreased F_max, F_o (force at turning point between early and late fatiguing), and F_o – F_min (late fatigue force, mostly contributed by type IIb fibers), whereas the F_min remained unchanged for both species. Specific forces were calculated by dividing functional parameters by their corresponding plantarflexor group muscle mass. The F_max-specific force values are comparable with previous publications (24, 27), which validated this in situ assay system. The conversion of the mass-based specific force (N/g) to CSA area-based specific force was 192–204 (kN/m²). The results indicated that both aged and adult rodents (rats and mice) shared similar specific forces.

To evaluate the correlation between assay parameters, we analyzed data generated from several independent in vivo studies of aged and adult rats and arranged the coefficients of determinants (R²) in a matrix (Table 2). Because aged rats showed profound muscle mass loss and muscle force reduction compared with mice, we focused on characterizing aging rats in the following analysis. Data were derived from body composition analysis, micro-CT, in situ assays, and muscle weight at necropsy. Plantarflexor muscle group mass was in good correlation with functional parameters related to fast, fatigable muscle function, such as F_max (0.47; in R² values), F_o (0.40), F_o – F_min (0.59), and S_2 (0.54), whereas it was moderately correlated with parameters related to fatigue-resistant muscles, such as F_max – F_o (0.21), F_min (0.00), and S_1 (0.39). The CSA from CT imaging was well correlated with muscle mass and was in moderate or good correlation with functional parameter.
ters, i.e., plantarflexor (0.80), $F_{\text{max}}$ (0.34), $F_o - F_{\text{min}}$ (0.50), and $S_2$ (0.47). Fat mass was highly correlated with body weight; however, body composition parameters were not correlated with any other parameters. A similar correlation pattern was also observed for data from mouse studies (data not shown).

Muscle functional parameters and specific force. Table 3 lists functional parameters, plantarflexor mass, and specific forces of individual animals from one study containing 22 of the 26-mo-old aged and 6 of the 13-mo-old rats. All parameters from aged rats showed significantly higher variability between individuals compared with younger adult rats, i.e., larger SD. Due to the variability among aged rats, they were divided into subgroups consisting of successful and unsuccessful (sarcopenic) cohorts. We adopted the current clinical definition to identify sarcopenic rats, i.e., 2 SD below the averaged muscle force from young rats. The 15 younger adult rats in this study showed averaged $F_{\text{max}}$ and $F_o - F_{\text{min}}$ values of 44.9 and 22.0 N with SD values at 7.5 and 7.9 N, respectively. Out of 94 aged rats, there were 34 that qualified by the cut-off values for $F_{\text{max}}$ and 24 that met the cut-off values for $F_o - F_{\text{min}}$. To evaluate whether the in situ assay data represented true muscle strength, the correlation between muscle mass and force parameters was analyzed. The $R^2$ showed good mass/
strength correlations on fast fatigue-related parameters, i.e., $F_{\text{max}}$ and $F_o - F_{\text{min}}$. Among the 109 aged and adult rats tested, 85 (78%) share similar $F_{\text{max}}$-specific force values, which is within the ±20% range (see Fig. 9, A and B). These 85 rats appeared to follow the expected muscle mass and force positive correlation, which validated this assay system. Those rats, which did not follow the expected mass vs. strength relationship, were all aged rats. Such a discrepancy could be due to other factors, such as change in muscle architecture or myofiber-type distribution or physiological condition.

**Histological analysis.** To better understand why some muscles deviated from the expected muscle mass vs. strength relation and to further evaluate whether the in situ assay system measured true muscle strength, we performed muscle histology analysis. Rat muscle cross-sections were stained for ATPase activity to analyze myofiber size and muscle structure.

We focused on analyzing aged (A) rats, e.g., A21, A35, and A85, from the “successful” cohort and aged rats, e.g., A20, A27, and A5, from the “unsuccessful (sarcopenic)” cohort and a 13-mo-old rat, young (Y)18 (Table 3). Rat Y18 was used as a comparator for the young cohort, and histology for this rat is shown in Fig. 6. The $F_{\text{max}}$ and plantarflexor mass relationship of rat Y18 was very close to the regression line; e.g., it followed the force vs. mass correlation. On the other hand, the $F_o - F_{\text{min}}$ vs. plantarflexor relation was significantly higher than the regression line, indicating significantly better fast-fatigable muscle performance (Table 3, and see Fig. 9, A and B). Fig. 6 illustrates that the slow-twitch fibers, i.e., types I and IIa fibers, are highly expressed in regions near the fibular bone, whereas the peripheral areas of the gastrocnemius muscle are mostly the fast-twitch fibers, i.e., types IIb/IX fibers (Fig. 6, A and B). The levels of types I and IIa fiber expression were most abundant in the P, followed by the lateral gastrocnemius (LG) and P adjacent LG (PALG; Fig. 6A). In the plantarflexor of 13-mo-old rats, type I fibers are mixed well with other fiber types, i.e., types IIa and IIb/IX fibers (Fig. 6, B and C). In the unsuccessful cohorts, a higher percentage of type I fibers is clustered together in aged rats (Fig. 7, A, C, and E) (30).

Rat A20 was one of the most sarcopenic rats, with muscle force significantly lower than expected, e.g., significantly lower $F_{\text{max}}$ and $F_o - F_{\text{min}}$ values than the expected regression line (Table 3, and see Fig. 9, A and B). The histological analysis showed that rat A20 had abnormally enlarged and oval-shaped myofibers with increased space between myofibers. In addition, there were many muscle bundles that contained a mixture of enlarged myofibers and smaller myofibers (Fig. 7, B and C). $F_{\text{max}}$ and $F_o - F_{\text{min}}$ values for rat A27 follow the force vs. mass regression line, which was significantly better than rat A20 (Table 3, and see Fig. 9, A and B). Although rat A27 had similar plantarflexor mass to rat A20, and its type

**Table 1. The averaged values of the functional parameters, plantarflexor mass, and specific forces of adult and aged rats and mice from multiple studies**

<table>
<thead>
<tr>
<th>Species</th>
<th>Groups</th>
<th>Age in mo (animal #)</th>
<th>$F_{\text{max}}$ (N ± SE)</th>
<th>$F_o$ (N ± SE)</th>
<th>$F_{\text{min}}$ (N ± SE)</th>
<th>$F_{\text{max}} - F_o$ (N ± SE)</th>
<th>$F_o - F_{\text{min}}$ (N ± SE)</th>
<th>PFX mass (g ± SE)</th>
<th>Spec. $F_{\text{max}}$ (N/g)</th>
<th>Spec. $F_o$ (N/g)</th>
<th>Spec. $F_{\text{min}}$ (N/g)</th>
<th>Spec. $F_{\text{max}} - F_o$ (N/g)</th>
<th>Spec. $F_o - F_{\text{min}}$ (N/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>13 (n = 15)</td>
<td>44.91 ± 1.93</td>
<td>37.29 ± 2.05</td>
<td>15.30 ± 2.53</td>
<td>7.63 ± 0.80</td>
<td>21.99 ± 2.05</td>
<td>3.59 ± 0.10</td>
<td>12.51</td>
<td>10.39</td>
<td>4.26</td>
<td>2.13</td>
<td>6.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24–26 (n = 94)</td>
<td>31.88 ± 1.10</td>
<td>25.99 ± 0.94</td>
<td>12.98 ± 0.66</td>
<td>5.89 ± 0.33</td>
<td>13.00 ± 0.73</td>
<td>2.65 ± 0.07</td>
<td>12.03</td>
<td>9.81</td>
<td>4.90</td>
<td>2.22</td>
<td>4.91</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>11 (n = 14)</td>
<td>4.40 ± 0.11</td>
<td>3.60 ± 0.17</td>
<td>1.26 ± 0.06</td>
<td>0.80 ± 0.12</td>
<td>2.35 ± 0.14</td>
<td>0.16 ± 0.03</td>
<td>27.50</td>
<td>22.50</td>
<td>7.88</td>
<td>5.00</td>
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<tr>
<td></td>
<td>26 (n = 10)</td>
<td>3.19 ± 0.16</td>
<td>2.68 ± 0.24</td>
<td>1.04 ± 0.13</td>
<td>0.72 ± 0.23</td>
<td>1.77 ± 0.12</td>
<td>0.13 ± 0.03</td>
<td>24.54</td>
<td>20.62</td>
<td>8.00</td>
<td>5.54</td>
<td>13.62</td>
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</tr>
</tbody>
</table>

$F_{\text{max}}$, maximum force; $F_o$, intermediate force; $F_{\text{min}}$, minimum force; $F_{\text{max}} - F_o$, early fatigue force; $F_o - F_{\text{min}}$, last fatigue force; PFX, plantarflexor; Spec., specific.
Table 2. Correlation coefficient \( (R^2) \) analysis of the body composition, muscle cross-sectional area, plantarflexor mass, and muscle force parameters of the adult and aged rats, expressed in coefficients of determinants \( (R^2) \) in a matrix

<table>
<thead>
<tr>
<th>Fat</th>
<th>Lean</th>
<th>BW</th>
<th>CSA</th>
<th>Fmax</th>
<th>Fc</th>
<th>Fmin</th>
<th>Fmax - Fc</th>
<th>Fc - Fmin</th>
<th>SL1</th>
<th>SL2</th>
<th>PFX</th>
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<tbody>
<tr>
<td>0.08</td>
<td>0.07</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
<td>0.02</td>
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BW, Body weight; CSA, cross-sectional area; SL 1, early slope; SL 2, late slope. All \( R^2 > 0.29 \) are bold, as this indicated correlations of the parameters.

I myofiber distribution pattern was also similar to that of rat A20, the architecture of types Iib/IIX fibers of rat A27 was significantly better than rat A20, e.g., no oval-shaped myofibers (Fig. 7, D and E). The muscle cross-section for rat A5 had lost the structural boundary among the four gastrocnemius regions, e.g., LG, medial gastrocnemius, P, and PALG. The types I and IIA fibers of rat A5 were clustered and segregated (Fig. 7A). In contrast, rat A5 myofiber architecture, e.g., myofiber shape and space between myofibers, was significantly better than rats A20 and A27. These factors may explain why rat A5 muscle force parameters, e.g., \( F_{\text{max}} \) and \( F_{c} - F_{\text{min}} \), were significantly better than rats A20 and A27 (see Fig. 9, A and B).

All of the successful aged cohorts shared normal myofiber architecture and uniform myofiber size, which were similar to adult rats (Fig. 8, A–C). Rat A35 exhibited normal myofiber architecture and normal muscle strength, which was close to the regression line. Rat A85 had the highest number of type I fibers, and more importantly, the distribution of type I fibers was evenly mixed with types IIA and IIB fibers, just as adult rats (Fig. 8C). This observation possibly explains why rat A85 had

Table 3. List of functional parameters, plantarflexor mass, and specific forces of individual animals from 1 study containing 22 of the 26-mo-aged and 6 of the 13-mo-old rats

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<th>( F_{\text{max}} - F_{c} ) (N)</th>
<th>( F_{c} - F_{\text{min}} ) (N)</th>
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The averaged force parameters of the aged (A) and younger adult (Y) rats from this study are listed in the bottom 2 rows (average ± SD). Bold ID numbers are animals that were used for histological comparison.

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significantly enhanced fatigue-resistant muscle function \( (F_{\text{max}}, F_{\text{max}} - F_0, \text{and } F_{\text{min}}) \), whereas \( F_0 - F_{\text{min}} \) force was similar to other rats in the successful cohort (Fig. 9, A and B). Conversely, \textit{rat} A21 had the least number of \textit{type} I myofibers and the least association with \textit{type} IIa myofibers among the three rats from the successful cohort (Fig. 8A). The histology may explain why \textit{rat} A21 had a higher \( F_0 - F_{\text{min}} \) value (fast-fatigable force) but a significantly lower \( F_{\text{min}} \) value (fatigue-resistant force; Fig. 9, A and B).

**DISCUSSION**

The in situ muscle contraction assays have been frequently used to characterize muscle function in multiple areas of muscle research (6, 24, 46, 56). In those assays, a force transducer was attached to the tendon of a muscle and aligned with the axis of contraction to directly measure its full strength, whereas the in situ setting kept blood circulation and nervous systems generally intact. This assay system maximized the efficient measurement of the strength of plantarflexor muscle contraction. On the other hand, several hurdles limited broader application of the protocol to routine in vivo efficacy studies, including slower workflow of the animal surgery procedures, assay system set-up, and muscle elongation during contraction. The plantarflexor muscle group elongates significantly during the first few contractions, which can compromise force output and therefore, compromise accuracy of force measurements (38, 45). We developed an in situ assay system, which incorporated automated DAQ, data analysis, and used curve fitting to collect the data for several parameters that characterize the contractile properties of myofiber subtype populations. This assay system not only significantly increased the assay throughput but also increased precision and robustness; therefore, it is applicable to routine preclinical studies for compound evaluation.

![Fig. 6. ATPase staining of gastrocnemius muscle groups from a 13-mo-old male, young (Y) rat Y18. The type I myofibers are stained as dark brown color, the type IIa myofibers are white, and the types IIb and IIX myofibers are medium brown. A: overview of ATPase staining on the whole gastrocnemius. The fibular bone is located on the right-hand side of the muscle, where most of the types I and IIa myofibers are located. MG, medium gastrocnemius; P, plantaris; LG, lateral gastrocnemius; PALG, P-adjacent LG. B: subregion of LG. The types I, IIa, and IIB/IIX are relatively evenly mixed in the region near the fibular bone. C: closer detail of the LG. The myofiber cross-sections are mostly irregular in shape and are structurally, tightly packed between myofibers.](image)

![Fig. 7. ATPase staining of gastrocnemius muscle groups from unsuccessful aged cohorts of the 26-mo-old male rats. A: image from a 26-mo-old aged (A) rat; ID: A5. The structural correlation of the gastrocnemius muscle subtypes of this rat has been lost, i.e., unable to distinguish each muscle type. The types I and IIa fibers are highly clustered, and the 2 fiber types are mostly segregated. The overall fiber-to-fiber connections remain tight. B and C: images of P and PALG from a 26-mo-old rat; ID: A20. B: most fibers are round shaped with increased space between fibers and contain mixtures of large and small fibers. C: type I fibers are also misshaped and clustered together. D and E: myofiber images in the PALG region of a 26-mo-old rat; ID: A27. D: mixture of enlarged and degenerated fibers in PALG. E: myofiber degeneration and the type I fibers are clustered together.](image)
A custom-built, adjustable assay stage was developed, which allowed for iterative and precise alignment of fully extended hindlimb bones and the plantarflexor muscle group with the motorized muscle lever via the Achilles tendon and a nonelastic Kevlar thread. Concurrently, the surgical procedure was optimized to efficiently expose the femur bone and the Achilles tendon, and the femur bone was clamped and anchored to ensure precise alignment between the Achilles tendon and the muscle lever. The assay stage was tailored to the overall workflow to increase throughput. To enhance assay precision, a LabVIEW-based proportional control algorithm was developed to ensure a constant muscle baseline tension on the muscle lever by automatically compensating muscle lengthening. A custom-developed, LabVIEW-based program was also used to automate data collection and storage, whereas curve fitting of the fatigue envelopes was developed in MATLAB. A double-sigmoidal equation had previously shown to provide reliable parameters for similar curves (13). As such, this in situ assay system has increased throughput to six mice or three rats/h and improved assay precision and robustness. We have used the in situ assay system for routine in vivo studies to evaluate drug efficacy at study endpoints. The specific forces generated from the assays are in agreement with previously published data (24, 27) and are in good correlation with the animals’ muscle mass. Among the aged and adult rats tested, 65% of muscles fall into ±20% of the regression line, indicating that most muscles followed the mass vs. strength roles. Those muscles with functional parameters, which deviated by >50% from the regression line, were also differentiated during the histological analysis. The agreement between functional and histological analysis demonstrates that the in situ assay system accurately reflects muscle functionality (24, 27, 40, 44).

Micro-CT is widely used to noninvasively monitor muscle size and mass in both clinical and preclinical studies. Many systems require manual image analysis, which limits throughput and slows down routine preclinical studies. The automation of this process, especially the development of a muscle segmentation algorithm in Definiens, has significantly facilitated the analysis. The good correlation between CSA values from maximum tibial circumference and muscle mass validates micro-CT as a reliable methodology to assess muscle size and mass. The correlation of functional parameters from the in situ assay to CSA values, as well as to muscle mass, was moderate to good, indicating that muscle size is a predictor for muscle function in most cases. The mass of the plantarflexor muscle group was more strongly correlated to the function of fast-fatigable muscle fibers (e.g., $F_{\text{max}}$, $F_o$, and $F_o - F_{\text{min}}$) than to the fatigue-resistant fibers (e.g., $F_{\text{min}}$ and $F_{\text{max}} - F_o$; Table 2). Furthermore, aged rats and mice lost significant fast-fatigable muscle force but lost less fatigue-resistant force (Table 1). These

Fig. 8. ATPase staining of gastrocnemius muscle groups from successful aged cohorts of the 26-mo-old male rats. A–C: images of myofibers of the LG of the aged rats, A21, A35, and A85, respectively. All 3 rats show tight myofiber organizations, and the type I fibers are relatively well mixed with types IIa and IIb/IIX fibers. Among the 3 rats, A21 has the least abundance of type I fibers, followed by A35 rats. A85 has the most-abundant type I fibers.

Fig. 9. Correlation of $F_{\text{max}}$ and $F_o - F_{\text{min}}$ with the plantarflexor muscle mass (plotted from 57 of the 26-mo-old aged rats and 15 of the 13-mo-old rats). A: correlation between $F_{\text{max}}$ and plantarflexor mass. There are 34 rats that are below the dashed line (30 N), 2 SD below the averaged $F_{\text{max}}$ force from adult rats, indicating sarcopenia. B: correlation between $F_o - F_{\text{min}}$ and plantarflexor mass. The red dots represent each individual rat included in the discussion. There are 24 sarcopenic rats, i.e., below the averaged $F_o - F_{\text{min}}$ (dashed line; 6.2 N).
observations are related to the fact that these muscles are predominantly composed of fast-fatigable fibers (~75%), which are affected more significantly by aging than fatigue-resistant fibers (2, 10, 30, 51). On the other hand, body composition parameters were poor predictors of muscle function (58).

ATPase-based, fiber-type staining techniques have been widely used on muscle cross-sections for preclinical and clinical studies and in the diagnosis of myopathies (7, 20, 25, 33, 55, 57). In this study, several histological features of muscle aging, including fiber degeneration and disorganization, were observed. All aged rats showed an increased proportion of type I over type II fibers, which is in agreement with the literature (30). However, the organization/distribution of type I fibers in relation to type II fibers appeared to be different among aged rats. Type I fiber grouping, likely due to motoneuron remodeling (47, 50), and the segregation of types I and IIa fibers may be a significant pathological phenotype. Muscle bundles, in some aged rats, contained a mixture of both reduced and enlarged fibers, and the fiber cross-section had changed to an oval shape and lost the tight contact with other fibers. The change of fiber morphology appeared to be correlated with muscle function and will be discussed further. The successful cohort in this study exhibited better organization of types I and IIa fibers, and most were of regular size and shape. However, the sarcopenic cohort typically showed clustered type I fibers and generally more disorganized muscle fibers.

The most significant example of a sarcopenic phenotype was observed in rat A20. It combined all of the above-mentioned properties of deteriorated muscle with poor muscle function. Although rat A27 exhibited a similar degree of types IIIa fibers segregation and misshapen fibers, its types IIb/IIx fibers appeared better organized, which may have led to slightly better muscle function. Rat A5 had the lowest muscle mass but functioned better than rats A20 and A27, especially when $F_{\text{max}}$ and $F_{\text{min}}$ parameters were compared. Although A5 had completely segregated types I and IIa fibers, it had the most well-organized myofibers of the three, which may explain its better performance than A20. Interestingly, the fatigue envelope of rat A5 lacked the typical fast-fatiguing phase, suggesting that its better performance may be due to more slow-twitch fibers.

Rats A21, A35, A85, and Y18 share similar plantarflexor muscle group mass and may provide a good example for comparison. Rat A85 exhibited the highest $F_{\text{max}}$, $F_{\text{max}} - F_{\text{rest}}$, and $F_{\text{min}}$ values among the four rats, which indicated a greater amount of fatigue-resistant muscle fibers in this animal. Indeed, histology showed that rat A85 had significantly more type I fibers than the other three rats; perhaps more importantly, these type I fibers were not clustered, and instead, they were evenly distributed with type IIa fibers. We hypothesized that an increased proportion of type I fibers may be beneficial for aged rats, if they are well distributed with type IIa fibers. A35, A85, and Y18 shared similar $F_{\text{rest}} - F_{\text{min}}$ values, indicative of a similar amount of types IIb and IIx fibers. However, A21 showed the highest $F_{\text{rest}} - F_{\text{min}}$ value but the lowest $F_{\text{min}}$ and $F_{\text{max}} - F_{\text{rest}}$ values among the four rats. This is agreeable with the histology data, which showed that A21 has the most-abundant types IIb/IIx myofibers and has the least amount of types I and IIa fibers (given A21 has similar plantarflexor mass).

Overall, the assayed aged rats showed a diverse histology with regard to fiber-type composition, fiber size, fiber grouping, and overall muscle architecture, which reflects their diverse range of functional parameters derived from the in situ assay. This supports the interpretation that an assessment of muscle function is necessary to evaluate drug efficacy, since muscle size is not sufficient to predict performance, and histology cannot be performed on all animals in a preclinical setting.

This paper describes an in situ assay, micro-CT imaging, and muscle histology procedures, which were customized and automated to study muscle performance, size, and architecture in a preclinical setting. The assays were confirmatory of each other, and the functional parameters derived from the analyzed fatigue envelopes, CSA, myofiber size, myofiber shape, myofiber subtype composition, and distribution patterns are useful tools. Potential therapies for sarcopenia should increase muscle mass and improve muscle quality, as defined by myofiber composition, fiber size, and organization, as well as overall muscle architecture. Connection of muscle function to muscle structure validated our custom in situ assay system as a reliable tool to assess muscle performance in aged animals, justifying its broader application in preclinical studies. It is clear that more research is needed to confirm the hypotheses proposed in this publication, but the demonstrated methodology, especially data mining and correlating assay parameters, can provide a validated approach for these investigations.

ACKNOWLEDGMENTS

We acknowledge the technical support from Ms. Xianlu Qu and John Reilly. We thank Dr. Boyd Scott for assistance in the preparation of this manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


