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Quantifying Myofiber Integrity using Diffusion MRI and Random Permeable Barrier Modeling in Skeletal Muscle Growth and Duchenne Muscular Dystrophy Model in Mice

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Abstract

Purpose—To measure the microstructural changes during skeletal muscle growth and progressive pathologies using the random permeable model (RPBM) with diffusion MRI, and compare findings to conventional imaging modalities such as three-point Dixon and T₂ imaging.

Methods—*In vivo* and *ex vivo* DTI experiments with multiple diffusion times (20–700 ms) were completed on wild-type (n=22) and muscle-dystrophic *mdx* mice (n=8) at various developmental time points. The DTI data were analyzed with the RPBM framework that provides estimates of the unrestricted diffusion coefficient (D_0), membrane surface-to-volume ratio (S/V), and membrane permeability (κ). In addition, MRI experiments included conventional measures, such as tissue fat fractions and T₂ relaxation.

Results—During normal muscle growth between week-4 and week-13, the *in vivo* S/V , fractional anisotropy (FA) and fat fraction correlated positively with age ($\rho=0.638$, 0.664 , and 0.686 , respectively), while T₂ correlated negatively ($\rho=-0.847$). In *mdx* mice, all DTI-RPBM parameters and fat fraction had significant positive correlation with age, while FA and T₂ did not have significant correlation with age. Histological measurements of the perimeter-to-area ratio (P/A) served as a proxy for the model-derived S/V in the cylindrical myofiber geometry, and had a significant correlation with the *ex vivo* S/V ($r=0.71$) as well as the *in vivo* S/V ($r=0.56$).

Conclusions—The present study demonstrates that DTI at multiple diffusion times with the RPBM analysis allows for non-invasively quantifying muscle fiber microstructural changes during both normal muscle growth and disease progression. Future studies can apply our technique to evaluate current and potential treatments to muscle myopathies.

Keywords

Diffusion time; random permeable barrier model; diffusion tensor imaging; surface-to-volume ratio; sarcolemma permeability

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INTRODUCTION

Patient management and drug discovery in a range of myopathies would greatly benefit from an objective noninvasive biomarker of myofiber integrity. The current gold standard, muscle biopsy, is invasive, prone to sampling errors, and cannot be repeated often. Other functional measures, e.g. manual muscle test used in clinical trials, offer limited accuracy and reliability, and do not provide local information on individual muscles. A non-invasive local biomarker free of the above limitations, and able to reflect microstructural changes in myofibers, has been lacking to-date. The lack of a reliable non-invasive tool for evaluating changes in muscle tissue microstructure has been noted as one of the major hindrances in assessing existing treatment methods and developing new therapies in various myopathies, such as muscular dystrophies (1) and idiopathic inflammatory myopathies (2).

T₁ and T₂ weighted magnetic resonance imaging (MRI) methods, including T₁ and T₂ mapping, have been used to study muscular anatomical features in disease processes (3–9). Specifically, T₁ weighted imaging has been used for evaluation of relative fatty infiltration (10), contrast uptake (11), and overall muscle cross-sectional area (12) as possible biomarkers of muscular dystrophy progression. T₂-weighted images have been correlated with distribution of disease activity and clinical assessment (13,14). Fat fraction measurement using the difference in proton resonance frequencies of water and lipids has been used to quantify fat replacement in muscular dystrophy (15). MRI has also been able to study age-related changes in muscle cross-sectional area and specific torque production in muscles (12). Overall, these conventional MRI methods have been used to characterize gross anatomical changes and MR relaxation properties. However, the ultimate potential of MRI to assess subtler changes in microstructural and functional properties of myofibers has not been fully realized. Such structural and functional changes at the cellular level plausibly occur before gross anatomical changes detectable with conventional MRI and other techniques.

Diffusion MRI, particularly diffusion tensor imaging (DTI), is one of the promising MRI methods to study muscular tissue properties. Diffusion MRI measures the Brownian motion of water molecules restricted by tissue microstructure (16,17). Conventional DTI captures the Gaussian properties of the diffusion signal or diffusion propagator, typically interpreted in terms of the major diffusion direction, i.e., the primary eigenvector of the diffusion tensor. DTI has been used to identify myofiber tracts (18–20) and to estimate functionally important muscle properties, such as the deviation of muscle shortening direction (21) and curvature pennation angle (22). DTI has also been used to visualize the complex myoarchitecture of the tongue (23–26) as well as the laminar structure of cardiac ventricles (27–29). However, while being sensitive, DTI alone is not specific about the microstructural properties at the cellular level, as the diffusion measurement is influenced by cellular structural properties, such as cellularity (30), cell size (31,32), extracellular volume fraction (33,34), and cellular membrane permeability (35).

Varying the diffusion time of the diffusion measurement creates an extra dimension that allows for better capturing skeletal muscle structural properties, such as membrane permeability and spacing (36). Overall, the time dependence of the diffusion coefficient signifies the presence of non-Gaussian contributions to the diffusion propagator (37). In

tissues, these effects arise due to cellular-size structure hindering or restricting the Brownian motion. By increasing the diffusion time, one can sensitize the measurement to the structure commensurate with the average diffusion length $L(t) \sim \sqrt{Dt}$, thereby exploring different scales of tissue architecture at the cellular level. Indeed, the Brownian motion of water molecules yields a root-mean-square displacement that increases from a few microns to about 50 microns with longer diffusion times ($t=1-1000$ ms).

Biophysical modeling is required to explain time-dependent DTI measurements in terms of the specific underlying microstructural features. Using *ex vivo* calf heart and tongue, it was demonstrated that the time dependence of the diffusion tensor could be used to estimate the diameters of myofibers (38). In the regions with myofibers in parallel, the axial diffusivity (λ_1) remained same for all diffusion times, whereas the radial diffusivities (λ_2 and λ_3) decreased by more than 30% between $t=32$ ms and $t=810$ ms. Such changes in the radial diffusivities were described using a model consisting of cylinders with restricted diffusion embedded in a nonrestricted compartment. While this method provided reasonable estimates of myofiber diameters, the assumption of impermeable myofiber membrane may not be valid when diffusion time t is as long as 800 ms.

Recently, Novikov *et al* (39) developed a model to quantify both cell diameter and membrane permeability based on time-dependent DTI. The random permeable barrier model (RPBM) provides estimates of average muscle fiber surface-to-volume ratio (and thereby fiber diameter), membrane permeability, and water diffusivity in sarcoplasm (40,41). This model has been validated using Monte Carlo simulations (39,40). It yields values for the fiber diameter which are generally expected in calf tongue and heart muscle (38) and in human calf muscle (35,41,42). However, there has not been a study to compare the fiber sizes and membrane permeability measured by the RPBM with corresponding histopathological markers.

In this study, we investigated the longitudinal changes of skeletal muscle in wild-type (WT) and Duchenne Muscular Dystrophy (DMD) model mice between postnatal week 4 and week 13 using the time-dependent DTI measurements and RPBM analysis. The range of muscle growth during this period was used to further validate the RPBM with histopathological measure of muscle fiber size and *ex vivo* imaging. The RPBM parameters were also compared with conventional MRI methods for fat fraction percentages and T_2 relaxation.

METHODS

Animals

All mice used in this study were maintained under protocols approved by Institutional Animal Care and Use Committee at the New York University School of Medicine. Male C57BL6 (n=22, C57BL/10ScSn, The Jackson Laboratory, ME) and *mdx* (n=8, male C57BL/10ScSn-Dmd^{mdx}/J, The Jackson Laboratory, ME) mice were used to observe myofiber size and sarcolemma permeability during normal muscle growth and myopathy, respectively. The WT mice were imaged at time points of 4 (n=4), 5 (n=4), 7 (n=7), 9 (n=4) and 13 (n=3) weeks old to measure skeletal muscle properties at these time points of normal muscular growth. All WTs were sacrificed immediately after the MRI study for a given time point for

ex vivo imaging and immunohistochemistry (IHC) staining of Wheat Germ Agglutinin (WGA). The *mdx* mice (n=8) were longitudinally studied at the same time points (n=8, 6, 6, 6, and 4 for week 4, 5, 7, 9 and 13 time points, respectively), while subsets of the *mdx* mice were sacrificed at 4 (n=2), 5 (n=2) and 13 weeks (n=4) for MRI-pathology correlations. The changes in the muscle between week 4 and week 13 in the WT and *mdx* mice were used to assess the association of the RPBW with other MRI and histopathological measures of the muscle.

MRI Data Acquisition

In vivo and *ex vivo* MRI exams were performed on a 7T Biospec micro-MRI system (Bruker Biospin MRI, Ettlingen, Germany) equipped with a Bruker BGA-9S 750 mT/m gradient coil. Mice were mounted on a custom 3D-printed mouse holder after general anesthesia was induced using 3% isoflurane in air and placed into a home-built quadrature volume coil (43). During the scan, the general anesthesia was lowered to 1.5% isoflurane in air to maintain a respiration rate of approximately 30 breaths/minute. Body temperature was also monitored during imaging sessions and the warm air was adjusted as needed to maintain $36\pm 2^{\circ}\text{C}$ (SA Instruments, NY, USA).

Each MRI session included a T_2 -weighted rapid acquisition with relaxation enhancement (RARE) sequence (TR/TE=2s/35.4ms, image matrix=128×128×10, voxel size=0.16×0.16 mm², slice thickness=0.8 mm with 0.2 mm gap, RARE factor = 9, NA=4, scan time=1 min 36 s) and a T_1 -weighed 3D FLASH sequence (TR/TE=40ms/3.6ms, flip angle=10°, image matrix =128×128×10, voxel size=0.16×0.16×1.0 mm³, NA=4, scan time=1 min 55 s) to locate the muscle groups of the lower legs. A diffusion weighed (DW) stimulated-echo acquisition mode (STEAM) pulse sequence with 3D echo planar imaging (EPI) readout with a chemical shift-based fat suppression (Gaussian pulse with bandwidth of 1054 Hz and pulse duration of 2.6 ms followed by a spoiler with 150 mT/m for 2 ms) was used to acquire images with diffusion gradients (b=1000 s/mm²) in twenty non-collinear directions (Supporting Table S1) and four images with low diffusion weighting (b=50 s/mm²). The DW-STEAM-EPI was run repeatedly for seven diffusion times *t* ranging from 20–700 ms with TR/TE=6s/27ms, image matrix =64×64×10, voxel size=0.34×0.34×1 mm³, NA=1, and scan time=12 min (Supporting Figure S1). The b-value was held constant near 1000 s/mm² by varying the diffusion weighting gradient strength as the diffusion times increased, particularly in the slab select gradients (38). For fat quantification, a gradient echo sequence was used to acquire images at six echo times (2.91–3.74 ms) with TR=5 ms, image matrix=128×128×10, and voxel size=0.16×0.16×1.0 mm³, NA=2, and scan time=2 min 52s. T_2 relaxation times were measured using a manufacturer supplied multi-spin-echo sequence with slice selective excitation and refocusing Gaussian-shaped pulses (32 echoes, echo-time increment=7.1 ms, TR=2.4 s, image matrix=128×128×10, voxel size=0.16×0.16×1.0 mm³, NA=1, and scan time=2 min 52 s). Overall, an MRI session per mouse/sample was about 2 hours. *Ex vivo* imaging samples provided a higher throughput as 4 samples were imaged simultaneously.

MRI Data Analysis

The diffusion-weighted images were co-registered to $b=0$ images to remove the eddy current and/or motion induced artifacts using a 3D affine transformation estimated by maximizing the mutual information between the images (44). The diffusion tensor was computed for each voxel using the b-matrix calculated to include the contribution of imaging gradients at each diffusion time (35,38). The $b=0$ images were not included in the diffusion tensor estimation in order to minimize the intravoxel incoherent motion effect. Subsequently, the eigenvalues and eigenvectors of each tensor were calculated. The average of the second and third eigenvalues at each diffusion *time* t was assumed as the measure of diffusion coefficient $D(t)$ perpendicular to the muscle fibers, to which the DTI-RPBM was fitted (39,41). The RPBM mimics the essential geometry of tight fiber packing in which muscle tissue is modeled with infinite planes, representing permeable membranes, randomly placed and oriented, so that they divide the sample into pores with random shapes. The model can be rationalized when myofibers are very densely packed with the intracellular space volume fraction over 90% as in skeletal muscles (45). Furthermore, fibers are locally ordered, such that their membranes form flat walls that are often extended over a few adjacent fibers.

The DTI-RPBM provided estimates of the following parameters:

- **The surface-to-volume ratio S/V** , a parameter adopted from the field of porous media, representing the density of membranes. S/V is the ratio of total membrane surface area S (conventionally counted twice, for each membrane face) within an imaging voxel to the voxel volume V . As the diffusion transverse to the fiber is effectively two-dimensional, S/V here equals to the ratio of (twice) the length of the borders between fibers to the sample cross-sectional area. The fiber diameter is then estimated as $a=4/(S/V)$. The S/V ratio is a model-independent way to quantify membranes in any sample, free of arbitrariness in the definition of the diameter in non-cylindrical fibers; the above definition implies a geometry of a square lattice, with a being equal to the side of the square.
- **The membrane permeability κ** to water molecules, assumed the same for all myofiber membranes.
- **The unrestricted diffusion coefficient D_0** , the self-diffusivity of water molecules in the absence of all membranes. We assumed D_0 is same as the axial diffusivity λ_1 within the diffusion times used in this study. The D_0 value of each voxel was fixed to the λ_1 averaged over the diffusion times between 20 and 200 ms in that voxel in order to minimize the bias from any measurement error in λ_1 values at individual diffusion times.

More detailed information on the DTI-RPBM model, parameter estimation and application in muscle studies can be found in a recent review by Fieremans et al (41). Regions of interests (ROIs) were manually drawn over either lower hindlimb muscle in the middle 3–4 slices and repeated for each sequence. In this study, we compared the DTI-RPBM measures to fat fraction percentages and T_2 values during the early muscle growth. Water and fat fractions were determined by the iterative decomposition of water and fat with an echo asymmetry and least-squares (IDEAL) data analysis method (46). Fat fractions were

reported in percentages unless specified otherwise. T_2 was calculated using a monoexponential fit to the multi spin-echo data above noise level (Supporting Figure S2). Note that DTI scans used fat suppression whereas the other sequences did not; diffusion measures represent the characteristics of lean tissue alone whereas both fat fractions and T_2 reflect the properties of the whole muscle tissue. The signal-to-noise ratio (SNR) of the DTI data was measured as the mean of the signal in the muscle divided by the standard deviation of the background data outside of the imaging object.

Immunohistochemical Staining and Measurements

After imaging, mice were administered ketamine/xylazine (150/10 mg/kg via an intraperitoneal injection) and transcardially perfused with phosphate-buffered saline (PBS) mixed with 5,000 units/L heparin followed by 4% paraformaldehyde (PFA). The dissected lower leg muscles were immersed in 4% PFA overnight at 4°C, and then washed twice with PBS for 30 minutes each. Tissues were then processed for cryo-sectioning with sucrose gradients prior to embedding in optimal-compound temperature media and storage (−80°C). Axial sections were cut at 5µm for IHC staining of WGA.

For IHC, heat antigen retrieval with citric acid buffer (pH=6) was done prior to staining protocol. The primary antibody was WGA Alexa Flour 488 Conjugate (1:1000; Life Technologies: W11261). Slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for nuclear visualization and imaged on a digital slide scanner (Hamamatsu Nanozoomer 2.0HT). Histology images were used to measure: myofiber area (A), perimeter (P) and the minimum Feret's Diameter (MF). The RPBM S/V ratio in the myofiber geometry corresponds to the ratio P/A (in the cross-section). For each mouse, this ratio P/A was quantified using the Analyze Particles function provided by ImageJ Software (47). We included as many fibers as possible in each section ($>> 100$ fibers/section). This approach gave us approximately 1,000 – 2,250 fibers per mouse.

Ex vivo MRI Scans

Ex vivo imaging sessions were performed for the WT mice to better correlate measures to the IHC data. After *in vivo* imaging and transcardial perfusions, one leg sample was immersed in 4% PFA overnight at 4°C, and washed similarly to the immunohistochemistry samples in PBS twice for 30 minutes each. Following the wash, the *ex vivo* samples were transferred to a fresh PBS for 2 days for full rehydration at 4°C. Before *ex vivo* imaging, the set of samples were allowed to get to room temperature and situated in a holder filled with Fomblin (Solvay Solexis Inc, Thorofare, NJ), which serves as a hydrophobic sealant to contain the water within the samples during the long imaging sessions. Four samples were scanned together in a container *using* the same 7T Biospec micro-MRI system. The same scan protocol used for the *in vivo* MRI scan was used for the *ex vivo* MRI scans. The *ex vivo* scans were conducted with the all animals used for the *in vivo* MRI study. All *ex vivo* scans were performed at room temperature (20±2°C).

Statistical Analysis

Spearman correlations were calculated between the MR measures and age. Pearson correlation was assessed between the DTI-RPBM parameters and the conventional MRI

measures. To compare the histology diameter measures to the MRI derived diameter measures, Pearson correlations were calculated between the S/V estimated from DTI-RPBM and that measured from the WGA stained slides (as P/A ratio). All statistical tests were conducted at the two-sided 5% significance level using GraphPad (GraphPad Software, Inc., CA).

RESULTS

Figure 1A and B show representative raw images and parametric maps from *in vivo* MRI studies with 13 week old WT and *mdx* mice. The *mdx* mouse showed a heterogeneous distribution of hyper-intensity areas in the T_2 weighted image and T_2 map. The directionally encoded color (DEC) maps show that the majority of the leg muscle fibers are aligned in parallel as reflected by homogenous blue or purple across the whole legs. Figure 2A shows an example plot of the diffusion tensor eigenvalues estimated at different diffusion times in a WT mouse, indicating restricted diffusion perpendicular to the fiber axis (λ_2 and λ_3), but less along the fiber axis (λ_1). The radial diffusivity at long diffusion times shows an asymptotically linear dependence of $D(t) = (\lambda_2 + \lambda_3)/2$ perpendicular to the myofibers on $1/t^{1/2}$ (Fig. 2B), in agreement with RPBM model predictions. Such particular functional form is indicative of the extended disorder class of random membranes (40). Similar trends can be observed with the *ex vivo* data shown in Figure 3. As shown in Supporting Table S2, the SNR of the *in vivo* diffusion weighted images ($b=1000$ s/mm²) was 21 ± 9 with the longest t (700 ms) and higher with shorter t values. The SNR of the *ex vivo* diffusion weighted images was 49.9 ± 17 when $t=700$ ms and higher for $t < 700$ ms.

DTI-RPBM parameters in WT mice

The *in vivo* S/V correlated positively ($\rho=0.638$, $p=0.001$) with age (Fig. 4B), whereas the *in vivo* D_0 and κ values had weak correlations with age ($\rho=0.372$ and 0.414 , respectively) that were not statistically significant (Fig. 4A and 4C). The correlations of the *ex vivo* DTI-RPBM parameters with age were weaker than those of the *in vivo* DTI-RPBM parameters and did not reach statistical significance (Fig. 5A–C).

Conventional DTI parameters in WT mice

As an example of conventional DTI measures, the mean diffusivity (MD) and fractional anisotropy (FA) measured at diffusion time of 50 ms are also shown in Figures 4 and 5. The *in vivo* FA correlated positively ($\rho=0.664$, $p=0.001$) with age, whereas the *in vivo* MD and RD values had much weaker correlations with age ($\rho=0.061$ and 0.206 , respectively). The correlations of the *ex vivo* DTI parameters with age were weak and did not reach statistical significance.

Conventional MRI parameters in WT mice

The T_2 and fat fraction were measured to compare with the DTI-RPBM values (Figures 4 and 5). The *in vivo* T_2 had the strongest correlation ($\rho=-0.847$, $p=0.001$) with age among the parameters used in this study (Fig. 4H). The *ex vivo* T_2 also had a significant correlation ($\rho=-0.499$, $p=0.035$) with age (Fig. 5H). The *in vivo* fat fraction correlated positively

($p=0.686$, $p=0.001$) with age, whereas the *ex vivo* fat fraction did not have a significant correlation with age ($p=0.203$, $p=0.390$) (Fig. 4I and Fig. 5I, respectively).

With the data combined from all time points, the *in vivo* S/V values showed moderate but significant ($p<0.05$) positive ($r=0.517$) and negative ($r=-0.550$) correlations with the *in vivo* T_2 and fat fraction, respectively (Supporting Figure S3). The *in vivo* κ values also correlated positively with the *in vivo* T_2 ($r=0.498$), but not with the *in vivo* fat fraction. There was no significant correlation between the *in vivo* D_0 and T_2 or between the *in vivo* D_0 and fat fraction. Among the pooled *ex vivo* data, the S/V and κ values showed no significant correlation with neither the T_2 nor fat fraction (Supporting Figure S4). The *ex vivo* D_0 moderately correlated with the *ex vivo* fat fraction ($r=0.453$).

Comparison with WGA staining in WT mice

Figure 6A and B show representative images of a WGA IHC stained cross-section of the lower leg muscles for a 4 and 13 week old WT mouse. The WGA stained slides were used to measure P/A . The P/A had a significant correlation with the S/V *in vivo* (Figure 7A, $r=0.56$, $p=0.025$) as well as *ex vivo* (Figure 7B, $r=0.71$, $p=0.010$). When converting S/V and P/A to diameters of cylinders, $a=4/(S/V)$, the corrections were 0.52 and 0.39 for *in vivo* and *ex vivo* data, respectively.

Muscle growth in *mdx* mice

The same *in vivo* imaging protocol and analysis were applied to measure *mdx* mouse muscles between week 4 and week 13 (Fig. 8). All three DTI-RPBM parameters had significant positive correlation with age; $p=0.499$ ($p=0.009$), 0.570 ($p=0.002$) and 0.404 ($p=0.041$) for D_0 , S/V and κ , respectively. Significant correlation was also observed with MD ($p=0.540$, $p=0.004$), RD ($p=0.458$, $p=0.019$), and fat fraction ($p=0.411$, $p=0.037$). FA and T_2 did not have significant correlation with age. Figure 9 provide examples of the WGA IHC stained myofibers for a 4- and 13-week old *mdx* mouse. There was no noticeable trend of P/A , MF, and $4/(P/A)$ between the two time points. A summary of MRI parameters measured with *in vivo* WT and *mdx* mice is provided in Table 1.

DISCUSSION

In this study, we investigated the feasibility of measuring the myofiber size using DTI data at multiple diffusion times and the RPBM analysis. We found that there are noticeable differences between the fiber sizes measured using *in vivo* and *ex vivo* DTI data; the fiber diameter estimates $a=4/(S/V)$ from the *ex vivo* data are smaller than those from the *in vivo* data. Furthermore, the fiber diameter estimates from the WGA-stained slides were in a similar range as those measured with *ex vivo* DTI and also showed a stronger correlation with those measured *ex vivo* compared to those measured *in vivo*. These results suggest that (i) voxel-averaged myofiber diameters can be assessed by the DTI-RPBM method and (ii) the myofiber diameters measured by histological methods or an *ex vivo* imaging can be substantially different from the actual fiber diameters in intact muscles.

The *in vivo* S/V estimates (around $0.2 \mu\text{m}^{-1}$) from our measurement with WT mice correspond to a fiber diameter of about $20 \mu\text{m}$, ranging from $14 \mu\text{m}$ to $24 \mu\text{m}$. These values

are within the range of mouse muscle fiber sizes reported in previous studies. White *et al* (48) investigated the muscle fiber growth during postnatal mouse development and reported a linear increase in the myofiber cross-sectional area from postnatal day 7 (P7, about 200 μm^2 ; diameter $\sim 16 \mu\text{m}$) to day 56 (P56, about 1350 μm^2 ; diameter $\sim 41 \mu\text{m}$). The mouse skeletal muscle fibers appear to be smaller than the human skeletal muscle fibers. Sigmund *et al* (35) used the same DTI-RPBM method using a 3T clinical scanner and reported human myofiber diameters between 30 μm and 60 μm . Myofiber diameters from other histologic studies were about 30–50 μm for human tongue muscles (49) and 30–60 μm for beef chuck (50). More recently, Fieremans *et al* utilized the RPBM method to track human calf diameters following an exercise regimen and noted an increase in size in the gastrocnemius medialis (41,51). Microstructural changes in the rotator cuff muscles, supraspinatus and infraspinatus, were also examined pre- and post-operatively and showed a decrease in myofiber size and atrophy secondary to mobility and activity following surgical repair. The fiber sizes measured by *ex vivo* DTI and histology in our study are in good agreement with these previous studies.

In our analysis, we assumed that the D_0 value in each voxel matches with λ_1 averaged over the diffusion times in that voxel. This approximation was based on the assumption that the myofiber length is substantially longer than the diameter such that the average displacement length of water molecules by self-diffusion during the diffusion times used in this study is shorter than the myofiber length. For $D \sim 2 \mu\text{m}^2/\text{ms}$ and $t \sim 700 \text{ ms}$, average diffusion length $L \sim 37 \mu\text{m}$. In contrast, the myofiber length at P7 is about 1000 μm and it grows to about 5000 μm at P56 (48). Hence, it is reasonable to assume that the diffusivity along the primary eigenvector direction represents the unrestricted diffusion coefficient D_0 . On the other hand, it is not fully understood whether any intracellular structures, such as z discs, could hinder or restrict water diffusion substantially. In the example data shown in Figures 1 and 2, λ_1 showed a small, but noticeable decrease over the diffusion times less than 100 ms. This change could be due to a dispersion of the orientations of muscle fibers within a voxel. It can also be induced by any intracellular or extracellular structures that have gaps smaller than the muscle diameter. Since our experiment was not designed to address this issue, we estimated D_0 by averaging the λ_1 values between 20 and 200 ms for simplicity. Further investigation is warranted to assess the diffusion time dependency of the axial diffusivity in skeletal muscles.

One of the unique parameters measured by the RPBM is the sarcolemma permeability, κ . An alternative non-invasive approach to measure cellular permeability *in vivo* is to use dynamic contrast enhanced MRI experiments using a bolus injection in cancer studies (52–55). It has been demonstrated that an infusion of contrast agent can be used to measure the sarcolemma permeability in animals (56). However, using human DCE-MRI, commonly performed with a bolus injection and a relatively short scan time of less than 10 min, it has been shown that the contrast enhancement in muscle is not high enough to observe the effect of water exchange to measure the sarcolemma permeability (57). Hence, the DTI-RPBM method demonstrated in this study may be a more practical approach to measure the sarcolemma permeability non-invasively.

Our data suggest that the permeability is higher at early age (4–5 weeks) than at later stages. Overall, the κ estimates were around 0.05 $\mu\text{m}/\text{ms}$ in both *in vivo* and *ex vivo* data, which are higher than those measured in human skeletal muscles (0.02–0.03 $\mu\text{m}/\text{ms}$). Following Benga's report on a group of water exchange proteins on red blood cell membranes (58), Peter Agre's definitive discovery of aquaporin in 1990 led to establishing the primary role that membrane water channels play in cell vitality and function (59). As the diffusion of water directly through a lipid bilayer is very slow, aquaporins facilitate the water exchange across the cell membrane that is crucial to cell function. Development of healthy skeletal muscle is accompanied by an increase in the number of aquaporin-4 (AQP4) (60), the major water channel of the neuromuscular system, causing an increase of sarcolemma permeability. In addition, the permeability measured by the DTI-RPBM method can also be affected by active trans-plasma membrane water cycling associated with ATP-driven ion cross-membrane transporters (61). Hence the permeability measured in our experiment can be a combination of both AQP4 expression level and ATP-driven membrane transport activity. It is interesting to note that the *ex vivo* κ values are similar to the *in vivo* values despite the absence of active water exchange processes. This could be because the cell membrane does not remain intact through the perfusion and fixation process. Further study is warranted to investigate the feasibility of using κ as a marker of AQP4 expression level as well as the metabolic status of cells.

Our data with the *mdx* mice also showed a similar trend observed in the WT mice, in which the changes in the fiber diameter measured in terms of the *in vivo* S/V measurement was observed in terms of the histological proxy, P/A or MF measurements in the WGA stained slides. The similarities between the WT and *mdx* mice may be explained by the lack of an exercise regimen, which helps to induce muscle tear injuries to the *mdx* mice. Current *mdx* studies generally incorporate an exercise program to initiate the muscle deterioration process with the *mdx* mice (62). This exercise challenge can also be part of a future study design to assess the DTI-RPBM in a wider range of pathologic changes. While the histologically measured fiber size may not be same as the actual fiber size *in vivo*, the histological method remains as the only clinically available method to measure muscle fiber size.

The DTI scan protocol used in this study was carefully selected to minimize the effect of noise on the estimation of the DTI-RPBM parameters. It was reported earlier that an SNR of 25 is required to have an accuracy of 5% in estimation of diffusion eigenvalues when 6 diffusion weighting directions are used (63). The importance of the SNR and the number of diffusion weighting directions in skeletal muscle DTI studies has also been discussed by Damon et al (64). The mean SNR of our data with diffusion time $t=700$ ms was 21.0 ± 9.0 . However, we used 20 diffusion weighting directions, instead of 6, resulting in close to 3 repetitions to increase SNR. In addition, it was shown earlier by a Monte Carlo simulation study that at least 20 diffusion sampling directions are necessary for a robust estimation of anisotropy (65). We also did not see any sign of eigenvalue repulsion due to noise (66) in our data as the primary eigenvalue is mostly constant. These observations support that the influence of noise is not a critical factor in our RPBM analysis.

In addition to the DTI and RPBM parameters, T_2 and fat fraction of the skeletal muscle were measured in this study. DTI and RPBM parameters reflect the characteristics of the lean

tissue only, while T_2 and fat fraction are for the whole tissue without fat suppression. It is interesting to note that T_2 of the muscle decreases with age while fat fraction increases. Since T_2 of subcutaneous fat is typically longer than that of a skeletal muscle (67), one could expect that an increase in fat fraction could lead to an increase in T_2 of the muscle. Instead the T_2 decreased with age in this study. For the same duration, the DTI-RPBM data indicate that the muscle fiber diameter increased (i.e., decrease in S/V); there is a moderate positive correlation between T_2 and S/V (Supporting Figure S3). The decrease in T_2 along with S/V and κ may indicate that the muscle growth in this time period is not only in size, but also in the structural complexity and heterogeneity. These observations in our study suggest that a comprehensive multiparametric approach using both conventional MR relaxation parameters and DTI-RPBM parameters would be necessary to elucidate the complex process of normal and abnormal muscle growth.

Muscular dystrophy is a class of genetic diseases that involve abnormal muscle degeneration and wasting due to the mutations in genes responsible for development of healthy muscle. The differences between the types of muscular dystrophy lie at the protein level in addition to onset and severity of symptoms. DMD is one of the most prevalent forms and is reported to have an incidence rate of 1 in every 5,000 newborn males (68–70). While this lethal X-linked genetic defect was identified in 1987 (71,72), there is still no effective treatment for DMD (70,73). Decrease in muscle fiber diameter and increase in muscle fiber necrosis clusters are the hallmarks of DMD, which are typically measured by histopathology of muscle biopsy (70). However, the lack of a reliable non-invasive tool for evaluating changes in muscle tissue microstructure in DMD has been one of the major hindrances in assessing existing treatment methods and developing new therapies (1). The DTI-RPBM method can provide non-invasive measures of muscle fiber diameter and membrane permeability. While the present study was focused on evaluating these measures in muscle growth and pathological validation, future studies are warranted to further evaluate the feasibility of using the DTI-RPBM method for quantitative assessment of disease progression and treatment response in muscular dystrophy.

This study has a few limitations. This study was conducted with a small cohort to demonstrate the feasibility of using the DTI-RPBM method to measure the muscle fiber changes during the early development of skeletal muscles, and needs to be followed up by a study including a larger sample size. The spatial resolution was not high enough to perform analysis for individual muscle groups. This could be partly addressed in future studies by using an RF coil with a higher sensitivity and longer scan time. Recent development of fast imaging techniques, such as simultaneous multi slice (SMS) imaging methods (74), may also help to improve the spatial resolution for a given scan time using parallel imaging. Another limitation of our study was that a longitudinal measurement of muscle growth in WT mice was not performed. Another limitation of the study was that ROI selection was manually performed in each sequence individually. This could be improved by using co-registration of all images and transferring the ROI selected on one image to the other images in future studies. Since the ROI was selected for the whole muscle over multiple slices, however, we assume that the error induced by any difference in the ROIs in this study would be not substantial. Our main focus was to compare the RPBM parameters with *in vivo* DTI and *ex vivo* DTI as well as histology at each time point. However, as demonstrated with the

mdx mice, it is possible to perform multiple *in vivo* scans with same mice which could increase the number of mice for all time points. Acquiring longitudinal measurements of microstructural changes in both WT and *mdx* mice will allow for a statistical comparison between the diffusion biomarkers. Future studies will be designed to take the advantage of *in vivo* longitudinal measurement of the DTI-RPBM method.

CONCLUSIONS

The present animal validation study demonstrates that DTI measurements at multiple diffusion times with the RPBM analysis can be used to measure muscle fiber size non-invasively. Our results suggest that the myofiber diameters measured by histological methods or *ex vivo* imaging can be substantially different from the actual fiber diameters in intact muscles. The histological measurements of the *P/A* correlated positively and strongly with the *ex vivo S/V* as well as the *in vivo S/V*. *In vivo* measurement of myofiber diameters using the diffusion time-dependent DTI method may help advance our understanding of the underlying change of muscle fibers during disease progression as well as current and prospective treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Representative parametric maps acquired for *in vivo* WT (A) and *mdx* (B) mice.

Figure 2.

The time-dependency of the diffusion tensor derived eigenvalues and mean diffusivity in the hindlimb (A). The blue line is a DTI-RPBM fit to the radial $D(t) = (\lambda_2 + \lambda_3)/2$. (B) $D(t)$ replotted as a function of $t^{-1/2}$ becomes asymptotically linear at smaller $t^{-1/2}$, i.e. at a larger t .

Figure 3. Representative weighted images and parametric maps acquired for *ex vivo* WT mice (A) with the corresponding eigenvalue plot (B) and radial $D(t)$ (C) similar to the *in vivo* acquisition.

Figure 4.

In vivo MRI parameters of WT mice during muscle growth between week 4 and week 13.

The association of the MRI parameters with age was assessed using the Spearman correlation coefficient (ρ).

Figure 5.

Ex vivo MRI parameters of WT mice during muscle growth between week 4 and week 13.

The association of the MRI parameters with age was assessed using the Spearman correlation coefficient (ρ).

Figure 6. Differences in immunohistological images from the youngest (A) and oldest (B) time points for a WT mouse. The green channel depicts the WGA staining and blue is the nuclear DAPI stain. Mean ($\pm$ standard deviations) histology measures P/A , MF and $4/(P/A)$ were estimated to correlate with DTI-RPBM parameters (C, D, F).

Figure 7.

Scatter plots between RPBm S/V ratio and histology proxy P/A for *in vivo* (A) and *ex vivo* (B) DTI data. Note the strongest correlation is between the *ex vivo* S/V and P/A measurements.

Figure 8.

In vivo MRI parameters of mdx mice during muscle growth between week 4 and week 13.

The association of the MRI parameters with age was assessed using the Spearman correlation coefficient (ρ).

Figure 9.

Immunohistological images from the youngest (A) and oldest (B) time points for a *mdx* mouse. The green channel depicts the WGA staining and blue is the nuclear DAPI stain. Histology mean P/A , MF and $4/(P/A)$ ($\pm$ standard deviations) values were estimated to correlate with DTI-RPBM parameters (C, D, F). Std were notably larger between the *mdx* groups rather than the WT.

Table 1
Summary of MRI parameters (mean $\pm$ standard deviation) measured with *in vivo* wild-type (WT) and *mdx* mice.

Age (weeks)	D_0 ($\mu\text{m}^2/\text{ms}$)	S/V (/um)	k ($\mu\text{m}/\text{ms}$)	MD ($\mu\text{m}^2/\text{ms}$)	FA	T_2 (ms)	FF (%)
WT							
4	1.75 ± 0.09	0.29 ± 0.58	0.06 ± 0.00	0.91 ± 0.06	0.69 ± 0.05	36.52 ± 2.65	2.74 ± 0.27
5	1.78 ± 0.19	0.28 ± 0.05	0.08 ± 0.03	1.00 ± 0.11	0.61 ± 0.03	36.06 ± 1.90	3.01 ± 0.19
7	1.77 ± 0.12	0.20 ± 0.05	0.04 ± 0.02	0.96 ± 0.89	0.63 ± 0.08	31.79 ± 0.62	3.44 ± 0.28
9	1.74 ± 0.07	0.17 ± 0.03	0.05 ± 0.02	1.03 ± 0.06	0.54 ± 0.02	31.48 ± 0.87	3.93 ± 0.13
13	1.54 ± 0.02	0.20 ± 0.05	0.04 ± 0.01	0.85 ± 0.05	0.59 ± 0.06	30.86 ± 0.46	3.27 ± 0.07
mdx							
4	1.66 ± 0.05	0.23 ± 0.03	0.06 ± 0.03	0.93 ± 0.08	0.59 ± 0.03	37.01 ± 2.54	5.44 ± 0.96
5	1.64 ± 0.04	0.18 ± 0.01	0.04 ± 0.01	0.93 ± 0.04	0.56 ± 0.03	35.95 ± 0.79	4.66 ± 0.79
7	1.74 ± 0.07	0.16 ± 0.24	0.03 ± 0.01	0.99 ± 0.05	0.57 ± 0.05	35.50 ± 1.41	5.84 ± 1.26
9	1.77 ± 0.08	0.20 ± 0.04	0.04 ± 0.2	1.00 ± 0.04	0.62 ± 0.03	36.80 ± 0.45	5.76 ± 2.47
13	1.73 ± 0.09	0.18 ± 0.03	0.05 ± 0.01	1.02 ± 0.07	0.54 ± 0.01	35.19 ± 1.48	3.80 ± 0.75