Automated Assessment of Dystrophin Expression in Muscular Dystrophy

G.D. Young[1], F. Aeffner[1], S.A. Moore[2], H. Lange[1], A.J. Milici[1]
[1]Flagship Biosciences Inc.; CO, USA; [2]Department of Pathology, The University of Iowa; IA, USA

Abstract

Quantification of skeletal muscle fiber parameters is challenging, but important for the evaluation of a variety of neuromuscular diseases. Various endpoints from individual fiber characteristics to whole tissue determinations may be required to better understand disease biology and/or treatment efficacy. In the context of muscular dystrophy studies, assessment of dystrophin expression in muscle fibers has largely been qualitative or semi-quantitative assessments of representative areas of an entire tissue section. To provide more robust quantitation of muscle fiber endpoints, an automated method (MuscleMap™) was created to quantify a number of group and individual muscle fiber parameters. To evaluate algorithm efficacy, MuscleMap was applied to a test cohort of dystrophic muscle biopsies. Duchenne and Becker muscular dystrophy (DMD and BMD, respectively) are rare genetic diseases that result from mutations in the DMD gene, which encodes the dystrophin protein, leading to progressive muscle degeneration, muscle weaknessand fatigue, and premature death. Muscle biopsy cryosections derived from DMD and BMD patients and from healthy control individuals were assessed using the MuscleMap algorithm. Numerous parameters relating to staining intensity, membrane staining completeness, and morphometric presentation of dystrophin in individual muscle fibers were quantified in dual label, immunofluorescence-stained sections. A number of parameters, including mean dystrophin staining intensity and dystrophin membrane staining completeness, were significantly different in DMD and BMD tissue when compared to normal controls, and are promising biomarkers for understanding biology of the disease. From early drug development to clinical trials, automated quantification of dystrophin expression in muscle fibers is an attractive endpoint given the mechanism of action for current promising therapies.

Materials and Methods

Patient Samples

Muscle biopsies were obtained for diagnostic testing from patients ranging in age from 2 to 51 years. Biopsies without diagnostic abnormalities served as normal controls (n=3; ages 2-20 years). Dystrophinopathy cases (n=6; ages 2-51 years) included patients with disease severity ranging from Duchenne muscular dystrophy to mild Becker muscular dystrophy. Each biopsy used for immunofluorescence staining was frozen in isopentane precooled to -160°C. Cross sections of each biopsy were cut at 10 µm thickness, air dried on glass slides, then dual label immunostained for merosin (laminin alpha-2) and dystrophin.

Slide Scanning

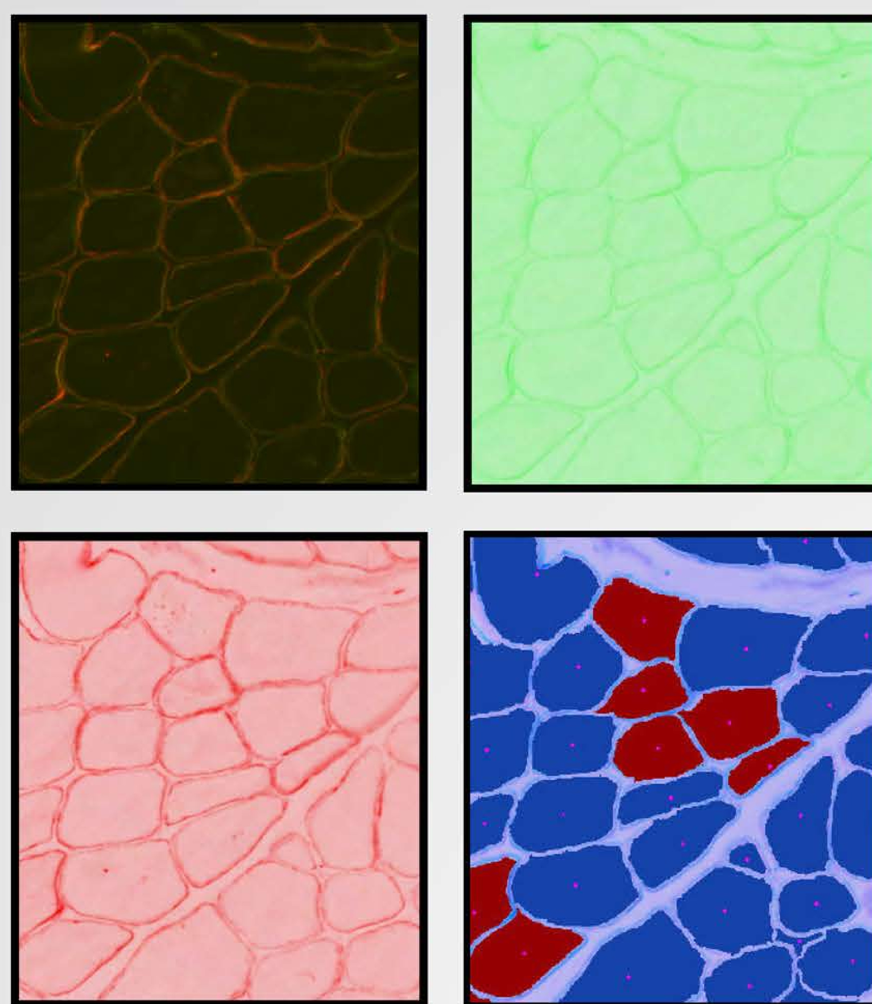
Upon receipt, slides were stored in the dark in a refrigerator until they could be scanned. Slides were warmed to RT and scanned with identical image capture settings at 20X in all three color channels using a 3DHistech panoramic scanner. The DAPI channel was primarily used to set focal planes and was not further analyzed.

Labeling Protocol

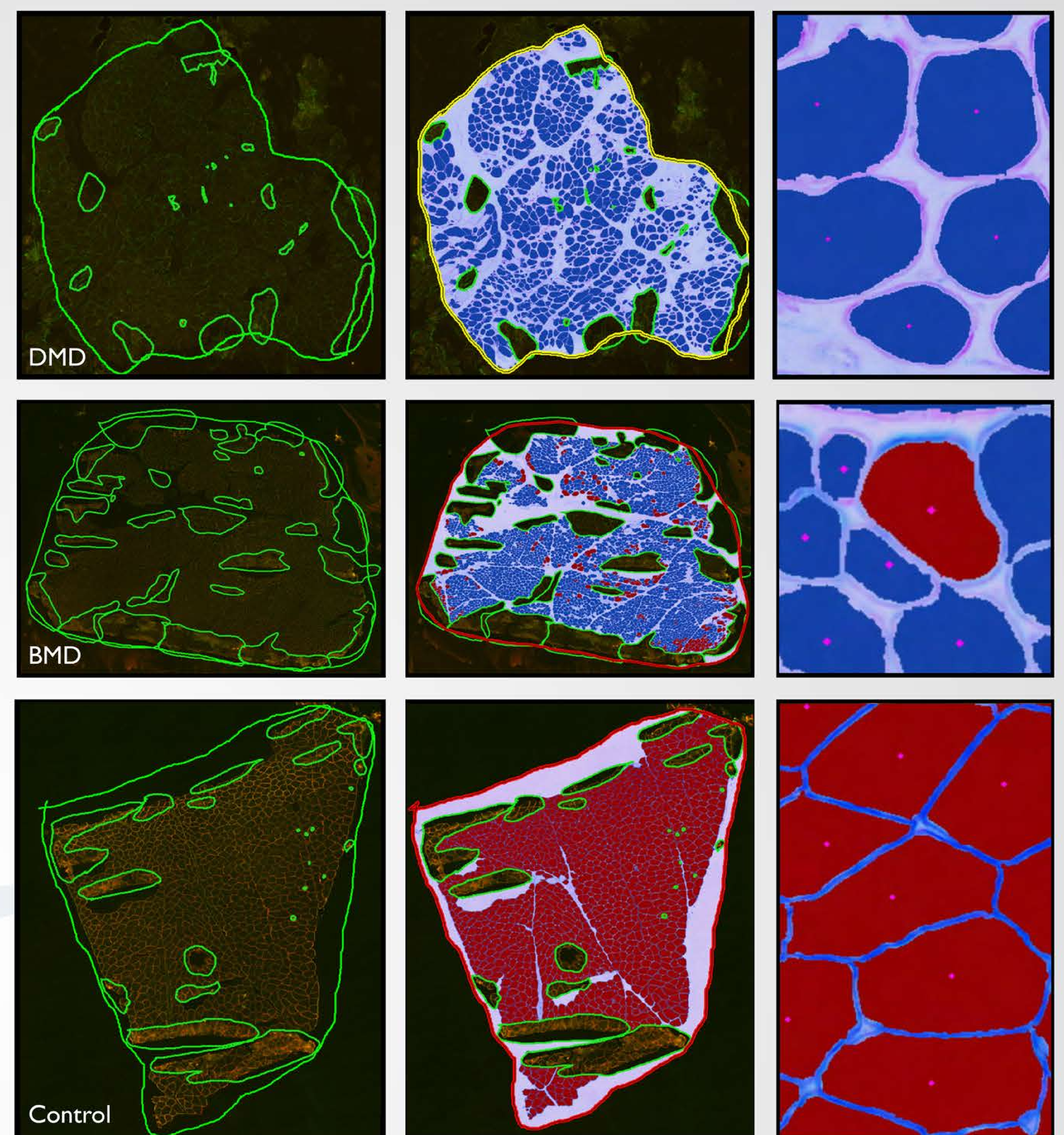
Sections were incubated with a cocktail of primary antibodies (Abcam #15277; final dilution 1:400 in PBS; Millipore #MAB1922; final dilution 1:100 in PBS) for 1 hr at room temperature (RT). Following washing steps, the sections were overlaid with a cocktail of secondary antibodies (Alexa Fluor® 488 goat anti-mouse IgG, Molecular Probes #A-11001; final dilution 1:400 in PBS; Alexa Fluor®594 goat anti-rabbit IgG, Molecular Probes #A-11037; final dilution 1:400 in PBS) for 30 min at RT. Sections were washed, coverslipped using Prolong Gold with DAPI (Invitrogen #P36931), sealed with nail polish and shipped to Flagship.

Image Analysis

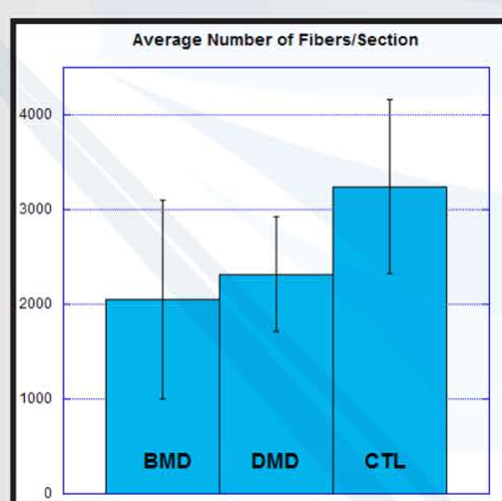
Scanned whole slide images were annotated to include the entire section and to exclude folds and artifacts. Image analysis was performed on whole section images using Flagship's MuscleMap algorithm. This algorithm used the Merosin (Alexa Fluor®488) label to identify muscle fibers based upon a number of morphological features. The algorithm then created a mask onto which the Dystrophin (Alexa Fluor®594) labeled membrane was quantified. The algorithm captured data on all sections, both on the whole slide as well as individual fiber level.



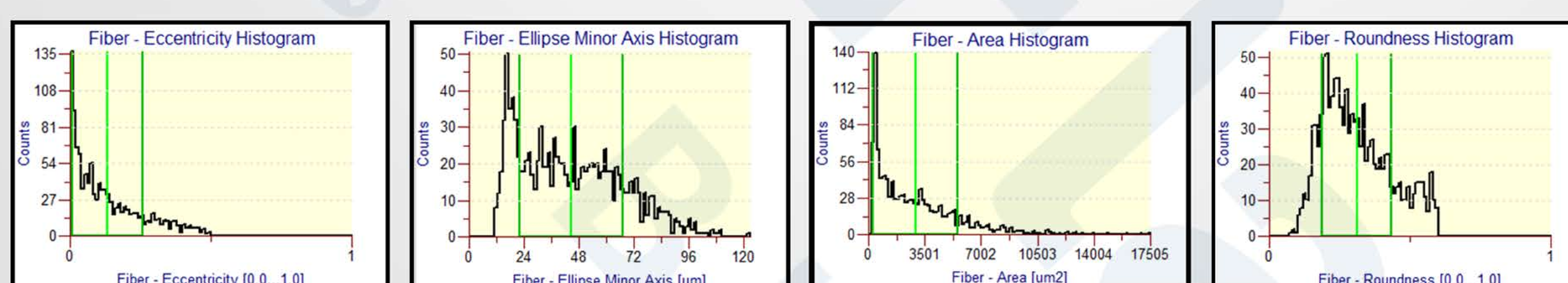
Overview of MuscleMap algorithm detection of individual muscle fibers. Starting with the original dual labeled fluorescent image (left top panel), the algorithm first splits the two color channels and defines muscle fibers based upon the merosin label) creating a membrane mask. This mask is then placed on the dystrophin labeled image (bottom left panel) allowing the algorithm to quantify membrane staining and classify fibers (bottom right panel – negative fibers are blue and positive fibers are red).



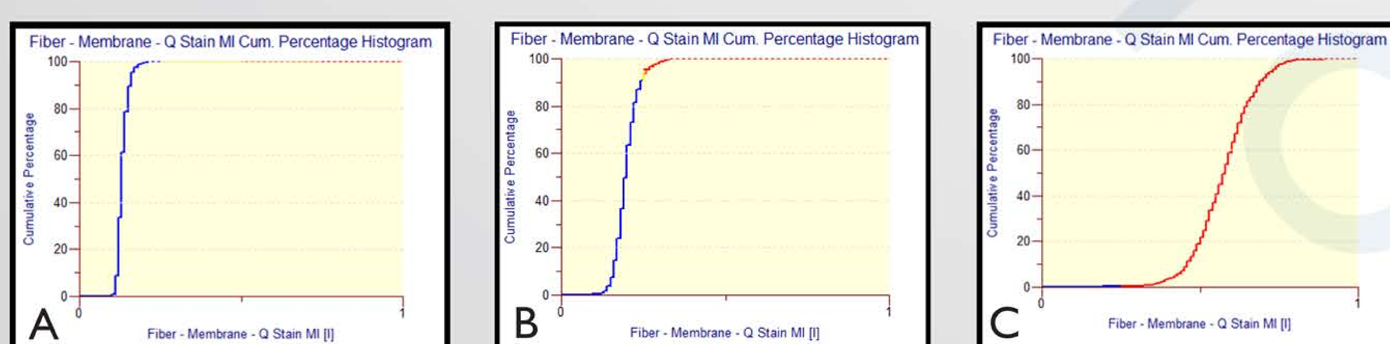
The original dual fluorescent scans of the entire DMD, BMD and Control muscle sections are shown in the left panels. These sections have been annotated to include analyzable tissue and to exclude folds, artifacts and non-muscle tissues such as intramuscular nerve bundles. In the middle panels, MuscleMap has been applied to the annotated images resulting in a false color markup demonstrating muscle fiber detection and classification. Fibers that are below threshold setting are shown in blue. Fibers that above dystrophin threshold settings are shown in red. High magnification of the MuscleMap determination of detection and classification is demonstrated in the panels on the right.



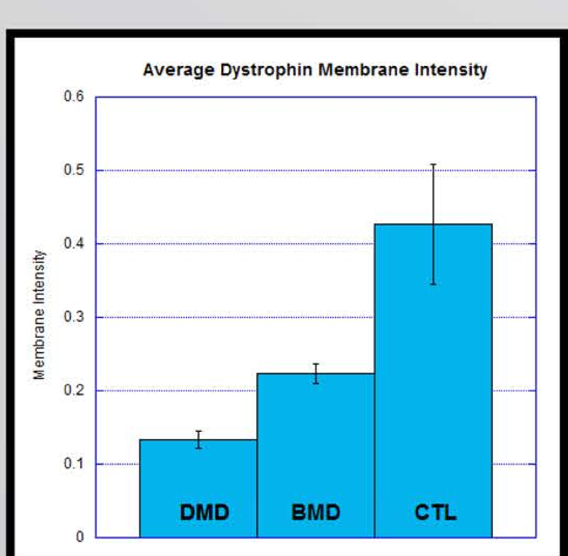
Average number of fibers detected in three sections from each condition ± SE.



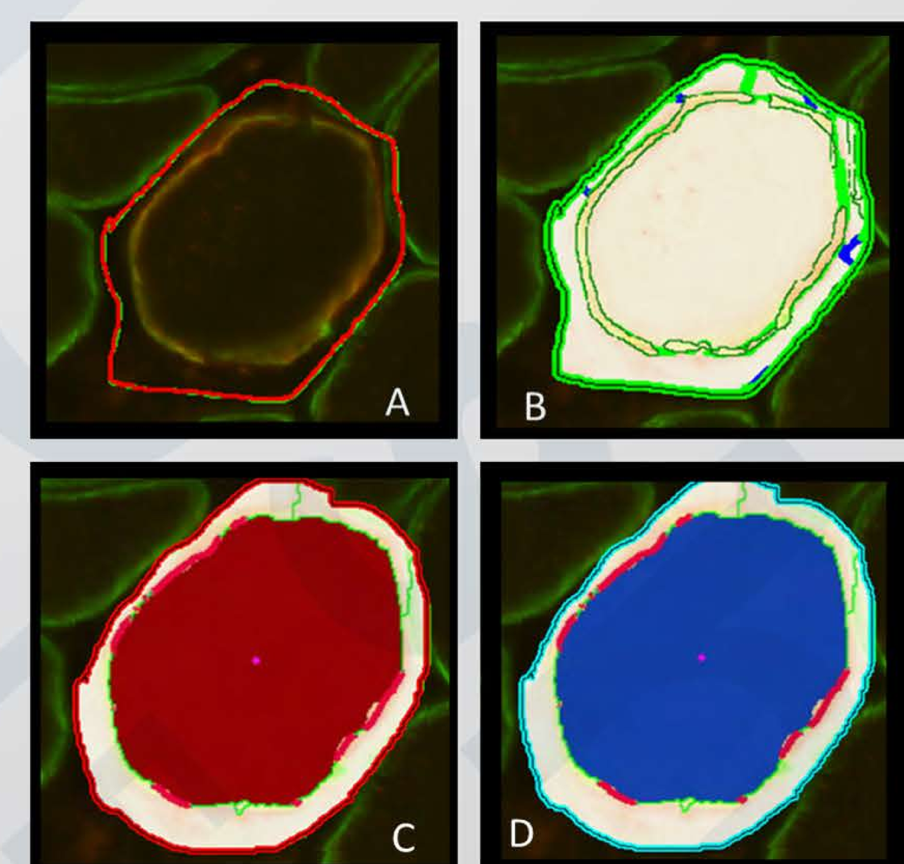
These graphs demonstrate some of the morphometric data that is collected for each fiber. The data was generated on a DMD sample.



Examples of the cumulative membrane intensity (MI) dystrophin staining from DMD (A), BMD (B) and Control (C) muscle biopsies. In these examples, there is a progressive shift to the right and increasing percentage of membranes classified above threshold (negative membranes are blue and positive membranes red) going from the DMD sample to the Control sample.



Quantification of the average membrane intensity demonstrates progressivly less dystrophin in Becker (BMD) and Duchenne (DMD) muscular dystrophy biopsies. This correlates with the severity of clinical phenotypes in BMD and DMD. n=3 for each condition. Error bars are standard error (± SE).



Demonstration of the effect of incorporating membrane completeness in fiber classification. In A, a single dual labeled muscle fiber is outlined and in B the mask of the membrane is demonstrated. In C, the MuscleMap algorithm was applied with a 30% membrane completeness cutoff resulting in the fiber being classified as positive (red). In D, the MuscleMap algorithm was applied with a 60% membrane completeness cutoff resulting in the fiber being classified as negative (blue). The portions of the membrane encircling the fiber above staining intensity threshold are shown in red while membrane segments below threshold are shown in green.

Results & Conclusions

- Flagship's proprietary algorithm MuscleMap can accurately identify muscle fibers in fluorescent dual labeled human muscle biopsies.
- MuscleMap algorithm generates a panel of quantitative data at the individual muscle fiber and whole tissue level.
- MuscleMap algorithm stratifies fibers as positive or negative based upon the intensity of the dystrophin labeling.
- MuscleMap is able to distinguish DMD from BMD from control muscle based on the quantification of dystrophin.
- Quantitative assessment of dystrophin expression and other muscle fiber parameters with MuscleMap may contribute to determinations of disease severity and therapeutic efficacy.
- MuscleMap has the potential to quantify other membrane-associated or cytoplasmic proteins in skeletal muscle.

Flagship
BIOSCIENCES