Development of Digital Tissue Image Analysis Solution for Muscle Biopsies in Support of Disease-Modifying Therapies for Duchenne Muscular Dystrophy

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Abstract

The continual expression of utrophin protein by pharmacological maintenance of utrophin transcription in dystrophin-deficient muscle fibres is potentially a disease-modifying treatment for Duchenne muscular dystrophy (DMD) regardless of the specific dystrophin mutation. The evaluation of molecular biomarkers of muscle regeneration and structural protein complexes, such as developmental myosin and dystroglycans respectively, may be important endpoints in future clinical trials of utrophin modulators. Building on a recently published manual quantification approach, which demonstrated a positive correlation between utrophin levels and the degree of muscle fiber regeneration in DMD and Becker muscular dystrophy (BMD) muscle biopsies, the development of fully automated processes is ongoing.

Here, we report the development of multiplex immunohistochemical (IHC) assays and digital tissue image analysis (tIA™) solutions for robustly quantifying utrophin, developmental myosin heavy chain, and beta-dystroglycan expression in DMD, BMD, and control muscle biopsies. The tIA™ approach enabled detection of biomarker signal features (e.g., cumulative intensities) and tissue morphometrics (e.g., fiber area and minimum diameter) in whole-slide images of muscle cryosections at an individual muscle fiber resolution.

The tIA™ solutions reproducibly demonstrated quantifiable differences in levels of utrophin, and regeneration between DMD, BMD, and control biopsies. These biomarkers may be informative endpoints for evaluating pharmacologic benefit in dystrophin-deficient muscle in future clinical trials of utrophin modulators and potentially other DMD therapeutic approaches.

Introduction & Objectives

Muscular dystrophies are a collection of progressive, muscle wasting diseases. The most prevalent and severe of these diseases is Duchene muscular dystrophy (DMD). Those afflicted with DMD suffer through extreme quality of life impairments and reduced life span. Patients are typically wheelchair-bound during their teenage years, require mechanical ventilation assistance in their twenties, and die due to cardiac or respiratory failure before they reach their early thirties.

DMD, and the related, but less severe, Becker muscular dystrophy (BMD), are caused by mutations in the dystrophin gene. These mutations result in loss or reduction of dystrophin expression, function, or both. In healthy muscle, dystrophin serves as a structural protein; loss of function compromises the structural integrity of the muscle, resulting in increased muscle damage and myonecrosis during the normal processes of contraction and relaxation (see Barresi 2011: Skeletal Muscle; 1:24 for review).

Utrophin is a dystrophin homologue that is normally expressed only in developing and regenerating myofibers, and at neuromuscular and musculotendinous junctions. As myofibers mature, utrophin is replaced by dystrophin. We and others have shown previously that utrophin can functionally substitute for dystrophin as a structural component of muscle (Tinsley et al. 1998: Nat Med; 4,1441-4, Guiraud et al. 2015: Hum Mol Gen: 1-13). These data suggest that utrophin upregulation in DMD patients may be an effective therapy to improve muscle integrity and ameliorate disease severity of DMD and BMD. To this end, Summit Therapeutics has developed a small molecule therapeutic called ezutromid, which is currently in DMD clinical trials, to assess the effectiveness of utrophin modulation.

In order to assess the efficacy of utrophin upregulation therapy, Flagship Biosciences has developed IHC assays and digital tissue image analysis (tIA™) tools to measure biomarkers of muscle health. Specifically, we have developed three duplex, IHC assays combining laminin alpha2 with utrophin, beta-dystroglycan or developmental myosin heavy chain respectively. The tIA™ tools allow quantification of biomarker expression and tissue morphometrics across whole-slide images of DMD, BMD, and control patient biopsies, allowing for a robust assessment of muscle health.

Methods

Samples

Frozen skeletal muscle biopsies from DMD, BMD, and non-DMD/BMD control patients (CTRL) were used. The control muscle originated from patients presenting with a clinical neuromuscular phenotype that warranted a muscle biopsy; however, these patients were ultimately not diagnosed with either DMD or BMD. In fact, control muscle biopsies had no histologic abnormalities. Samples were previously stained with monoplex, IHC assays for utrophin, beta-dystroglycan, and developmental myosin heavy chain. The whole-slide images were semi-quantitatively evaluated by a board-certified, DVM pathologist to approximate percentages of biomarker-positive myofibers. Samples were obtained from the University of Iowa, and Paul D. Wellstone Muscular Dystrophy Cooperative Research Center. Sample details are listed in Table 1.

Samples			Patient Age at Time of Biopsy, y	Patient Sex	Biopsied Muscle	DMD Mutation	~%POS myofibers		
Diagnosis	Estimated Severity	Sample ID					Utrophin	Beta-dystroglycan	MHCd
DMD	Severe	DMD 1	6	M	unknown	dup ex 2	41	99	13
DMD	Severe	DMD 2	6	M	quad	dup ex 50-55	22	100	15
BMD	Mild	BMD 1	22	M	deltoid	probable in frame del	27	100	1
BMD	Mild	BMD 2	4	M	quad	del ex 14-27	6	99	2
Control	Healthy	CTRL 1	14	M	quad	NA	4	100	0
Control	Healthy	CTRL 2	9	F	unknown	NA	4	100	0

Immunohistochemistry

Duplex IHC assays were developed and optimized for laminin alpha2 (clone 4H8-2, Enzo Life Sciences ALX-804-190) with utrophin (clone DRP3/20C5, Leica Biosystems), beta-dystroglycan (clone 43DAG1/8D5, Leica Biosystems), or developmental myosin heavy chain (MHCd Clone RNMY2/9D2, Leica Biosystems). In each duplex assay, laminin alpha2 expression is visualized by a yellow chromogen and utrophin, beta-dystroglycan, or MHCd by blue chromogens. All staining was performed using the Leica Biosystems BONDRX autostainer.

Image Analysis

IHC-stained slides were scanned on an Aperio ScanScope CS brightfield scanner at 20x magnification to generate whole-slide images. Flagship's proprietary software un-mixes the chromogen colors, generating new images that distinguish yellow and blue staining. MuscleMap™ algorithms then quantify the blue signal intensities in the regions defined by the yellow stain (myofiber membrane or cytoplasm).

Results

Figure 1. Duplex IHC Assays for Assessment of Utrophin, Beta-dystroglycan, and MHCd in Skeletal Muscle.
DMD, BMD, and CTRL skeletal muscle sections were stained with duplex, IHC assays for laminin alpha2 (yellow), to outline myofibers, and either a marker of muscle regeneration (utrophin or MHCd, blue) or a marker of the dystrophin-associated protein complex (beta-dystroglyan, blue). Zoomed images are at 10x magnification; scale bar represents 100 µm. Insets are fit, whole-slide images of the tissue sections.

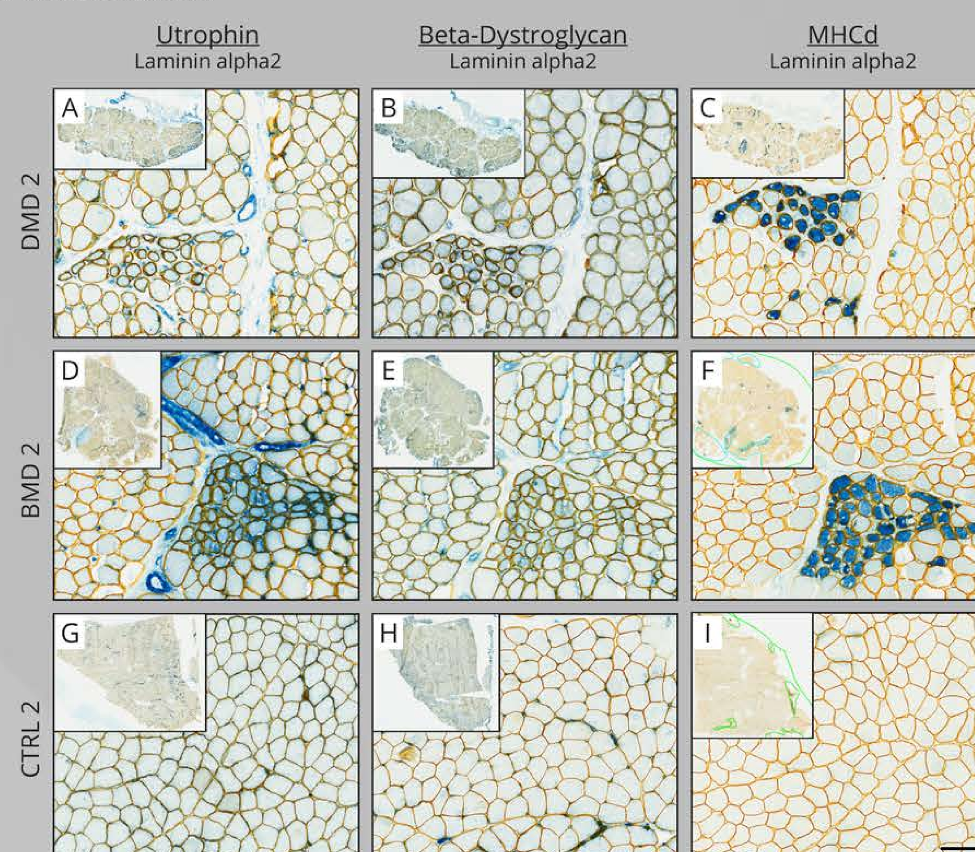


Figure 2. Digital Tissue Image Analysis of Whole-Slide Images.
Whole-slide images were annotated prior to analysis to remove artifacts (tissue folds, tears, etc.). Panel A shows an annotated, fit image of a section from BMD 2 stained with the laminin alpha2 and MHCd duplex assay. The annotated image is analysed by MuscleMap™ to identify and classify myofibers according to their biomarker expression (B). This allows for precise biomarker quantification in thousands of myofibers. Staining intensity thresholds were then determined by a board-certified, DVM pathologist to classify myofibers by biomarker expression on a 0-3+ scale. Myofibers were artificially colored according to their classification (0, blue; 1+, yellow; 2+, orange; 3+, red). The algorithms are undergoing final review which may result in threshold modifications in subsequent studies. Zoomed images C and D are at 10x magnification; scale bar represents 100 µm.

Figure 3. tIA™ of Duplex, IHC Assays Distinguishes Biomarkers within the same Cellular Compartment
Images of sections from DMD 2 stained with the duplex, IHC assays were digitally processed to separate the yellow and blue signals (A-C, and E-G). MuscleMap™ algorithms were developed to identify myofibers through detection of laminin alpha2 signal from the yellow chromogen. The location of the yellow signal defines the region for quantification of the blue signal as either the membrane, for utrophin and beta-dystroglycan, or the cytoplasmic boundary, for MHCd quantification (see Fig. 2 for example). Staining intensity thresholds were then determined by a board-certified, DVM pathologist to classify myofibers by biomarker expression on a 0-3+ scale. Myofibers were artificially colored according to their classification (0, blue; 1+, yellow; 2+, orange; 3+, red). The algorithms are undergoing final review which may result in threshold modifications in subsequent studies. Zoomed images are at 10x magnification; scale bar represents 100 µm.

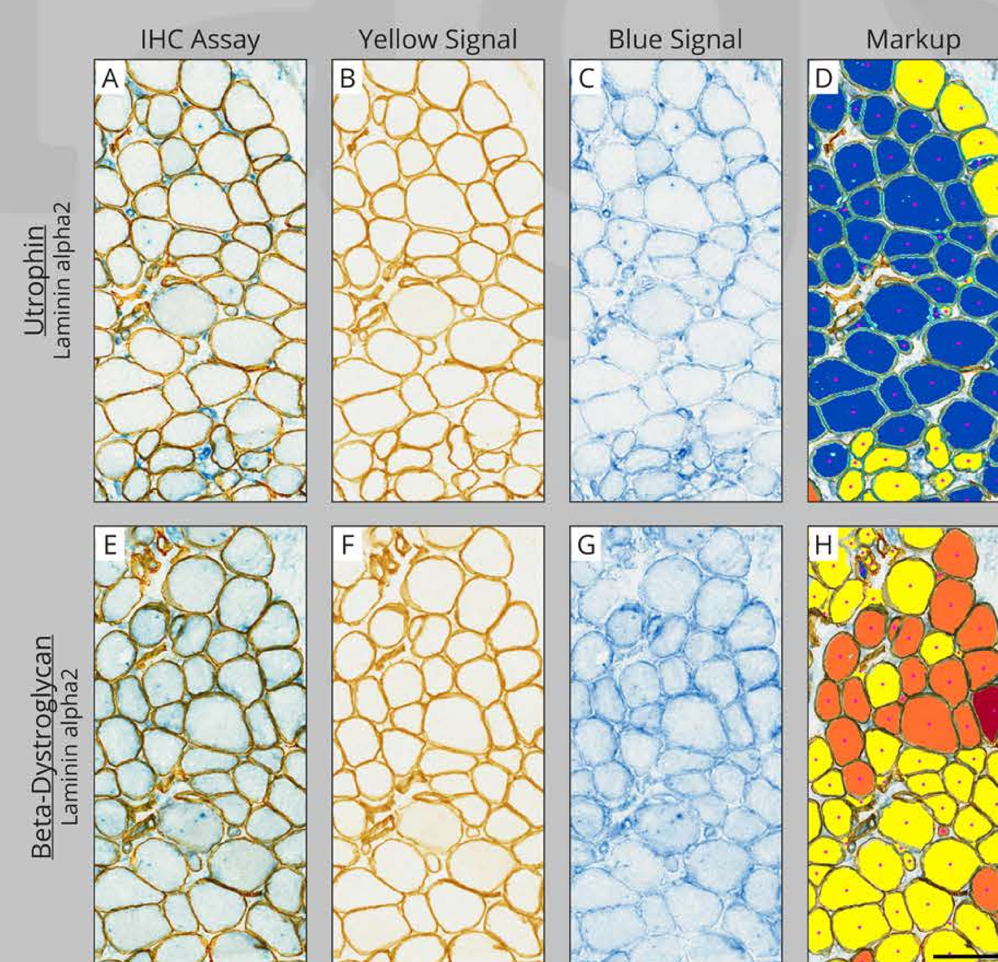
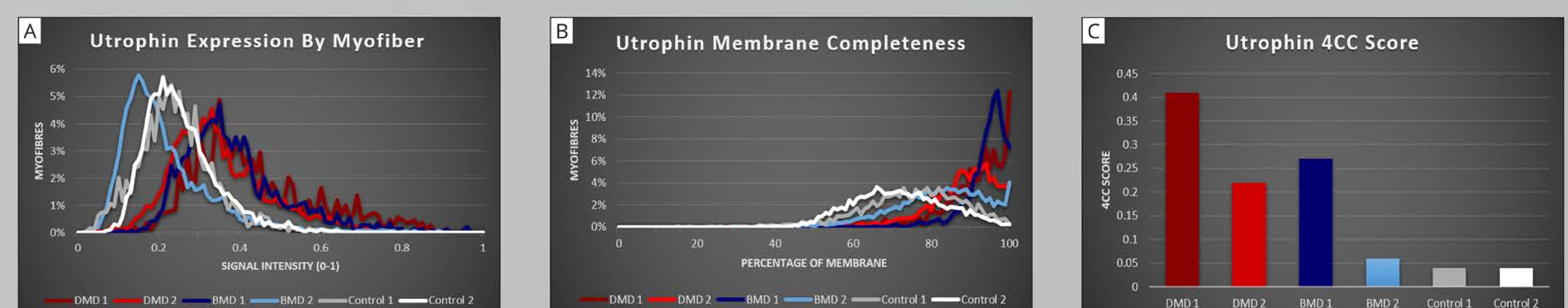


Figure 4. MuscleMap™ Quantifies Differential Expression of Utrophin in DMD, BMD, and Control Skeletal Muscle
The MuscleMap™ algorithms collected biomarker expression data from each analysed image. Signal intensity histograms (A) showed that the dynamic range of biomarker signal fell within the detectable limits for each disease state; signal was neither saturated nor too faint to quantify differences from the control samples. With utrophin (A) we observed a right shift in signal intensity from CTRL to DMD as more myofibers expressed higher levels of this biomarker. Average signal intensity also grossly showed this trend (Mean utrophin signal intensity for DMD 1, 0.43; DMD 2, 0.35; CTRL 1, 0.24; CTRL 2, 0.25). MHCd samples had a subtle right shift with increased disease severity and beta-dystroglycan showed no difference in signal intensity between samples (Data not shown). For utrophin, both signal intensity and the percentage of membrane expressing the biomarker (membrane completeness) varied both within sample and between sample types (B). To achieve a more comprehensive understanding of such complex expression patterns, Flagship has developed the 4CC analysis method. The 4CC method accounts for both membrane completeness and signal intensity for each myofiber to derive a summary score for individual images. This tool can clearly differentiate samples by taking into account multiple aspects of biomarker expression, as demonstrated for utrophin in C.



Conclusions

- Here, we have shown three duplex, IHC assays and tIA™ solutions that enable quantification of utrophin, beta-dystroglycan, and MHCd in whole-slide images to robustly assess muscle health in DMD, BMD, and control biopsies.
- We have demonstrated that MuscleMap™ can measure biomarker expression and muscle fiber morphometrics in all muscle fibers in a biopsy in a quantitative fashion. This tool can aid pathologists in evaluating the disease status of skeletal muscle biopsies and the efficacy of novel neuromuscular therapeutics.

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