Assessment of Mean Fluorescence Signal Intensity of Muscle Fibers Expressing Dystrophin

Louise Rodino-Klapac, PhD
Asst. Professor of Pediatrics
The Ohio State University
Center for Gene Therapy
Principal Investigator Neuromuscular Disorders
The Research Institute at Nationwide Children's Hospital
Columbus, Ohio, USA

Rationale

- To quantify dystrophin levels associated with sarcolemma
  - Indirect immunofluorescence – digital images
  - Primary antibodies MANDYS106 or DYS2.

- Simple, straightforward high throughput approach
  - Single antibody
  - Screen large tissue sample size
  - Output is average percent dystrophin across all fibers analyzed and not individual fibers.

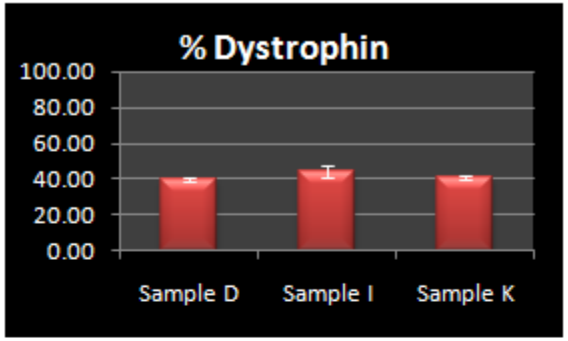
Reproducibility Study

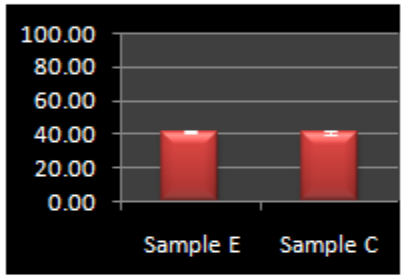
- *Goal – Determine how variations in sectioning, staining and image analysis impact dystrophin quantification*
  - BMD – same case for all experiments
  - Age-matched Normal control (NC)
  - No Primary Ab control for NC and BMD for background subtraction
  - All Images taken at the same time
  - Bioquant analysis done at the same time
  - Blinded Study

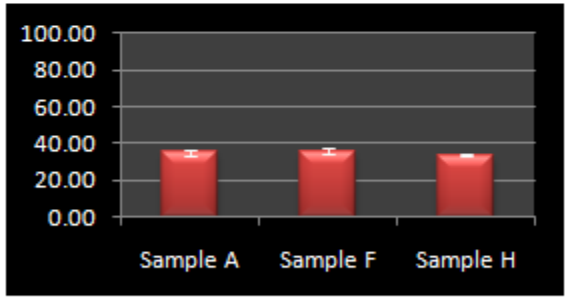
Experimental Design

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Mean Dys	SD
		Sectioned			Stained					
Experiment 1										
	Sample D								39.72	1.24
	Sample I								43.97	3.42
	Sample K								40.80	1.28
Experiment 2										
	Sample E								41.36	0.85
	Sample C								40.46	1.16
Experiment 3										
	Sample A								35.26	1.32
	Sample F								35.92	1.32
	Sample H								33.94	1.08
Experiment 4										
	Sample B								49.85	3.19
	Sample J								45.60	3.22
	Sample G								47.91	2.19

Overall Mean 41.34 ±5.2







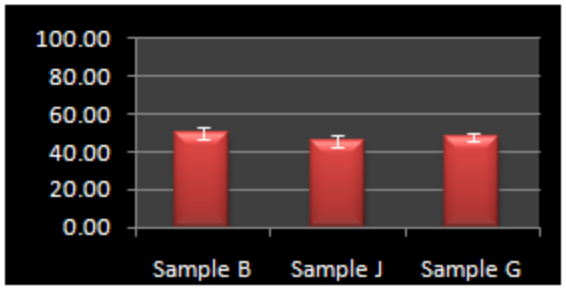
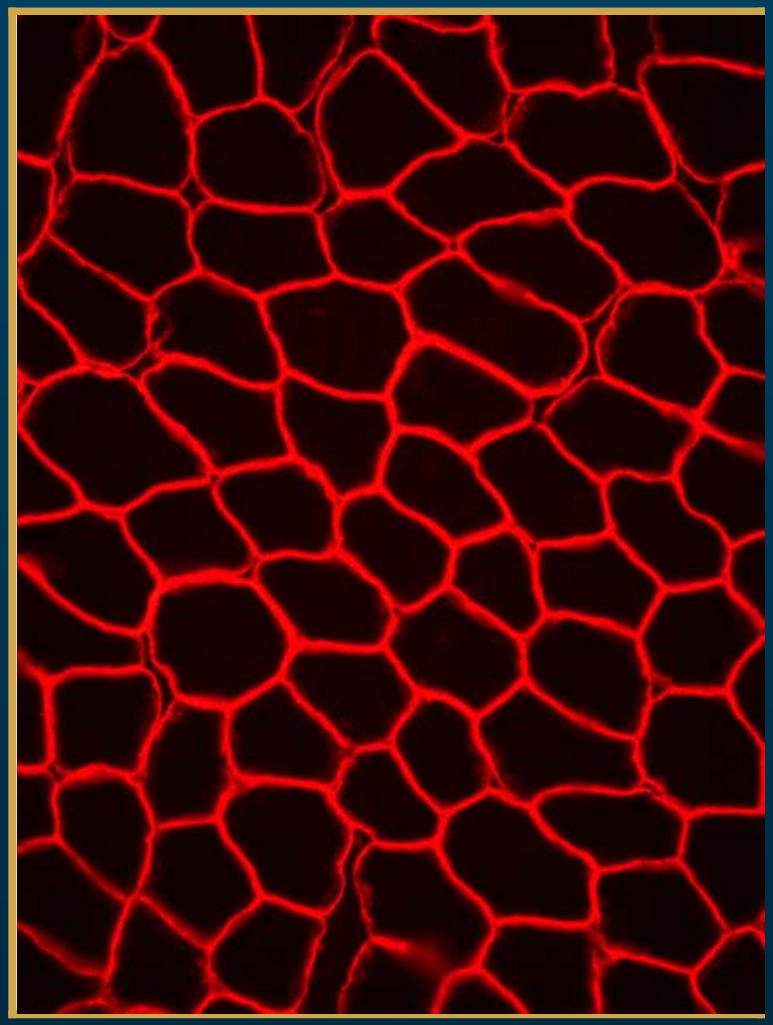


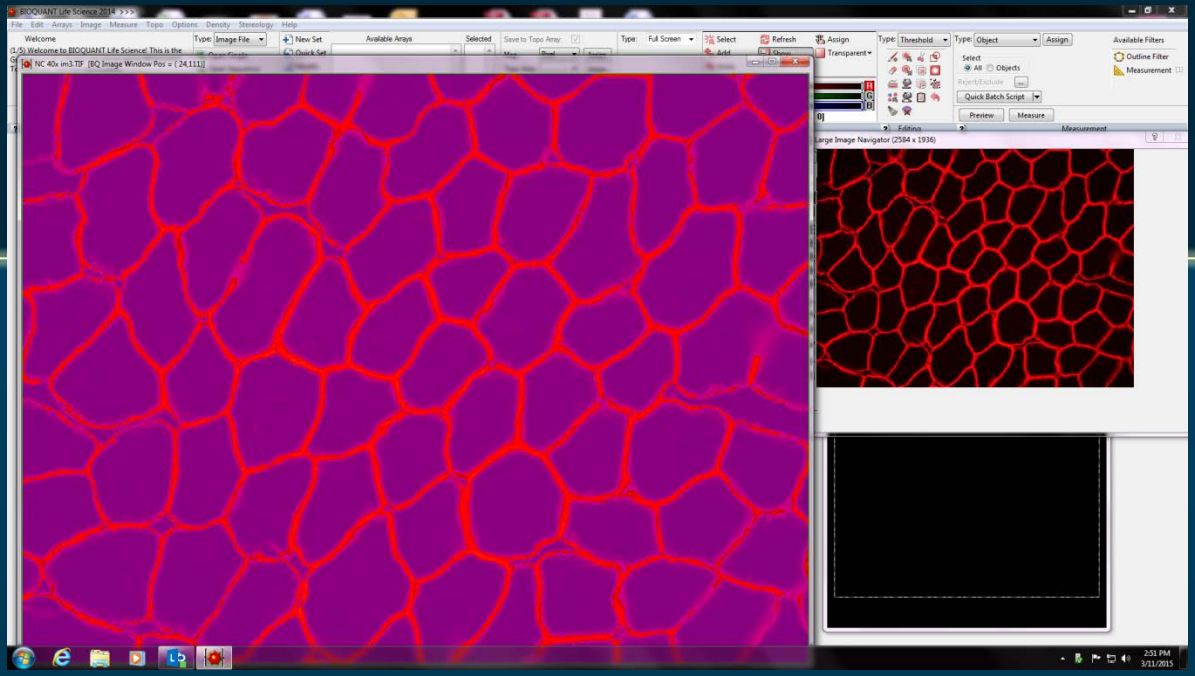
Image Capture Procedure

- First, the positive controls stained in the same batch as the patients are photographed using the auto exposure feature in AxioVision Rel 4.8 software
  - These exposure conditions are fixed, used to photograph all negative controls and patient slides stained in the same batch
- 4 randomly selected areas (one random field from each quadrant of sections) are photographed at 40x (edge areas of the sections and areas of sectioning/handling artifacts were avoided)
- Images are saved as RGB.tif files

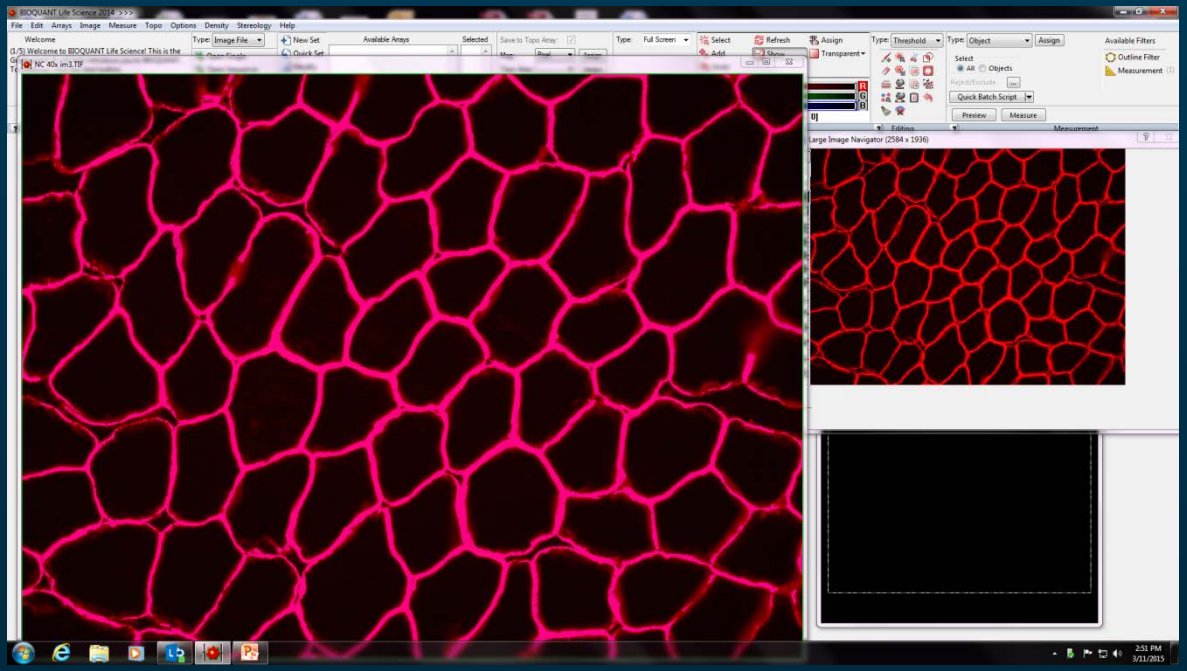


Bioquant Method – Bioquant® Life Science

- The minimum and maximum pixel intensity Threshold is set manually by the operator to define the sarcolemmal membrane using the normal tissue control images.
- The same minimum and maximum pixel intensity settings are using for all subsequent test images.
  - % CV for normal controls is 6.7%
- There is no exclusion of revertant fibers in any samples.
- The software algorithm determines the fluorescent signal intensity within the defined Thresholded region and averages it across the entire image.



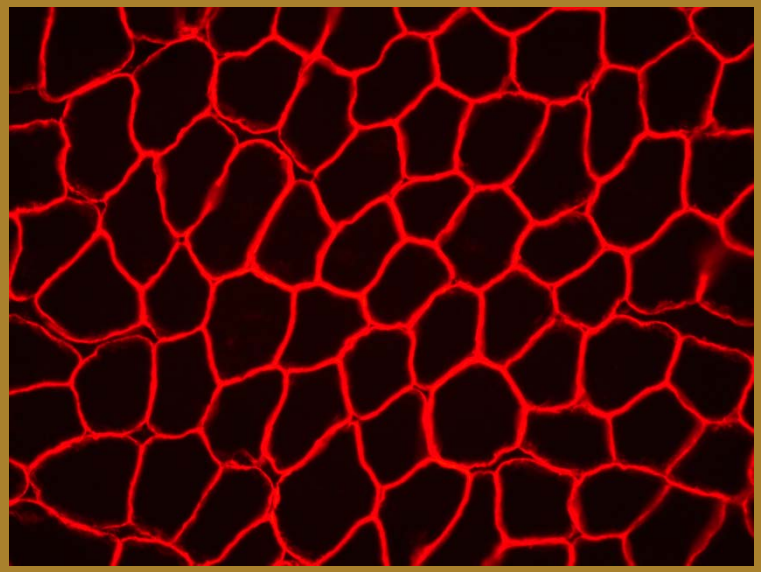
1. Define Threshold



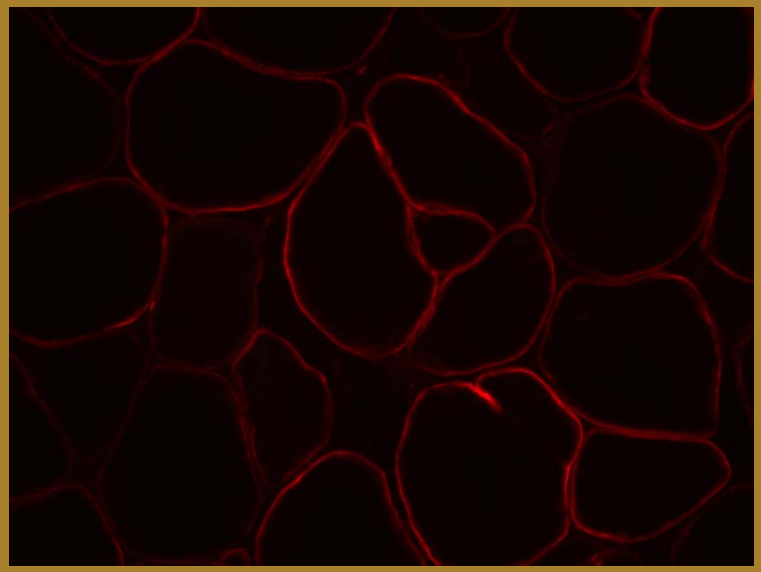
2. Invert Image
3. Use Measure function to measure average fluorescence intensity at the membrane.
4. Value is in Arbitrary Intensity Units
5. Apply threshold parameters to all images

Bioquant Procedure

Threshold set using Normal Control



Same Threshold used for all samples



- 4 - 40 X images /block
- 2 blocks = 8 images/time point per patient
- 8 x 3 biopsies = 24 images per patient
- 24 x 12 patients = 288 images analyzed

(0.72 mm^2 area screened per biopsy)

Calculation of Percent Intensity

- Percent Intensity is calculated for each test sample relative to the normal tissue control field density value.
- Background signal is measured from negative control images and subtracted from test sample and normal control values in final calculation of Percent Intensity.
- Calculation of Percent Intensity relative to Normal:
  - Percent Intensity is calculated for each test sample as the background-subtracted average field density for each test image divided by the averaged normal signal value multiplied by 100 to achieve a percentage of normal value.
    - **% Dystrophin = $Avg^{Sample}/Avg^{Normal}$ x 100**

Conclusions

- Simple and straightforward methodology using standard protocols, designed to generate data by screening large amounts of tissue for a reliable assessment of a drug effect.

- Automeasure features of Zeiss Image acquisition and Bioquant software eliminates operator bias and accelerates the process of large data acquisition.

- Drawbacks/Improvements: standardizing threshold underestimates the percentage of dystrophin in some images. As it currently stands, maintaining threshold affords the most reproducible and unbiased data acquisition.

Acknowledgements

Nationwide Children's Hospital

- Sarah Lewis
- Kim Shontz
- Jerry R. Mendell
- Louise Rodino-Klapac
- Linda Lowes
- Lindsay Alfano
- Kandice Roush
- Loren Bird
- Christopher Walker
- Katie Campbell
- Xiomara Rosales
- Ana Maria Gomez
- Maria Rouhana
- Kevin Flanigan
- John R. Kean
- Marco Corridore

Collaborators

- Steve Wilton

Sponsor - Sarepta Therapeutics

- Ed Kaye
- Pete Sazani
- Ryszard Kole