

DMD_M.1.2.001

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Quantitative determination of muscle fiber diameter (minimal Feret's diameter) and percentage of centralized nuclei

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Author	Judith Dubach-Powell Santhera Pharmaceuticals (Switzerland) Ltd Hammerstrasse 47, 4410 Liestal; now at Novartis, Basel, Switzerland
Working group members	Michael Erb (Santhera Pharmaceuticals (Switzerland) Ltd, Liestal, Switzerland; now at CSL Behring AG, Bern, Switzerland) Maaïke van Putten (Leiden University Medical Center, Department of Human Genetics, Leiden, The Netherlands) Christian Thirion (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany; now at Sirion Biotech GmbH, Munich, Germany) Elisabeth Barton (Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, USA) Miranda Grounds (School of Anatomy and Human Biology, The University of Western Australia, Crawley, Australia) Markus A. Rüegg (Biozentrum, University of Basel, Basel, Switzerland)
SOP responsible	Michael Erb
Official reviewer	Markus A. Rüegg

TABLE OF CONTENTS

OBJECTIVE..... 3

SCOPE AND APPLICABILITY 3

CAUTIONS..... 3

 Advantages..... 3

 Disadvantages 3

MATERIALS 4

METHODS 4

 Embedding of muscle..... 4

 Cryosectioning 4

 Staining..... 5

 Automated digital morphometry..... 5

 Background 5

 Procedure for digital image acquisition 6

 Determination of muscle fiber diameter (e.g, minimal “Feret’s diameter”)..... 7

 Centralized Nuclei analysis 10

EVALUATION AND INTERPRETATION OF RESULTS 11

 Muscle fiber diameter variability..... 11

 Centralized myonuclei 12

REFERENCES 12

APPENDIX 12

 Fiber diameter..... 12

 Percentage of centralized nuclei 13

1 OBJECTIVE

This document describes a method and provides reference values for the quantitative and reliable measurement of pathology-relevant histological parameter of dystrophic muscle in the *mdx* mouse.

2 SCOPE AND APPLICABILITY

The pathologic processes in dystrophic muscles include marked degeneration and regeneration of muscle fibers. These processes can be quantified by the measurement of the diameter of the muscle fibers and by the determination of the fraction of muscle fibers with centralized nuclei (indicative of muscle regeneration).

The method described relies on the determination of the muscle fiber size exemplified by using the minimal 'Feret's diameter' of a muscle fiber cross-section. Unlike other morphometric parameters of muscle fiber size, the minimal 'Feret's diameter' is very robust against experimental errors such as the orientation of the sectioning angle. Moreover, the minimal 'Feret's diameter' reliably discriminates between dystrophic and normal phenotypes in a representative set of muscles. Alternative parameters are suggested in case the assessment of the minimal 'Feret's diameter' is not possible. In addition, the percentage of centralized nuclei is determined as a measure indicative of regeneration in dystrophic muscle.

Once the digital images of entire muscles are available, additional measurement parameters can easily be implemented (e.g. total muscle cross-sectional area). In combination with other staining procedures, additional pathological parameters might be assessed with minor modification of the system (e.g. necrotic area, macrophage infiltration, etc.).

3 CAUTIONS

3.1 Advantages

If a motorized, computer-controlled microscope is used, a digital imaging of an entire muscle is possible in a fully automated fashion. The automated image analysis system allows for identification of cell boundaries and the analysis of all fibers of a muscle cross section (typically 2,000-5,000 muscle fibers) in a reasonable amount of time (0.5-1h) and avoids the need of the manual "circling" of muscle fibers. The analysis of the entire muscle guarantees for unbiased results. All types of "minimal fiber diameter" measures provide comparable results and are very robust against experimental errors such as the orientation of the sectioning angle. The use of the minimal 'Feret's diameter' is recommended.

3.2 Disadvantages

The automated image acquisition and analysis requires a fully automated digital fluorescence microscope and appropriate image analysis software, enabling all steps of image acquisition and analysis (e.g. the computer program CellP is a reasonable choice because of automation, ease of use, flexibility and price). If a manual microscope is used analysis of fiber

DMD_M.1.2.001

size and the percentage of centrally nucleated fibers can be determined with Mayachitra Imago. Comparable results can be achieved in this way.

4 MATERIALS

Superfrost plus Slides : Millian (SFPlus-42)

Tissue-Tek O.C.T: Sakura (Cat. No. 4583)

Para-formaldehyde: Fluka (Cat. No. 76240)

Triton X 100: Sigma (Cat. No. T8787)

Alexa Fluor 488 conjugate wheat germ agglutinin (WGA): Molecular Probes (Cat. No. W-11261; use 1mg/mL in PBS as 1000 x stock solution)

DAPI: Sigma (Cat. No. D-9542; use 1mg/mL in PBS as 1000 x stock solution)

FluorSaveReagent™: Calbiochem-Novabiochem, San Diego, CA, USA

Gum Tragacanth: Sigma-Aldrich (Cat. No. G1128)

CellP software (Olympus) or Mayachitra Imago

Stock solutions:

10% gum tragacanth: a homogenous paste of 10% gum Tragacanth is prepared by mixing it with water, freeze-thawing and re-mixing. Batches prepared in advance can be stored at -20°C.

PBS (e.g. 7.2g Na₂HPO₄, 1.2g KH₂PO₄, 40g NaCl and 1g KCl; dissolved in 1l H₂O)

4% PFA in PBS (prepared in batches by overnight stirring and stored in 50ml aliquots at -20°C).

5 METHODS

5.1 Embedding of muscle

After removal, the muscles (e.g. Diaphragm, Quadriceps, TA, EDL, Gastrocnemius, etc) are mounted on a small mound of 10% Gum Tragacanth that is placed on a cork disc. Ensure that the muscles are totally covered by the gum and that they are placed with the distal end of the muscle facing down and the proximal side up.

Place a cold resistant beaker of isopentane into liquid nitrogen and allow cooling to -150°C. When the correct temperature is attained 'sludge' will appear in the bottom of the isopentane. Freeze the embedded muscle by placing it into the cooled isopentane for 20 seconds (longer contact times can result in the formation of cracks in the samples; insufficient time can result in freezing artifacts). Place the muscle sample in the -20°C or in the -80°C freezer for short or long term storage, respectively.

5.2 Cryosectioning

To achieve a thermal equilibration before cryosectioning, store the samples overnight in the -20°C freezer and place them into the cryostat for at least 20 minutes before further processing. Mount the sample on the round metallic mount of the cryostat with Tissue-Tek O.C.T. The knife should be pre-cooled to -20°C and the muscle sample to -24°C. Make 12 µm-thick section and collect them on warm (RT) slides (Superfrost plus). Store unstained slides at -20°C.

DMD_M.1.2.001

5.3 Staining

The following staining procedure is used to stain membrane-bound and extracellular epitopes as well as nuclei:

- Bring the slides to room temperature
- Incubate the slides with 4% para-formaldehyde dissolved in PBS (pH 7.2) for 5 minutes (fixation)
- Place the slides in 0.1% Triton-X100 in PBS for 5 minutes (permeabilization)
- Wash with PBS 3 times for 5 minutes each (e.g. in a staining beaker)
- Incubate in the fluorescent dye* overnight at 4°C (e.g. in a staining beaker)
- Wash 3 times for 10 minutes each with PBS in the dark (e.g. in a staining beaker)
- Allow the slides to dry for 5 minutes at room temperature
- Add 2 drops of FluorSaveReagent™ to the slides and mount the cover slip. Avoid the formation of air bubbles.
- Place at 4°C for 30 minutes before viewing.

* While in principle any membrane- or extracellular-matrix-specific immunofluorescence staining might be suitable for the staining of cell boundaries, the staining with fluorescence-labeled WGA is easy, robust and reliable.

5.4 Automated digital morphometry

5.4.1 Background

The minimal “Feret’s diameter” (“a” in the figure overleaf) is defined as closest possible distance between the two parallel tangents of an object (i.e. muscle fiber). The determination of the minimal “Feret’s diameter” and the determination of the number of “holes” within individual measurement objects are two of the many selectable built-in morphometric measurement parameters in professional image analysis programs.

In case the minimal “Feret’s diameter” cannot be used (i.e. not available as image analysis tool) other “minimal” diameter algorithms (e.g. minimal inner diameter, minimal out diameter) are recommended.

DMD_M.1.2.001

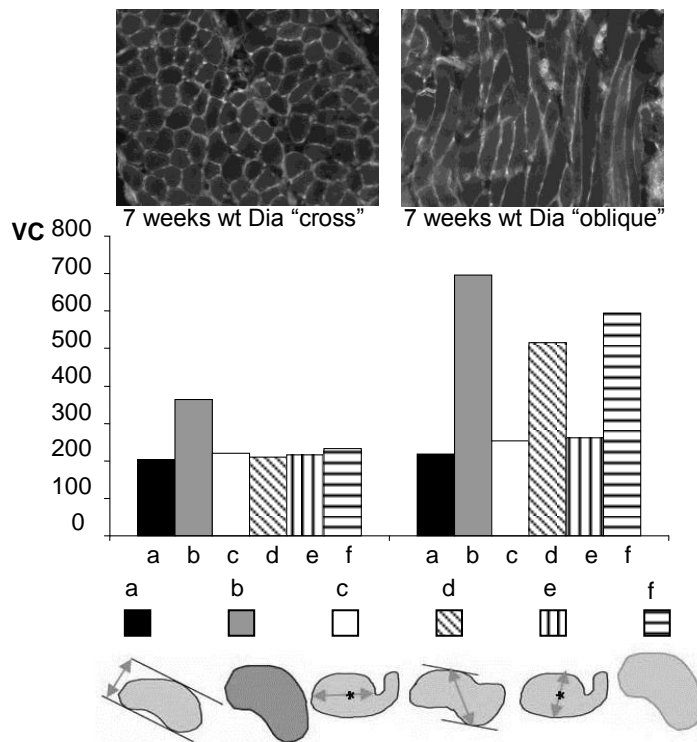


Figure 1: Determination of the sensitivity of each geometrical parameter to changes in the angle of sectioning. The size of the muscle fibers was determined on a cross-section (left) and a slightly oblique section (right) from the same diaphragm (Dia) muscle of a 7 week old wild-type mouse using six different geometrical parameters (a: minimal "Feret's diameter"; b: "area"; c: "minimal inner diameter"; d: "minimal diameter"; e: "minimal outer diameter"; f: "perimeter"). The variance coefficients of the fiber size in the cross-section and the oblique section were calculated using each of the six geometrical parameters and plotted in a bar histogram. Note that the variance

coefficient (VC) measured using the minimal "Feret's diameter" (black bars) least influenced by the sectioning angle. The "minimal inner diameter" and "minimal outer diameter" are also insensitive to the plane of sectioning and are recommended as substitute parameters for the "Feret's diameter"

The variance coefficient (VC) of all muscle fiber minimal diameters of a given muscle cross-section provides for a numerical expression of fiber size variability. Together with the percentage of fibers with centralized nuclei, this information is a reliable and robust histological assessment of the pathology associated with dystrophin-deficiency. Moreover, by measuring several hundred fibers per muscle sample (e.g. up to 3,000 fibers per diaphragm and up to 2000 per tibialis anterior muscle) this method allows for the detection of even subtle differences between muscle samples of differently treated mice.

5.4.2 Procedure for digital image acquisition

A digital image of the muscle section is acquired, ideally in an automated manner, covering the entire muscle. This digital image is then analyzed to deliver the pathology-relevant morphometric parameters: 1) fiber diameter and 2) percentage of centralized nuclei.

DMD_M.1.2.001

Work step	Degree of automation*	Comment
Image acquisition	Fully automated	Alternative: manual process
Multiple image alignment	Fully automated	Alternative: manual process
Border and particle detection	High degree of automation	Automated border detection, needs visual control and minor revision
Fiber diameter determination	Fully automated	Dependent on software used
Centralized nuclei detection	Fully automated	Dependent on software used
Image and data archiving	Fully automated	Dependent on software used

* using CellP Software

Image acquisition is most efficiently done using a motorized microscope (e.g. Olympus IX series), equipped with a digital camera and a XYZ object table and under the control of an appropriate program allowing automated image acquisition, image analysis and storage (e.g. CellP). If such a configuration is used, a fully automated documentation of an entire muscle is possible. Fluorescence microscopic images of both the WGA and the DAPI labels are acquired using a digital camera coupled to a fluorescence microscope and overlaid to a composite image (Image 1). When there is no access to a motorized microscope, images slightly overlapping each other can be acquired manually with a conventional microscope.

5.4.2.1 Determination of muscle fiber diameter (e.g., minimal “Feret’s diameter”)

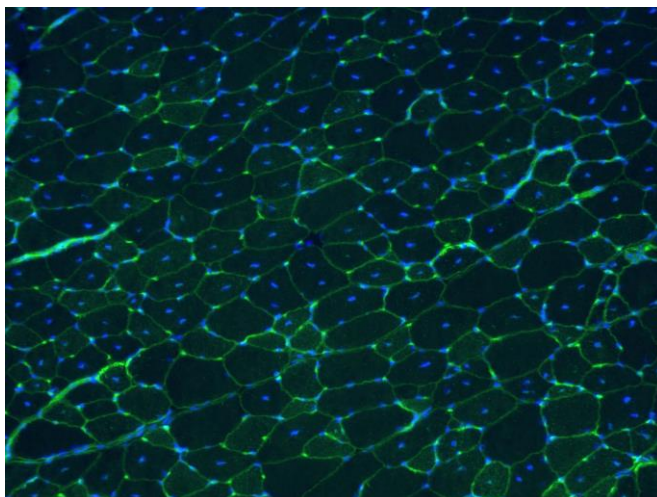


Image 1: Fluorescence staining of a dystrophic muscle using fluorescein-labelled wheat germ agglutinin (extracellular matrix and membrane outer layer) and DAPI staining (nuclei). Note that the frame shows only a small part of a muscle.

DMD_M.1.2.001

5.4.2.1.1 *Multiple image alignment*

If overlapping images are acquired, they can be assembled to one picture covering the entire muscle, allowing the analysis of a few thousand muscle fibers (not shown). If using the CellP program, image acquisition and multiple image alignment can be performed in a fully automated manner very efficiently. When images are captured manually, those can be aligned with Mayachitra Imago, archived and directly analyzed.

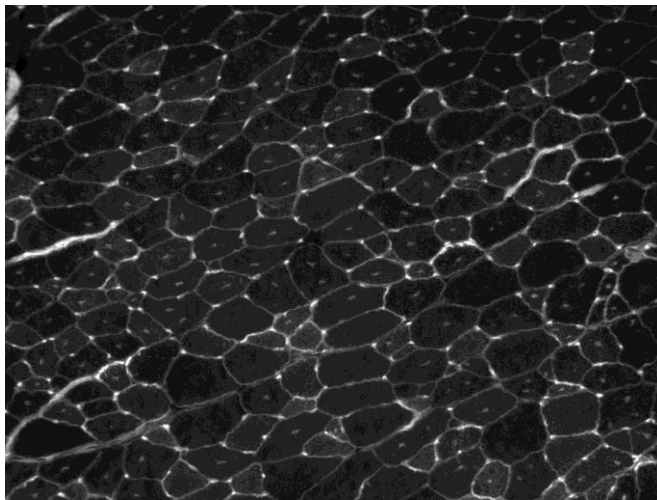


Image 2: The green channel (WGA staining) separated from Image 1. Note that WGA also stains the nuclei

5.4.2.1.2 *Border detection and fiber Feret's diameter determination*

- Separate the green channel of Image 1 using the appropriate command (Image 2).
- Identify cell boundaries on Image 2 using an appropriate algorithm or filter generating an image where the boundaries are represented by lines (Image 3). Usually, attempts to identify cell boundaries on the basis of gray value thresholds deliver insufficient results, particularly if diseased muscle samples are analyzed. Therefore the use of more sophisticated boundary detection algorithms is necessary if a manual identification of boundaries should be avoided. If using the CellP program, perform the OPER/Define filter/Separator... menu to detect cell boundaries: control the boundaries, delete false ones if any, and draw missing ones if necessary.

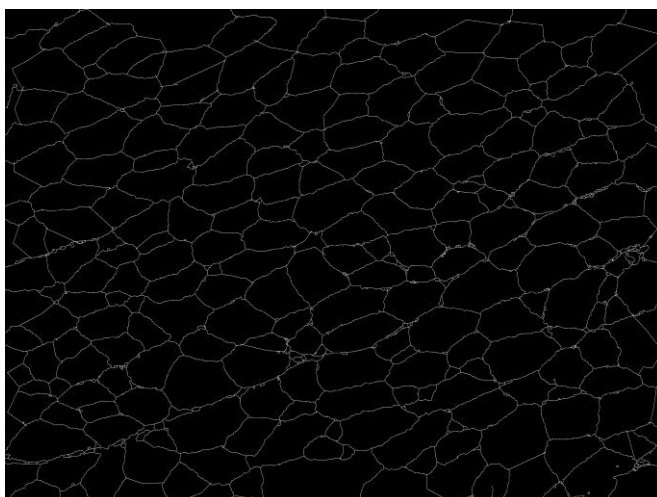


Image 3: Cell boundaries identified by the internal "Separator" filter of CellP. Note that objects (cells) touching the image border are omitted

- Apply Image 3 as a mask to Image 1. This manipulation enables the faultless detection of muscle fibers by setting the

DMD_M.1.2.001

thresholds to exclude the white lines. A broadening of the threshold to exclude areas with strong WGA staining enables the omission of fibrotic areas. With the above manipulation, the cell boundaries and the fibrotic areas are combined to a unified separating criterion between neighboring muscle fibers. Alternatively, instead of the original image 1 (color), the color-separated image of the WGA staining (Image 2) can also be combined with the boundary lines and processed analogously.

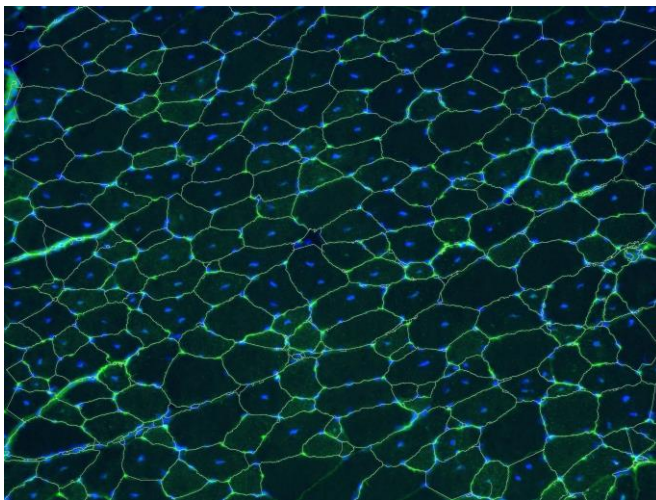


Image 4: Combined image of the boundary drawing and the original fluorescence image

- Perform particle determination: control the particles found and eliminate obvious erroneous particles if any (Image 5).

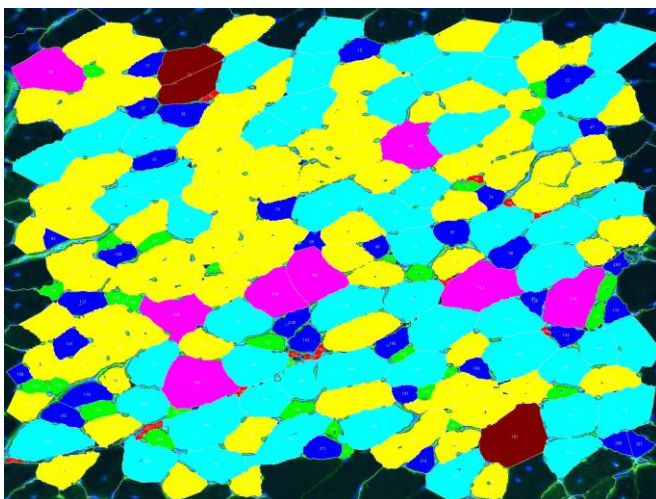


Image 5: identified particles (with optional diameter dependant color coding)

- After particle identification the software offers various algorithms for calculation including minimal diameter of fiber area.
- Save the image along with the corresponding result sheets. When using Mayachitra Imago, each fiber is segmented based on an interactive segmentation algorithm that segregated the foreground (cytoplasm) from the background (membranes), given the information provided

DMD_M.1.2.001

by the user in terms of mouse-drawn scribbles. The number of fibers and area (μm^2) are then computed automatically. The integration of the Feret's diameter is still under progression.

5.4.2.2 Centralized Nuclei analysis

- Expand the cellular boundaries from Image 3 to an extent that is sufficient to mask the peripheral nuclei (Image 6).

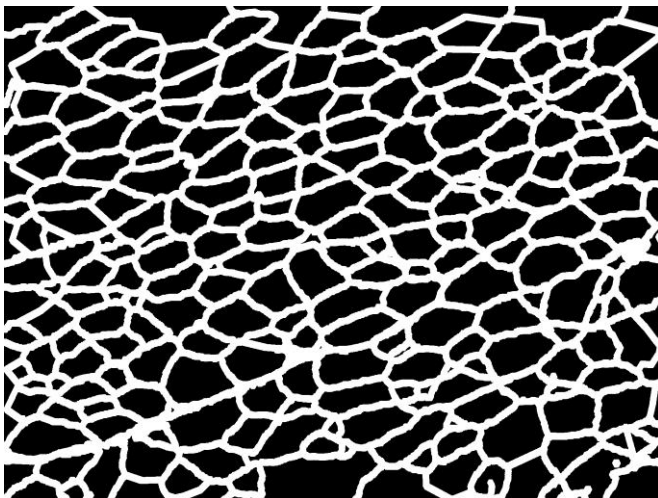


Image 6: The muscle fiber borders from Image 3 have been expanded by several dots, providing a mask covering the peripheral nuclei.

- Mask peripheral nuclei by superimposing Image 6 (as a mask) on Image 1 resulting in Image 7. Instead of the original Image 1 (color), the separated blue channel of Image 1 (black and white; corresponding to the nuclei staining with DAPI) could also be combined with Image 6.

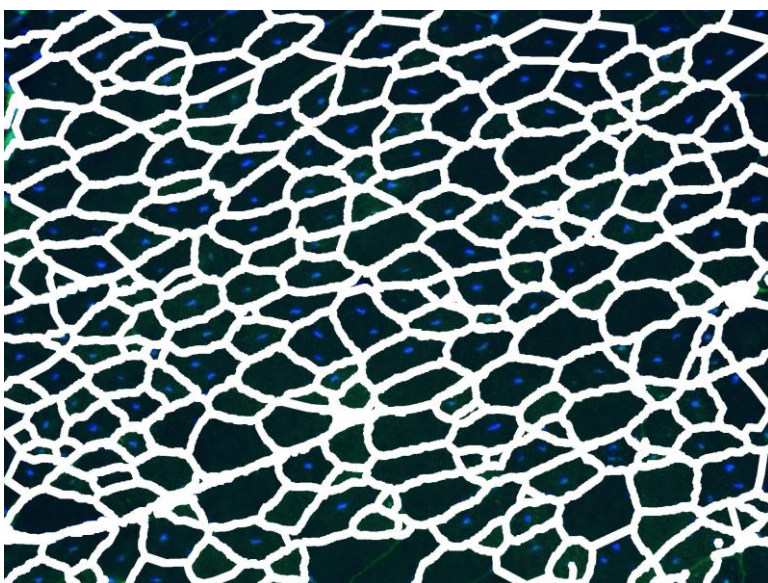


Image 7: Image of a muscle with the cell boundaries and the peripheral nuclei masked. Centralized nuclei are visible with DAPI stain.

and nuclei). Perform particle determination, and count the number of “holes” within each particle. The number of “holes” corresponds to the number of centralized nuclei within a given muscle fiber.

- Define an upper threshold that allows the determination of the inner part of the fibers and the exclusion of all elements with a certain signal intensity (borders and nuclei).

DMD_M.1.2.001

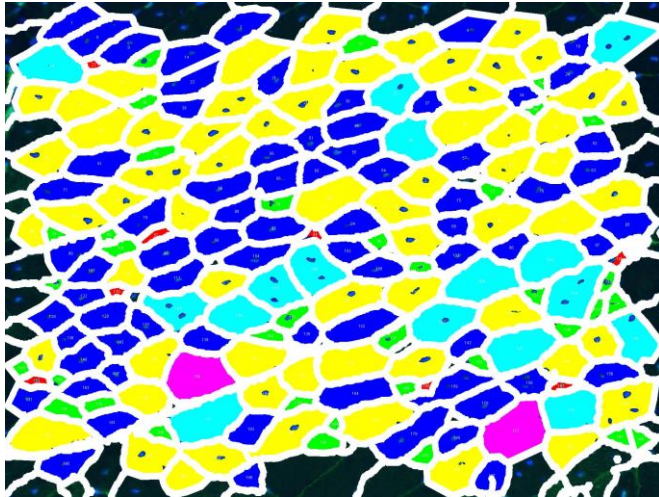


Image 8: Final Image of a muscle with the centralized nuclei indicated as “holes” within the particles (with optional diameter dependant color coding).

Save the image along with the corresponding result sheets. Typically the results show a list of all objects (=muscle fibers) identified, along with the number of “holes” (=centralized nuclei) in each fiber.

Using separate software (such as Excel) this list can be used to calculate the percentage of fibers with centralized nuclei whereby each fiber with 1 or more holes should be considered as a centrally nucleated fiber. Using Mayachitra Imago, the number of centrally nucleated fibers is assessed based on the average intensity of each fiber computed from the nuclear channel. In healthy fibers this is close to zero and higher in centrally nucleated fibers due to the DAPI-stained central nuclei. Fibers exceeding a threshold are considered centrally nucleated.

6 EVALUATION AND INTERPRETATION OF RESULTS

6.1 Muscle fiber diameter variability

Dystrophic muscles typically show a higher variability of the muscle fiber diameter. Therefore, the variance coefficient (VC) of the muscle fiber minimal diameter is higher in *mdx* muscle in comparison to wild-type muscle. Typically, VC in *mdx* muscle is approximately twice as high as in wild-type animals. VC of the muscle fiber minimal diameters is defined as:

variance coefficient $Z = 1000 \times \text{standard deviation of muscle fiber minimal diameters} / \text{mean muscle fiber minimal diameter}$.

A change in VC can be expressed as the percentage in change of the variance coefficient towards the wild-type value:

$$100 \times (Z_{(mdx \text{ controls})} - Z_{(mdx \text{ test conditions})}) / (Z_{(mdx \text{ controls})} - Z_{(wild \text{ type})})$$

6.2 Centralized myonuclei

Muscle fibers of ‘healthy’ muscles rarely contain centralized nuclei. Therefore, the percentage of fibers with centralized nuclei should be below 3% in 3 week-old wild-type or *mdx* mice as well as in 7 week-old wild-type mice. However, by 7 weeks of age, *mdx* mice contain up to 66% of fibers with centralized nuclei (Briguet et al., 2004). The number of fibers with centralized nuclei is counted and is expressed as percentage of all fibers (CN in %).

A change in the relative number of centralized nuclei and can be expressed as follows:

$$100 \times (\text{CN}_{(\text{mdx controls})} - \text{CN}_{(\text{mdx test conditions})}) / (\text{CN}_{(\text{mdx controls})} - \text{CN}_{(\text{wild type})})$$

7 REFERENCES

Briguet et al., (2004) Histological parameters for the quantitative assessment of muscular dystrophy in the *mdx* mouse. *Neuromuscular Disorders* 14: 675–682.

8 APPENDIX

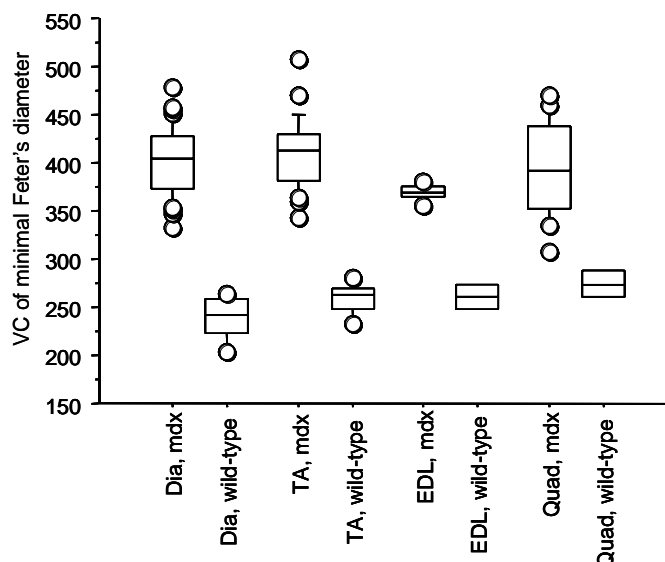
8.1 Fiber diameter

Historical data is available for both the minimal ‘Feret’s diameter’ and centralized nuclei parameters from wild type and *mdx* mice at 7 weeks of age for a set of different muscles.

Fig. 1: Analysis of minimal Feret diameter:

Variability of the variance coefficient (VC) measurements determined with the minimal ‘Feret’s diameter’ method of 7 week-old *mdx* mice

(y-axis: variance coefficient of the minimal ‘Feret’s diameter’ = 1000 x standard deviation of the minimal ‘Feret’s diameter’ / mean minimal ‘Feret’s diameter’). Box blot settings: 10th, 25th, 50th (median), 75th and 90th percentiles; values above 90th and below 10th percentiles are shown as points.



DMD_M.1.2.001

Table 1: VC of minimal Feret diameters obtained in 7 week-old *mdx* mice.

Comparison of Fiber Feret Analysis of different muscles from wild type (WT) and *mdx* mice (same data as in Fig. 1).

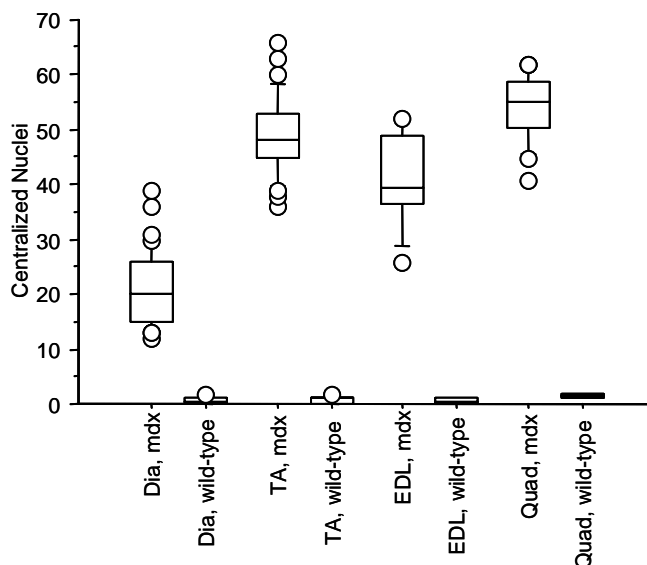
	7w <i>mdx</i> Dia	7w WT Dia	7w <i>mdx</i> TA	7w WT TA	7w <i>mdx</i> EDL	7w WT EDL	7w <i>mdx</i> Quad	7w WT Quad
Mean	402	237	409	259	369	260	393	274
Stdev	34	22	35	15	8	16	48	17
min- max	333 - 479	204 - 264	343 - 509	233 - 282	357 - 382	242 - 279	308 - 470	254 - 295
n	43	12	33	11	8	4	19	4

n indicates the number of mice analysed. For each measurement all fibers of an entire muscle have been analysed (typical muscle fiber numbers per muscle are: Diaphragm >2500; TA: >2000; EDL: >750 and Quadriceps: >3000). Dia = diaphragm; TA = tibialis anterior; Quad = quadriceps; EDL = extensor digitorum longus

8.2 Percentage of centralized nuclei

Fig. 2: Percentage of centralized nuclei

Percentage of muscle fibres with centralized nuclei (y-axes: percentage of muscle fibres with centralized nuclei). Box blot settings: 10th, 25th, 50th (median), 75th and 90th percentiles; values above 90th and below 10th percentiles are shown as points.



DMD_M.1.2.001

Table 2: Percentage of centralized nuclei

Comparison of centralized nuclei analysis of different muscles from wild type (WT) and *mdx* mice

	7w <i>mdx</i> Dia	7w WT Dia	7w <i>mdx</i> TA	7w WT TA	7w <i>mdx</i> EDL	7w WT EDL	7w <i>mdx</i> Quad	7w WT Quad
Mean	21	1	49	1	41	0	54	1
Stdev	7	0	7	0	9	0	6	1
min- max	12 - 39	0 - 2	36 - 66	0 - 2	26 - 52	0 - 1	41 - 62	1 - 2
n	43	12	33	11	8	4	19	4

Data from same muscle samples as Fig.1 and Table 1