MDC1A_M.1.2.002

Please quote the use of this SOP in your Methods.

# Quantitative determination of minimal Feret's diameter, including the evaluation of the percentage of centralized nuclei, fiber numbers, cross-sectional area and the percentage of fibrosis

| | |
|---|---|
| SOP (ID) Number | MDC1A_M.1.2.002 |
| Version | 1.0 |
| Issued | April 19th,2011 |
| Last reviewed | January 28th, 2014 |
| | |
| Authors | Markus A. Rüegg & Sarina Meinen |
| | Biozentrum, University of Basel, Basel, Switzerland |
| | |
| Working group members | Valerie Allamand (Inserm, U974, Institut de Myologie, Paris, France) |
| | Jeffrey B. Miller (Neuromuscular Biology & Disease Group, Boston University School of Medicine, USA) |
| | Madeleine Durbeej (Faculty of Medicine, Lund University, Lund, Sweden) |
| | Dean J. Burkin (Department of Pharmacology, University of Nevada, Reno, NV, USA) |
| | Anne M. Connolly (University of Washington School of Medicine, St Louis, MO, USA) |
| | Janice Dominov (University of Massachusetts Medical School, Worcester, MA, USA) |
| SOP responsibles | Markus A. Rüegg, Sarina Meinen |
| Official reviewer | Valerie Allamand |

**TABLE OF CONTENTS**

## 1    OBJECTIVE

This document describes a method for the quantitative and reliable measurement of some of the pathology-relevant histological parameters in the dystrophic muscle of the $dy^W/dy^W$ mouse.

## 2    SCOPE AND APPLICABILITY

Some of the hallmarks of muscles from MDC1A patients and mouse models are variation in muscle fiber diameters, a high proportion of small fibers due to cycles of de- and regeneration of muscle fibers as well as loss of muscle fibers. These parameters can be quantified by the measurement of the diameter of the muscle fibers (which at the same time counts the exact fiber numbers per muscle). In addition, lamininalpha2-deficient muscles show impaired regeneration capacity which may be improved by treatment approaches. This parameter can be assessed 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, fiber numbers per muscle). In combination with other staining procedures, additional pathological parameters might be assessed with minor modification of the system (e.g. percentage of fibrosis per cross-section, 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 2,000-6,000 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. Most 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 are reasonable choices because of automation, ease of use, flexibility and price; there is also the possibility to implement it on ImageJ). If a manual microscope is used analysis of fiber 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) or ThermoScientific Menzel Gläser (J1800AMNZ)
- Tissue-Tek O.C.T: Sakura (Cat. No. 4583)
- Paraformaldehyde: Fluka (Cat. No. 76240)
- Triton X 100: Sigma (Cat. No. T8787)
- Alexa Fluor 488 conjugated 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)
- Mounting Media: FluorSaveReagent ™ (Calbiochem-Novabiochem, San Diego, CA, USA), or other aqueous-based mounting media (Celvol, Mowiol,..)
- Cork plates (cut into quadrates of approx. 1.5cm x 1.5cm)
- Isopentane: Merck 1060561000 or Sigma-Aldrich 27,034-2
- PapPen Liquid Blocker (NANDAI Trading, Japan)
- Gum Tragacanth: Sigma-Aldrich (Cat. No. G1128)
- CellP software (Olympus) or Mayachitra Imago

**Stock solutions:**

10% gum tragacanth:        prepare 10% gum tragacanth in distilled water, mix thoroughly, incubate at 37°C for 30-60 minutes, mix thoroughly, freeze-thaw the mixture and mix

thoroughly again. If stored at +4°C add little amount (a pinch) of Thyme powder to prevent contaminations. For long-term storage -20°C is recommended.

PBS (e.g. 7.2g $Na_2HPO_4$, 1.2g $KH_2PO_4$, 40g NaCl and 1g KCl; dissolved in 1l $H_2O$)

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 dissection, the muscles (e.g. Diaphragm, Triceps, Quadriceps, TA, EDL, Gastrocnemius, etc) are mounted on a small mound of 10% Gum Tragacanth that is placed on a cork disc. For bigger muscles (Triceps, Quadriceps, TA) prepare a little hole in the gum tragacanth and stick the muscle into it. Smaller muscles (Soleus, EDL) are looped around the forceps, stuck into the Gum and then stretched by pulling out the forceps. 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. Alternatively, muscles can be embedded in Tissue-Tek O.C.T. freezing medium in cryomolds (e.g Tissue-Tek 4565 or 4566).  Use the minimum amount of O.C.T. possible to cover the muscles, thus allowing rapid freezing to occur.

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 20-40 seconds (longer contact times can result in the formation of cracks in the samples; insufficient time can result in freezing artifacts) and then transfer the muscle sample to dry ice. For short-term or long-term storage keep the samples in -20°C or -80°C freezer, 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 -22°C. Make 7-12 µm-thick sections and collect them on warm (RT) Superfrost Plus slides. Let the sections dry at RT for 1hr and then store the unstained slides at -20°C.

### 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 and surround the sections with a liquid blocker (PapPen).

- Fixation: Incubate the slides with 4% Paraformaldehyde dissolved in PBS (pH 7.2) for 15 minutes.

- Permeabilization: Place the slides in 0.1% Triton-X100 in PBS for 5 minutes.

- Wash with PBS 3 times for 5 minutes each (e.g. in a staining beaker)

- Staining: Incubate the sections with Alexa Fluor 488 conjugated WGA (1:500 to 1:1000 dilution in PBS)* overnight at 4°C. Keep in the dark.

- 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 ™ or other appropriate mounting media to the slides and mount the cover slip. Avoid the formation of air bubbles.

- Press the slides under heavy weight (to get rid of excess of mounting media) 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 outer diameter) are recommended.
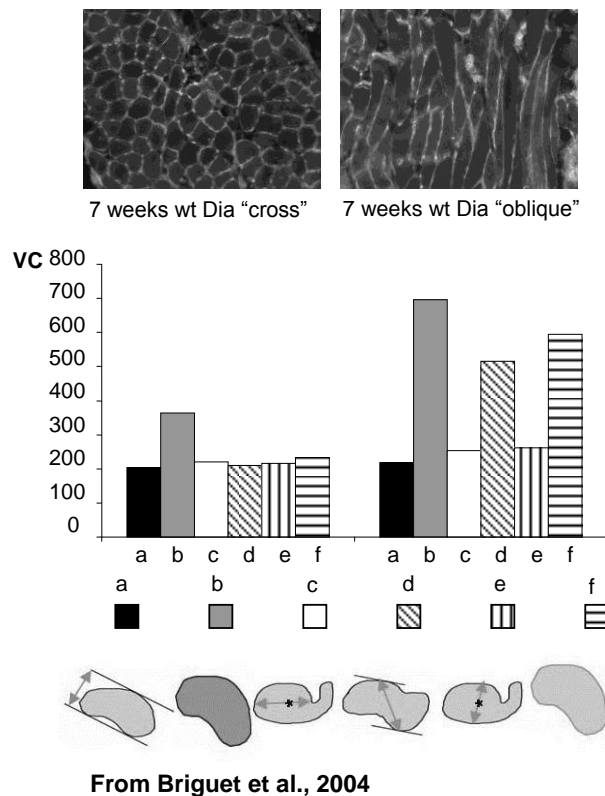
*Figure 1: Determination of the sensitivity of each geometrical parameter to changes in the angle of sectioning. The size of the muscle fibers was determined on a cross-section (left) and a slightly oblique section (right) from the same diaphragm (Dia) muscle of a 7 week old wild-type mouse using six 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) is 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. In addition, the shift of the muscle fiber size distribution towards small caliber fibers is very significant in the MDC1A mouse models (see APPENDIX, Fig. 1).

Together with the percentage of fibers with centralized nuclei, this information is a reliable and robust histological assessment of some of the pathology associated with laminin alpha2-deficiency. Moreover, by measuring several hundred fibers per muscle sample (e.g. up to 3,000 fibers per diaphragm and up to 6,000 per triceps brachii muscle) this method allows for the detection of even subtle differences between muscle samples of differently treated mice.

Since morphological features appear to be equally divided throughout the length of the muscle (e.g. from tendon to tendon) in $dy^W/dy^W$ mice, it is sufficient to take pictures at one location within the muscle (van Putten et al 2010). However, it is important to analyze equal regions/locations in all of the muscles. Best consistency is achieved if mid-belly

sections are analyzed. At least 3 muscles of different individual mice should be analyzed per experimental group.

### 5.4.2  Procedure for digital image acquisition

A digital image of the muscle section is acquired (a 10x magnification is appropriate), 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.

| 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.
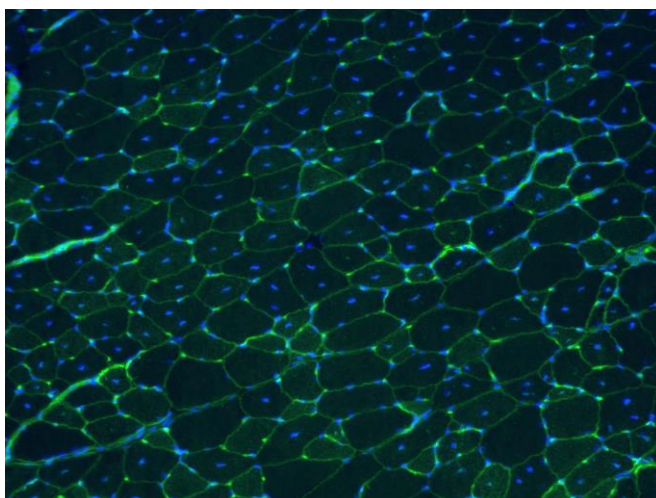


### 5.4.2.1 Determination of muscle fiber diameter (e.g., minimal "Feret's diameter")

*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.*

*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, image acquisition and multiple image alignment can be performed in an automated manner very efficiently. When images are captured manually, those can be aligned with Mayachitra Imago, archived and directly analyzed.
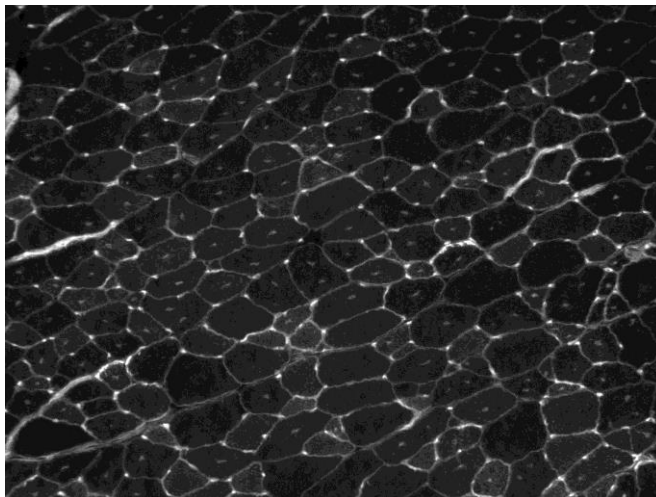


*Image 2: The green channel (WGA staining) separated from Image 1. Note that WGA also stains the nuclei*

*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).

• To visualize a shift of the fiber size distribution (see Section 6.2.) to smaller fiber sizes in dystrophic muscles, relative numbers of fibers in a given diameter class (e.g. 5µm/class) are normally presented. The appropriate diameter classes (5µm/class is recommended) can be pre-set in the microscope analysis software to automatically obtain the numbers of fibers in a given class. Alternatively, this evaluation can be performed manually in Excel.

• 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.
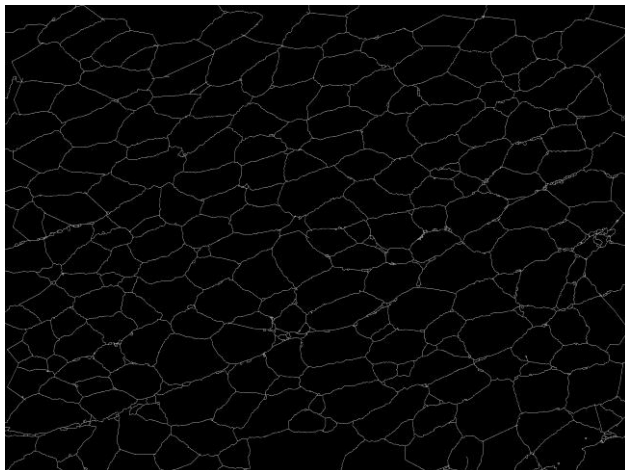


*Image 3: Cell boundaries identified by the internal "Separator" filter of CellP. Note that objects (cells) touching the image border are omitted*

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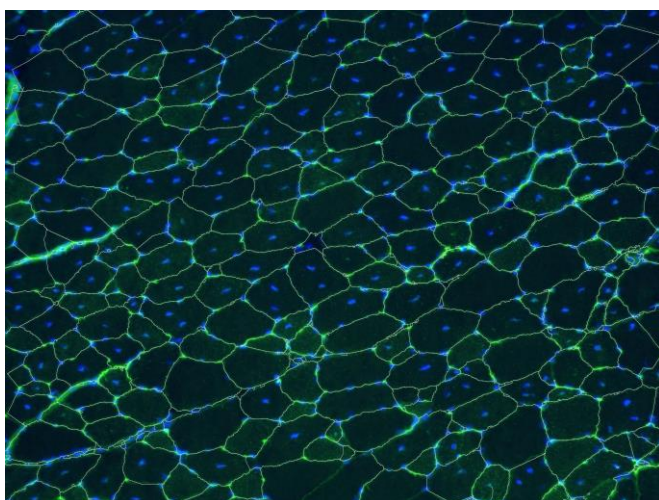


*Image 4: Combined image of the boundary drawing and the original fluorescence image*

- Perform particle determination: control the particles found and eliminate obvious erroneous particles if any (Image 5).
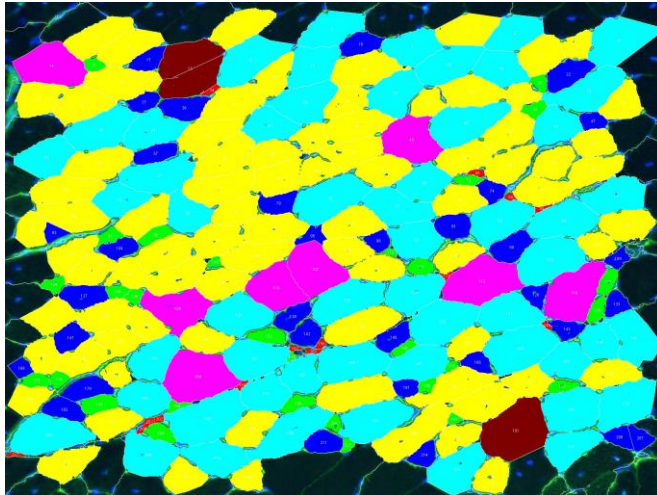
*Image 5: identified particles (with optional diameter dependant color coding)*

- 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) form the background (membranes), given the information provided by the user in terms of mouse-drawn scribbles. The number of fibers and area ($\mu m^2$) 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).
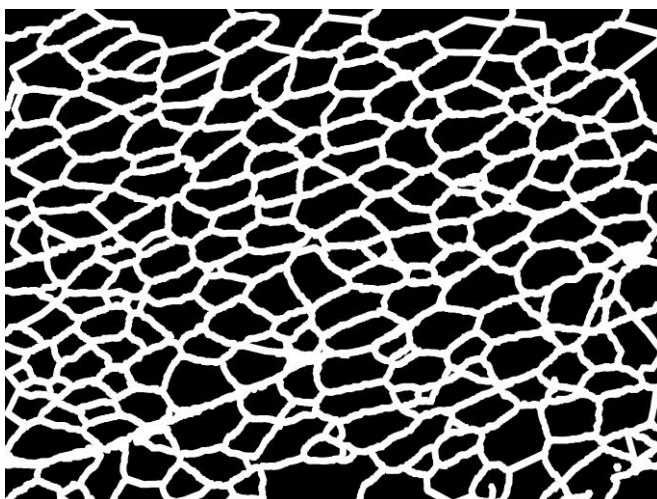


*Image 6: The muscle fiber borders from Image 3 have been expanded by several dots, providing a mask covering the peripheral nuclei.*

- 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.
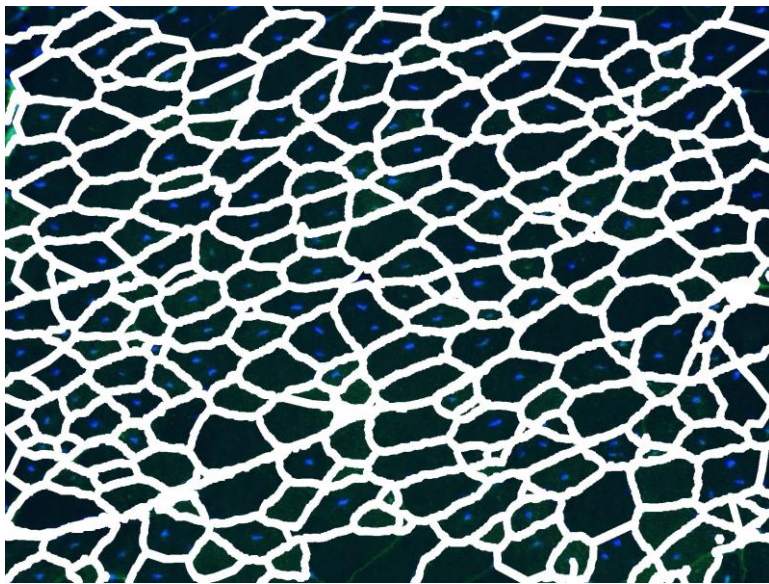


*Image 7: Image of a muscle with the cell boundaries and the peripheral nuclei masked. Centralized nuclei are visible with DAPI stain.*

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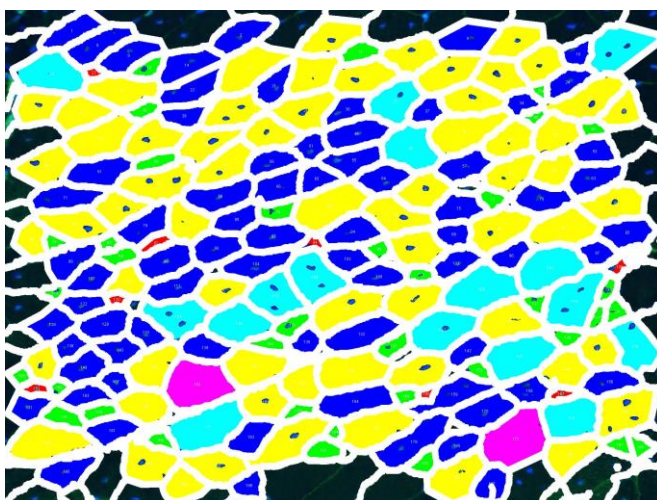


*Image 8: Final Image of a muscle with the centralized nuclei indicated as "holes" within the particles (with optional diameter dependant color coding).*

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) the result sheet 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 due to de-/regeneration processes. Although in $dy^W/dy^W$ mice this parameter is not as prominent as the shift of the fiber size distribution to smaller fibers, the variance coefficient (VC) of the muscle fiber minimal diameter is higher in $dy^W/dy^W$ muscle in comparison to wild-type muscle. Typically, VC in $dy^W/dy^W$ muscle is approximately 1.5-1.8 times as high as in wild-type animals. VC of the muscle fiber minimal diameters is defined as:

Variance coefficient Z = 1000 x standard deviation of muscle fiber minimal diameters/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:

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

### 6.2    Fiber size distribution

A hallmark of muscles from MDC1A patients and mouse models is a high proportion of small, rounded fibers (due to cycles of de- and regeneration of muscle fibers). To visualize the shift to smaller fiber sizes in $dy^W/dy^W$ when compared to wild-type control animals, the fiber diameters are assigned to classes of for example 5μm (recommended) per class. If not pre-defined in the microscope analysis software, this classification can be done manually in Excel. The number of fibers in each diameter class (e.g. 0-5μm, 5-10μm, 10-15μm, ...) is then

normalized to the total fiber number per muscle. The average per genotype (or treatment) is then used to represent relative numbers of fibers in a given diameter class (5 µm/class) in a diagram (see Figure 1, APPENDIX).

## 6.3 Fiber number per muscle cross-section

Muscles of MDC1A patients and mouse models are characterized by loss of muscle fibers due to fiber degeneration and failure of complete regeneration. The above described microscopic analysis provides the total number of fibers per muscle. Typically, a 10week-old $dy^W/dy^W$ triceps brachii muscle contains half of the fibers counted in a wild-type control muscle (see Figure 2, APPENDIX)

## 6.4 Centralized myonuclei

Muscle fibers of 'healthy' muscles rarely contain centralized nuclei. Therefore, the percentage of fibers with centralized nuclei should be below 3% in wild-type muscles. Although regeneration is impaired in $dy^W/dy^W$ muscles, the number of fibers with centralized nuclei is increased when compared to healthy control muscles. A potential treatment option that improves the regeneration capacity of $dy^W/dy^W$ muscles may increase central nucleation in fibers of $dy^W/dy^W$ muscles. The number of fibers with centralized nuclei is counted and is expressed as percentage of all fibers (CN in %; see Figure 3, APPENDIX)

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

$$100 \times (CN_{(dyW\ controls)} - CN_{(dyW\ test\ conditions)}) / (CN_{(dyW\ controls)} - CN_{(wild\ type)})$$

## 6.5 Cross-sectional area and the percentage of fibrosis

A prominent feature of MDC1A-diseased muscles is extensive fibrosis arising from replacement of muscle tissue. In addition, the area per cross-section is heavily reduced in $dy^W/dy^W$ muscles (see Figure 4, APPENDIX). The WGA/DAPI staining procedure and the subsequent alignment of the images to one picture covering the entire muscle, complies all requirements to manually defining and quantifying the fibrotic area per cross-section using microscopic analysis software or Photoshop as a tool. Fibrosis is normally expressed as percentage of fibrotic area per total cross-sectional area (see Figure 5, APPENDIX).

## 7    REFERENCES

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

Moll et al., (2001) An agrin minigene rescues dystrophic symptoms in a mouse model for congenital muscular dystrophy. Nature 413: 302-307

Bentzinger et al., (2005) Overexpression of mini-agrin in skeletal muscle increases muscle integrity and regenerative capacity in laminin-alpha2-deficient mice. FASEB Journal 19: 934-942

Meinen et al., (2007) Linker molecules between laminins and dystroglycan ameliorate laminin-alpha2-deficient muscular dystrophy at all disease stages. The Journal of cell Biology 176: 979-993
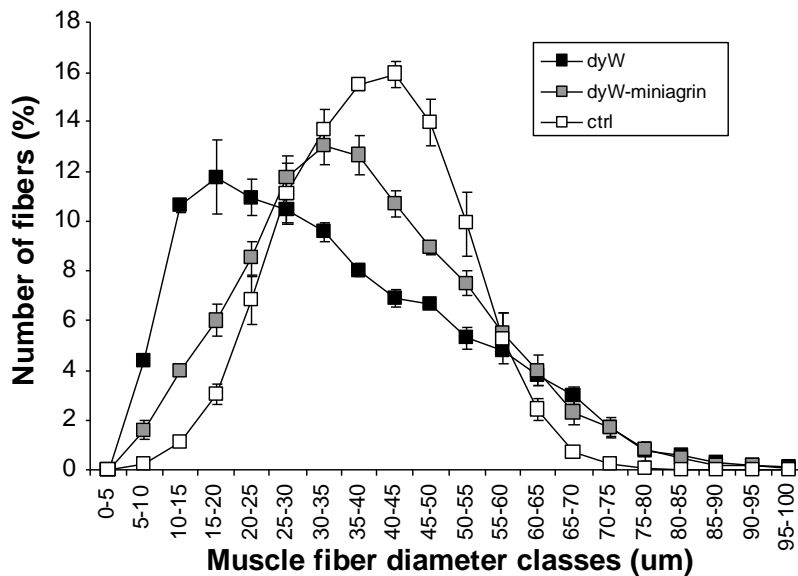
## 8 APPENDIX



**Figure 1.** Fiber size distribution of triceps brachii muscles from 12 week-old $dy^W/dy^W$ mice ($dy^W$), $dy^W/dy^W$ mice expressing the mini-agrin in muscles ($dy^W$-mini-agrin) and control mice (ctrl). Values represent relative numbers of fibers in a given diameter class (5µm/class). The fiber size distribution in $dy^W/dy^W$ muscles is shifted to smaller fibers when compared to control. Expression of mini-agrin, which restores the mechanical stability of laminin aplha2-deficient muscles (Moll et al. 2001, Bentzinger et al., 2005; Meinen et al., 2007) normalizes the distribution of fiber diameters towards control.
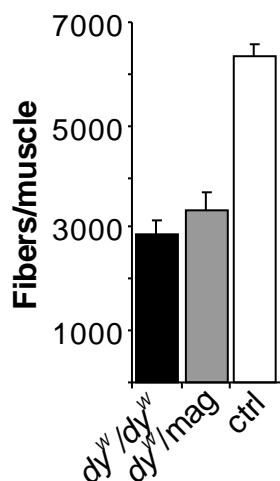


**Figure 2.** Fiber numbers per cross-section of triceps brachii muscles from 12 week-old $dy^W/dy^W$ mice ($dy^W$), $dy^W/dy^W$ mice expressing the mini-agrin in muscles ($dy^W$/mag) and control mice (ctrl). In $dy^W/dy^W$ muscle, around half of the fibers have been lost due to muscle destruction. Around up to 20% of the muscle tissue of in $dy^W/dy^W$ cross-sections has been replaced by fibrotic tissue. Mini-agrin expression (Moll et al. 2001, Bentzinger et al., 2005; Meinen et al., 2007) prevents the loss of muscle fibers only marginally.
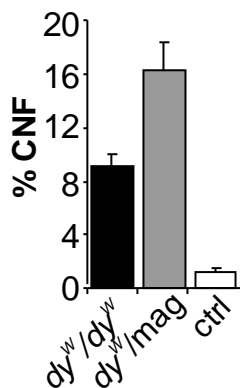
**Figure 3.** Fibers containing centralized nuclei expressed as percentage of the total number of fibers per triceps brachii muscle from 12-week-old $dy^W/dy^W$ mice ($dy^W$), $dy^W/dy^W$ mice expressing the mini-agrin in muscles ($dy^W$/mag) and control mice (ctrl). The dystrophic $dy^W/dy^W$ muscles undergo cycles of de-/regeneration but fail to successfully complete muscle regeneration. Mini-agrin expression (Moll et al. 2001, Bentzinger et al., 2005; Meinen et al., 2007) restores the regenerative capacity of $dy^W/dy^W$ muscles.
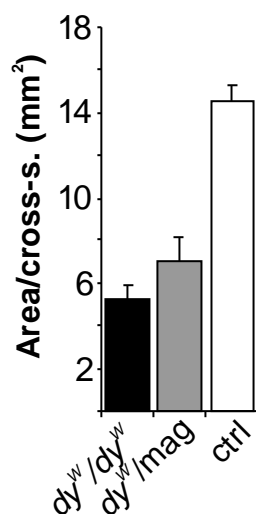


**Figure 4.** Area of triceps brachii cross-sections from 12 week-old $dy^W/dy^W$ mice ($dy^W$), $dy^W/dy^W$ mice expressing the mini-agrin in muscles ($dy^W$/mag) and control mice (ctrl). The area of $dy^W/dy^W$ cross-sections is reduced to approx. one-third of that of control muscles. Mini-agrin (Moll et al. 2001, Bentzinger et al., 2005; Meinen et al., 2007) slightly increases the cross-sectional area of $dyW$ muscles.
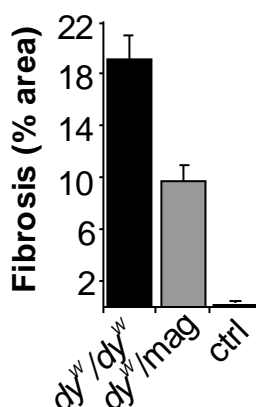


**Figure 5.** Percentage of fibrotic area per cross-section of triceps brachii muscles from 12 week-old $dy^W/dy^W$ mice ($dy^W$), $dy^W/dy^W$ mice expressing the mini-agrin in muscles ($dy^W$/mag) and control mice (ctrl). Approximately 20% of the muscle tissue of in $dy^W/dy^W$ cross-sections has been replaced by fibrotic tissue. Expression of mini-agrin restores the mechanical stability of laminin aplha2-deficient muscles (Moll et al. 2001, Bentzinger et al., 2005; Meinen et al., 2007) which reduces muscle damage and subsequently halves the extent of fibrosis.