Use of Small Animal Models for Duchenne and Parameters to Assess Efficiency upon Antisense Treatment

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Abstract

Duchenne muscular dystrophy (DMD) is a rare genetic disease affecting 1 in 5000 newborn boys. It is caused by mutations in the *DMD* gene with a consequent lack of dystrophin protein that leads to deterioration of myofibers and their replacement with fibro-adipogenic tissue. Using antisense oligonucleotides (AONs) to modify out-of-frame mutations in the *DMD* gene, named exon skipping, is currently considered among the most promising treatments for DMD patients. The development of this strategy is rapidly moving forward, and AONs designed to skip exons 51 and 53 have received accelerated approval in the USA. In preclinical setting, the *mdx* mouse model, carrying a point mutation in exon 23 of the murine *Dmd* gene that prevents production of dystrophin protein, has emerged as a valuable tool, and it is widely used to study in vivo therapeutic approaches for DMD. Here we describe the methodology for intravenous delivery of AONs targeting dystrophin through tail vein of *mdx* mice. Furthermore, the most relevant functional analyses to be performed in living mice, and the most informative histopathological and molecular assays to evaluate the effect of this treatment are detailed.

Key words Antisense oligonucleotides, Duchenne muscular dystrophy, Dystrophin, Exon skipping, *mdx*

1 Introduction

Duchenne muscular dystrophy (DMD) is due to mutations on the *DMD* gene and the consequent loss of the dystrophin protein in skeletal and cardiac muscles [1]. The loss of dystrophin makes the muscle fibers extremely fragile and susceptible to cycles of tissue degeneration and regeneration causing depletion of muscle stem cells and massive deposition of fat/connective tissue finally leading to muscle weakness, respiratory insufficiency, and cardiac failure [2]. Mutations of the *DMD* gene often lead to the disruption of the reading frame. Exon skipping, based on the selective removal of exons flanking the out-of-frame mutations using antisense oligonucleotides (AONs), has been used to re-frame the mRNA transcript to allow the expression of a partially functional Becker

muscular dystrophy (BMD)-like protein [3, 4]. To study this approach in vivo, AONs targeting exon 23 of the mouse *Dmd* gene have been tested by local and systemic delivery in *mdx* mice, the most used animal model of DMD, from neonatal to very late (>15-month old) stages of development [5–8].

In this chapter, we focus on systemic intravenous administration of AONs that allows their body-wide distribution. We provide a detailed description of intravenous infusion of AONs through the mouse tail vein. Phosphorodiamidate morpholino oligomers (PMOs) with or without the conjugation with a cell-penetrating peptide have been used for this approach [4–8]. We also list methods for assessing the animal locomotor activities, forelimb strength, treadmill and wheel exercise, and in situ muscle physiology. *Post-mortem* protocols for RT-PCRs measuring the level of exon skipping for *DMD* exon 23, Western blot for quantifying the level of dystrophin protein, and immunofluorescence for evaluating muscle histology and the effect of the treatment on muscle size, muscle fibrosis, and myofiber cross-sectional area are additionally described.

2 Materials

2.1 PMO and PMO Conjugate

1. PMO-DMD inducing skipping of murine *Dmd* exon 23 (5'-GGCCAAACCTCGGCTTACCTGAAAT-3') is synthesized and conjugated to an arginine-rich cell-penetrating peptide (so-called B-peptide: RXRRBRRXRBRXB) at the 3'-end of the PMO by Sarepta Therapeutics Inc. (*see Note 1*).
2. BPMOs, as lyophilized powder, are kept at -20°C .
3. Resuspended BPMOs (at 10 mg/ml), in sterile ddH₂O, are kept at 4°C .

2.2 Animals

1. All animal procedures are performed in accordance with the UK Animals (Scientific Procedures) Act, 1986.
2. *Mdx* (C57BL/10ScSn-Dmdmdx) and C57BL/10 mice are bred in an authorized animal facility and are maintained in a standard 12-h light/dark cycle with free access to food and water. Both *mdx* and C57BL/10 mice are also available from The Jackson laboratory (<https://www.jax.org>).

2.3 Intravenous Tail Vein Injection

1. BPMOs (as described in Subheading 2.1).
2. 0.9% saline.
3. BD Microfine Insulin 0.3-ml syringes with 30G needles attached.
4. 70% ethanol.
5. ddH₂O.

6. Heat chamber (set at 40 °C).
7. Mouse restrainer.
8. Clean cages with bedding, food, and water.

2.4 Postmortem Tissue Processing

1. CO₂ chamber.
2. Liquid nitrogen (N₂).
3. Isopentane, isopentane container.
4. 70% ethanol.
5. Dissection tools (i.e., scalpels, scissors, tweezers, forceps).
6. Raymond lamb cork disc for cryostat use (20 mm x 3 mm), cork holder.
7. Optimal cutting temperature (OCT) embedding medium.
8. 1.5-ml tubes.
9. Scales to weigh mice and samples harvested.

2.5 RNA Extraction and RT-PCR

1. RNeasy Fibrous Tissue kit (QIAgen).
2. Tungsten Carbide beads, 3 mm (QIAgen).
3. TissueLyser II (QIAgen).
4. ND-1000 NanoDrop spectrophotometer.
5. GoScript Reverse Transcription System.
6. GoTaq Polymerase.
7. Reverse transcription master mix: 2.5 mM MgCl₂, 0.5 mM dNTPs, 1x GoScript Reverse Transcription buffer, 8 units of GoScript Reverse Transcriptase, ddH₂O up to 10 µl/reaction.
8. PCR master mix: 200 nM forward/reverse primer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1x GoTaq buffer, 1.25 units of GoTaq Polymerase, ddH₂O up to 21 µl/reaction.
9. Agarose.
10. 1x TAE buffer: 40 mM Trizma base, 20 mM glacial acetic acid, 1 mM Na₂EDTA, pH 7.6.
11. GeneTools Image Analysis software 4.02 (Syngene) or ImageJ (NIH, free download at <https://imagej.nih.gov/ij/download.html>).
12. PCR primers, see below:

Primer name	Sequence (5'–3')
mDmd SDY 20x21 (F)	GTAAGGATGAAGTCAACAGATTG
mDmd ASDY 25 (R)	TCCCACTGAGTGTTAAGCTC
mDmd ASDY 24 (R)	GCCATCCATTTCTGTAAG

2.6 Protein Extraction and Western Blot

1. Lysis buffer: 0.15 M NaCl, 0.05 M HEPES, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.01 M EDTA, protease inhibitor (Roche, 1 tablet/50 ml). Aliquot and store at -20°C .
2. Tungsten Carbide beads, 3 mm.
3. TissueLyser II.
4. DC Protein Assay.
5. NuPAGE 3–8% Tris–Acetate gels, Tris–Acetate running and transfer buffers.
6. HiMark Pre-stained Protein Ladder.
7. HyBond nitrocellulose membrane, 0.45 μm pore size.
8. 1x Phosphate-buffered saline (PBS) solution: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH 7.4.
9. Blocking buffer: 5% skimmed milk, 1x PBS, 0.2% Tween-20.
10. Washing buffer: 1x PBS, 0.1% Tween-20.
11. Primary antibodies: Mouse monoclonal anti-dystrophin 6C5 (Novocastra Laboratories), rabbit anti- α tubulin (Abcam).
12. Secondary antibodies (LI-COR Biosciences): goat anti-mouse IRDye800, goat anti-rabbit IRDye680.
13. Odyssey Infrared Imaging System (LI-COR Biosciences).

2.7 Immunohisto- chemistry Staining and Histological Analyses

1. OTF 5000 cryostat (Bright).
2. Superfrost[®] Plus microscopy slides, coverslips.
3. Dako (hydrophobic) pen.
4. 100% acetone, 100% ethanol, 100% xylene.
5. 1x PBS solution: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH 7.4. Store at room temperature.
6. 4% paraformaldehyde (PFA): dissolve 40 g PFA in 700 ml of 1x PBS at 60°C . Top up with 260 ml dH_2O to cool down solution. Add few drops of 5 M NaOH and adjust to pH 7.4. Aliquot and store at -20°C .
7. 0.3% Picro sirius red: 0.3 g sirius red in 100 ml of saturated picric acid solution, filter through filter paper.
8. 0.5% acetic acid: 100% acetic acid diluted in tap water.
9. Mouse-On-Mouse (MOM) Basic kit (Vector Laboratories).
10. Blocking buffer: MOM blocking reagent (supplied with the MOM kit, 1 drop in 1.25 ml 1x PBS), 1% bovine serum albumin, 1% goat serum, 0.1% Triton X-100.
11. Washing buffer: 1x PBS, 0.05% Tween-20.

12. Primary antibodies: mouse monoclonal anti-dystrophin 6C5 (Novocastra Laboratories), rabbit polyclonal anti-laminin (Abcam), rabbit polyclonal anti-collagen VI (Abcam).
13. Secondary antibodies (Life Technologies): goat anti-mouse Alexa488 and goat anti-rabbit Alexa568.
14. 4',6-Diamidino-2-phenylindole (DAPI).
15. 0.1% *p*-Phenylenediamine (PPD) anti-bleaching solution: dissolved in water, stored at -20°C , and protected from light.
16. Mowiol solution: stir 4.8 ml glycerol, 2.4 g Mowiol in 6 ml water for 2 h, at room temperature. Add 12 ml of 0.2 M Tris buffer pH 8.5, 0.02% sodium azide. Incubate solution in $50\text{--}60^{\circ}\text{C}$ water bath for 10 min, stirring frequently. Centrifuge at $5000 \times g$ for 15 min. Collect supernatant, aliquot and store at -20°C (*see Note 2*).
17. Mounting solution: mix 1 part of 0.1% PPD solution with 9 parts of Mowiol solution, maintain at room temperature, protect from light and use within a day.
18. DPX mountant (Sigma Aldrich).
19. Microscope (e.g., Axio Observer D1, ZEISS).
20. Image acquisition and analysis software (e.g., ZEN software, Fiji—free downloaded at <https://imagej.net/Fiji/Downloads>).

3 Methods

3.1 Intravenous Tail-Vein Injection (*See Note 3*)

1. Dilute BPMO in sterile 0.9% saline at a desired concentration, immediately prior to injection, and keep on ice during the procedure.
2. Weigh mouse and calculate the dose of BPMO needed in a total volume of $200\text{ }\mu\text{l}$ /mouse.
3. Place mouse in a heat chamber for about 10 min.
4. Load BPMO solution into syringe, remove all air bubbles.
5. Transfer the mouse into a restrainer leaving the tail outside.
6. Hold the tail all the time to avoid the mouse withdrawing its tail.
7. Close the restrainer tightly so that the mouse can breathe but barely move back and forth (*see Note 4*).
8. Turn restrainer on its side, this will also turn the mouse on the side.
9. Wipe the tail with 70% ethanol.
10. Identify the lateral veins (the tail has one vein on each side).

11. Hold the tail on your left hand (for right-handed researchers). Hold the needle (with bevel up) on your right hand and inject BPMO. If injection fails, try few more times on that side prior to changing to the other side.
12. Slowly remove the needle and apply gentle compression (with a thumb) until bleeding ceases.
13. Place mouse into a clean cage.

3.2 Functional Tests

TREAT-NMD has provided exhaustive experimental protocols for DMD animal models (<http://www.treat-nmd.eu/resources/research-resources/dmd-sops/>). Below are four functional tests we use to assess muscle function, usually 1 or 2 weeks before the experiment ends:

1. Behavioral and locomotor measurements using open field animal activity monitoring system (SOP ID No.: DMD_M.2.1.002).
2. Use of treadmill and wheel exercise to assess dystrophic state (SOP ID No.: DMD_M.2.1.003).
3. Use of grip strength meter to assess the limb strength of *mdx* mice (SOP ID No.: DMD_M.2.2.001).
4. Measuring isometric force of isolated mouse skeletal muscles in situ (TREAT-NMD, SOP ID No.: DMD_M.2.2.005).

3.3 Postmortem Tissue Processing

1. Weigh mouse before sacrificing by CO₂ exposure and confirm the death by neck dislocation.
2. From each mouse, we usually collect heart, diaphragm, extensor digitorum longus, gastrocnemius, soleus, and tibialis anterior.
3. Tissues from one side of the body are frozen immediately in liquid N₂ for RNA and protein extraction while tissues from the other side are embedded in OCT and subsequently frozen in liquid N₂-cooled isopentane for cryo-sectioning (*see Note 5*).
4. All samples are kept at −80 °C until use.
5. If required, collect total blood by cardiac puncture into 1-ml syringe (*see Note 6*). Transfer the blood into an ice-cold 1.5-ml tube, keep on ice for at least 4 h or overnight prior to serum extraction: (1) centrifuge at $8000 \times g$ for 10 min at 4 °C; (2) transfer supernatant to fresh tubes; (3) repeat centrifugation; (4) collect the supernatant and store at −80 °C.

**3.4 RNA Extraction
and RT-PCR
Quantifying Exon
Skipping Efficiency**

1. Place samples on ice immediately after taking them out of -80°C freezer.
2. Add lysis buffer RLT provided with RNeasy Fibrous Tissue kit and wait until samples thawed down.
3. Disrupt and homogenize samples with 3 mm Tungsten Carbide beads at 25 Hz for 2 x 2 min (*see Note 7*) on a TissueLyser II.
4. Follow manufacturer's instructions to complete RNA extraction process (*see Note 8*).
5. Quantify RNA concentration using a ND-1000 NanoDrop spectrophotometer.
6. Make first strand cDNA: 500–1000 ng RNA, 0.5 μg oligo dT, 0.5 μg random primers, ddH₂O up to 10 μl /reaction. Run program: 70°C for 5 min, 4°C for at least 5 min.
7. Reverse Transcription: add 10 μl of the master mix into 10 μl of first strand cDNA. Run program: 25°C for 5 min, 42°C for 1 h, 70°C for 15 min (*see Note 9*).
8. Mix 4 μl cDNA (made in **step 7**, equivalent to 100–200 ng cDNA) with 21 μl PCR master mix and run PCR programs as below.
9. First-round PCR program: 94°C for 2 min, 30 cycles (94°C for 1 min, 57°C for 1 min, 72°C for 1 min), 72°C for 5 min, 4°C for infinity.
10. Nested PCR program: 94°C for 2 min, 30 cycles (94°C for 1 min, 54°C for 1 min, 72°C for 45 sec), 72°C for 5 min, 4°C for infinity.
11. PCR products are loaded onto 2% agarose gels, with HyperLadderIV used as a size standard. Expected sizes: 577 bp (unskipped), 346 bp (skipped exon 23), 218 bp (skipped exons 22 + 23).
12. Densitometric analysis of gel electrophoresis results can be done using GeneTools Image Analysis or ImageJ software.
13. Evaluate the efficiency of dystrophin exon skipping as the percentage of the density of skipped products against the total density of unskipped and skipped products.

**3.5 Protein
Extraction and
Western Blot
Quantifying Dystrophin
Expression**

1. Place samples on ice immediately after taking them out of -80°C freezer.
2. Add lysis buffer (*see Note 10*) and wait until samples thawed down.
3. Disrupt and homogenize samples with 3 mm Tungsten Carbide beads at 25 Hz for 2 x 2 min (*see Note 7*) on a TissueLyser II.
4. Centrifuge at $14,000 \times g$, 10 min, 4°C .

5. Transfer the supernatant to fresh pre-chilled 1.5-ml tubes. Repeat centrifugation if required.
6. Quantify the total protein with *DC* Protein Assay following the manufacturer's instructions.
7. Reduced protein samples (100–200 µg) are resolved on NuPAGE 3–8% Tris–Acetate gels with HiMark Pre-stained Protein Ladder used as a size standard.
8. Run gel at 150 V for 1.5 h (or until the dye reaches the bottom of gel) in Tris–Acetate buffer and blot onto HyBond nitrocellulose membranes for 2 h at 30 V.
9. Block in blocking buffer for 1 h, at room temperature, with gentle agitation.
10. Primary antibody incubation for overnight at 4 °C (with gentle agitation) with either mouse monoclonal anti-dystrophin 6C5 (1:100) or rabbit anti- α tubulin (1:2500), diluted in blocking buffer (*see Note 11*).
11. Wash for 3 x 5 min in washing buffer with gentle agitation.
12. Secondary antibody incubation for 1 h at room temperature (with gentle agitation) with goat anti-mouse IRDye800 and goat anti-rabbit IRDye680 (1:10,000, diluted in blocking buffer), protect from light afterwards.
13. Wash for 3 x 5 min in washing buffer with gentle agitation.
14. Analyze on an Odyssey Infrared Imaging System (ImageJ software can be used alternatively). Expected size: 427 kDa for dystrophin and 50 kDa for α tubulin.
15. The values of dystrophin intensity are normalized to the values of corresponding α -tubulin intensity and subsequently quantified based on a standard curve of dystrophin (*see Note 12*).

3.6 Immunostaining for Dystrophin, Laminin, or Collagen VI

For histological analyses, muscles are sectioned and co-immunostained for dystrophin/laminin to assess the percentage of dystrophin positive fibers and the muscle cross sectional area or immunostained for collagen VI to assess the amount of muscle fibrosis.

1. Section frozen tissue on an OTF 5000 cryostat at 10-µm thickness through the muscle length. Cutting temperature is between –24 and –20 °C.
2. Collect transverse sections onto Superfrost® Plus microscopy slides, keep at –80 °C for long-term storage (*see Note 13*) or proceed to immunostaining (as below).
3. Air-dry samples for 15 min.
4. Fix in ice-cold acetone for 10 min.

5. Air-dry for 10 min. During this time, limit with a Dako (hydrophobic) pen the area to be stained.
6. Rehydrate samples in 1x PBS for 2 x 2 min.
7. Use a Mouse-on-Mouse (MOM) Basic kit (*see Note 14* for collagen VI staining) and block samples in blocking buffer for 1 h, at room temperature (*see Note 15*).
8. Wash 3 x 5 min in washing buffer with gentle agitation.
9. Incubate with MOM diluent buffer (supplied with the kit, 80 µl reagent in 1 ml of 1x PBS) for 5 min, at room temperature. Tap off excess.
10. Incubate with mouse monoclonal anti-dystrophin 6C5 (1:50) and rabbit polyclonal anti-laminin (1:400) (or with rabbit polyclonal anti-collagen VI, 1:300), diluted in MOM diluent, for overnight, at 4 °C.
11. Wash 3 x 5 min in washing buffer with gentle agitation.
12. Incubate with goat anti-mouse Alexa488 (1:500) and goat anti-rabbit Alexa568 (1:500), diluted in 1% goat serum, 1x PBS, 0.05% Tween-20, for 1 h, at room temperature. Protect from light afterwards.
13. Wash 3 x 5 min in washing buffer with gentle agitation.
14. Incubate with 1 µg/ml DAPI diluted in 1x PBS for 15 min, at room temperature.
15. Rinse with 1x PBS.
16. Add mounting solution (50–100 µl/slide) and cover with coverslips.
17. Air-dry slides and store at 4 °C.

3.7 Picro Sirius Red Staining for Detecting Collagen I and III

Deposition of muscle fibrosis can also be assessed following staining with picro sirius red that stains collagen in red and cytoplasm in yellow:

1. Air-dry tissue sections for 15 min.
2. Fix in 4% PFA for 10 min.
3. Wash 2 x 5 min in tap water and hydrate in dH₂O for 5 min.
4. Immerse in 100% ethanol for 5 min.
5. Air-dry completely for at least 20 min (*see Note 16*).
6. Stain with 0.3% picro sirius red for 1 h.
7. Rinse quickly in dH₂O.
8. Fix staining in 0.5% acetic acid for 2 x 5 min.
9. Dehydrate in 100% ethanol for 3 x 5 min.
10. Clear in 100% xylene for 2 x 10 min.

11. Mount in DPX mountant and cover with coverslips.
12. Air-dry slides and store at room temperature.

3.8 Histological Analyses

1. Immunostainings are visualized under an inverted fluorescence Axio Observer D1 microscope and taken by an AxioCam MR3 camera combined with ZEN imaging software (*see Note 17*).
2. Ideally a mosaic image of the whole muscle section is acquired by overlapping and stitching individual images of that section together.
3. For quantifying dystrophin expression: use ZEN software to score the mean intensity of dystrophin and subsequently normalize to the mean intensity of laminin of the same section.
4. For quantifying dystrophin-positive fiber numbers: use ImageJ, manually count the number of positive fibers, and evaluate as the percentage of the number of total fibers within the same image field that are positive with laminin staining (*see Note 18*).
5. For quantifying the frequency distribution of fiber size: see TREAT-NMD, SOP ID No.: DMD_M.1.2.001.
6. For quantifying the level of central nucleation: use ImageJ, manually count the number of fibers having centralized nuclei (as shown by DAPI staining) and evaluate as the percentage of the number of total fibers within the same image field that are positive with laminin staining.
7. For quantifying muscle fibrosis: use ZEN software to score the mean intensity of collagen VI and evaluate as the percentage of wild-type values obtained in the same way. For measuring the collagen area positive with collagen VI or sirius red staining: use ImageJ and select the area covered by the section. Then using the threshold function, highlight only the portion of the section positive for collagen staining and evaluate as the percentage of the total area of the muscle cross-section (*see Note 19*).

4 Notes

1. BPMOs have been used to describe these experiments; however, other chemistries can be used if preferred (e.g., unconjugated PMO, vivo-PMO by Genetools, 2OMePS, etc.).
2. Mowiol may not be dissolved completely, but the pellet must be colorless. After centrifugation, collect and aliquot the supernatant and store at -20°C until use. Do not disturb the pellet.
3. Four to 6 weeks after in vivo delivery, the efficacy of the AONs reaches a plateau. Intravenous injections can be repeated

weekly for several weeks to boost the antisense efficacy and make a more robust exon skipping.

4. In case the mouse is particularly weak or very old, a general anesthesia may be preferable to avoid the mouse feeling too distressed by the procedure. In this case, in addition to the standard materials you will need: anesthesia chamber, isoflurane, isoabsorber, 100% oxygen, heat pads. Place mouse into an anesthesia chamber and induce with 5% isoflurane in 100% O₂ until the animal goes into deep anesthesia (heart beats slowly and regularly). Transfer the mouse onto a heat pad, with anesthesia maintained at approximately 2.5% isoflurane in 100% O₂. The animal is ready for injection following loss of pedal withdrawal reflex and eye-blink reflex. Monitor the state of anesthesia throughout the procedure. Turn the mouse on the side, identify the tail vein, and proceed with the injection.
5. Cover samples on cork discs with silver foil before immersing in liquid N₂ for additional tissue protection.
6. The volume of total blood depends on how quickly you reach this stage after the animal dies (also depends on animal strain); we usually get 200–600 µl/mouse.
7. Homogenization can be extended to maximum 10 min if required.
8. All centrifuge steps are performed at 8000 × *g*.
9. Store cDNA at –20 °C, if can't proceed to PCR.
10. Recommended volume for diaphragm, heart, tibialis anterior (25–30 mg): 200–300 µl; for gastrocnemius (70–90 mg): 300–400 µl; for extensor digitorum longus or soleus: 50–100 µl.
11. To avoid cross-reactivity, cut the membrane between the fourth and fifth bands (counted from top to bottom) equivalent to 117 and 71 kDa bands. Incubate top segment with mouse anti-dystrophin and bottom segment with rabbit anti-α tubulin.
12. For dystrophin standard curve, different protein amounts extracted from C57 mice are mixed with those from *mdx* mice (muscle type-matched) such that in each 100–200 µg protein mixture there is 100, 75, 50, 25, 0% of C57 dystrophin.
13. Transverse sections can be maintained at room temperature during sectioning for few hours but must be stored at –80 °C afterwards.
14. The MOM kit is not strictly needed in case of Collagen VI staining. In this case, a blocking with 5% skimmed milk in 1x PBS, 0.05% Tween-20 is sufficient. Similarly, 2.5% milk in 1x

PBS, 0.05% Tween-20 can be used to dilute primary and secondary antibodies.

15. We usually use 200–350 μ l reagent/slide, depending on the number of sections on each slide.
16. If samples are not completely dry, the cytoplasm of myofibers will also be colored in orange.
17. Select the mid-belly muscle section (for hind-limb muscles), usually the biggest section on the slide, or nearly the biggest one, which shows the best sectioning and staining. This is important as suboptimal sectioning forms artefacts that interferes with the analysis.
18. Determine the area of a single image field or of the whole muscle section using rectangle or polygon tool of ZEN software, respectively. Express data as the number of dystrophin-positive fibers per mm^2 . This method takes less time and effort but is still informative.
19. Analysis should be performed in a blinded manner and by a single operator to avoid bias.

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