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Histopathology in Hematoxylin & Eosin stained muscle sections

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1 OBJECTIVE

This document describes a method and provides reference values for the histological characterisation of dystrophic muscle from dy^W/dy^W mice.

2 SCOPE AND APPLICABILITY

Hematoxylin and eosin (H&E) is the most widely used stain in medical diagnosis. It is useful to determine the integrity of a tissue and thus is well suited to assess the dystrophic skeletal muscle pathology on transverse muscle sections. This SOP is written as a guide for those who wish to get a qualitative and eventually quantitative overview of the extent of the pathology in dy^W/dy^W muscles.

Hallmarks of the pathology of muscles from MDC1A patients and mouse models that can be visualized and quantified using H&E staining include the extent of fibrotic area, adipose tissue, the variation in muscle fiber diameters, the presence of small or rounded fibers (de-/regeneration), centralized nuclei (indicative of regeneration) as well as the presence of inflammatory cells.

3 CAUTIONS

As with all histological staining, great care must be taken in preparation of the samples:

- Accurate removal of the muscle(s) from the mouse will ensure that the appropriate portion of the appropriate muscle is actually being consistently analysed.
- Fixation (or freezing) should be done carefully to minimize shrinkage of tissues (or ice-artifact). It is important to identify areas of ice-artifact in frozen sections and to distinguish this from actual areas of muscle necrosis.
- Accurate embedding is essential as all sections must be embedded the same way.
- Bad cutting (e.g. a blunt blade which leaves knife scores in the section) or folding of the muscle section can make it hard to analyse a section accurately.

4 MATERIALS

- Cork plates (cut into quadrates of approx. 1.5cm x 1.5cm)
- Isopentane: Merck 1060561000 or Sigma-Aldrich 27,034-2
- Gum Tragacanth: Sigma-Aldrich (Cat. No. G1128)

- Tissue-Tek O.C.T: Sakura (Cat. No. 4583)
- Superfrost Plus Slides: Millian (SFPlus-42) or ThermoScientific Menzel Gläser (J1800AMNZ)
- PapPen Liquid Blocker (NANDAI Trading, Japan)
- Para-formaldehyde (PFA): Fluka (Cat. No. 76240) or 4% Paraformaldehyde (Sigma P6148)
- Meyers Hematoxilin (Merck 1.09249.0500)
- Eosin G-Lösung 0.5% wässrig (Merck 1.09844.1000)
- Glacial Acetic Acid (Merck 100063.1000)
- 100% ethanol, 90% ethanol, 70% ethanol
- Xylene
- Xylene-based mounting media: DePeX mountant (BDH, 361254D)

Stock solutions:

100ml of 4% neutralized PFA: Heat ~50ml ddH2O to 60°C and add 4g of PFA while stirring (in a hood!). Then add 1M NaOH until PFA is dissolving (pH= 7.4-7.5). Fill up to 100ml with 2xPBS (end conc. 1x). Filter the PFA solution and check pH 7.4-7.5.

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 -80°C for long-term and at -20°C for short-term storage.

5.3 H&E Staining

Background:

Most histological dyes are classified either as acid or as basic dyes. An acid dye exists as an anion (negatively charged) in solution, while a basic dye exists as a cation (positive charge). H&E is also a charge-based staining. **Hematoxylin** acts as a basic dye. Any substance that is stained by the basic hematoxylin-metal complex dye is considered to be basophilic; it carries acid groups which bind the basic dye through salt linkages. The basophilic structures are usually the ones containing nucleic acids, such as the ribosomes and the chromatin-rich cell nucleus, and the cytoplasmatic regions rich in RNA. When using hematoxylin, basophilic structures in the tissue appear purple-blue. **Eosin** acts as an acid dye and therefore stains basic materials in red-pink. Most of the cytoplasm is eosinophilic as well as intracellular or extracellular proteins. The eosin staining will allow the non-nuclear tissue components to be clearly differentiated from each other, e.g. muscle from collagen. Some structures do not stain well, e.g. basal lamina, and hydrophobic fat-rich structures (adipocytes, myelin) tend to remain clear.

Protocol:

1. Preparation: Bring the slides to room temperature and surround the sections with a liquid blocker (PapPen) to prevent running away of the incubation solutions.
2. Fixation: Incubate the slides with 4% paraformaldehyde dissolved in PBS (pH 7.2) for 5 minutes.
3. Washing: Put the slides in a glass chamber and wash with running tap water (not cold!) for ~1 minute to remove rests of formaldehyd. Drain (not dry!) the slides on a tissue before proceeding.
4. Hematoxylin Staining: Incubate the slides with Mayer's hematoxylin solution for 5 minutes to stain the nuclei dark.

5. Blueing: Put the slides in a glass chamber and wash under warm running tap water for ~10 minutes. Hematoxylin is used in combination with the metallic salt aluminum sulfate that is needed for oxidation and for coupling it to the tissue. In an alkaline solution aluminum sulfate combines with -OH of water to form aluminum hydroxide. In the presence of excess acid, aluminum hydroxide cannot be formed due to lack of –OH ions. Because most hematoxylin-metal formulae are fairly acid, the nuclei will at first be stained in purple color. Since tap water is considerably more alkaline than the pH of most alum hematoxylins (2.6 - 2.9), washing the sections in tap water in order to neutralize the acid and free the OH group will change the color of the nuclei to blue. This procedure is known as ***Blueing*****.**
6. Eosin staining: Add acetic acid (1:100) to the 0.5% Eosin solution (Merck 1.09844.1000) and incubate the slides 10 minutes to stain the fibers red.
7. Washing: Put the slides in a glass chamber and wash 3x for 1minute with ddH2O to remove excess of Eosin.
8. Dehydration: Drain successively in
   - 70% EtOH for 1 minute
   - 90% EtOH for 30 seconds
   - 100% EtOH for 30 seconds
   - Xylene for 30 seconds
9. Mounting: Mount the slides with 1-2 drops of a xylene-based mounting media (e.g. Depex) and cover with cover slides, avoid bubbles.
10. Press the slides under heavy weight for 10 minutes at room temperature
11. Store the slides at room temperature

5.4 Image analysis and quantification

5.4.1 Image acquisition

The morphological features of the muscle are identified using digital images of the stained section. Images are acquired using a bright-light microscope (10-20x magnification objective is appropriate), digital camera and image capture software (e.g. Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software). More sophisticated digital slide scanners (e.g. Aperio Scanscope) can be used to collect images although not all researchers will have access to these machines. Slightly overlapping images of a transverse muscle section can be tiled together to provide a single digital image of the entire muscle cross sectional area.

5.4.2 Manual Quantifications

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, i.e. the middle (van Putten et al 2010). For consistency it is necessary to keep the location constant in all muscles and to mention which part of the muscle is analyzed.

Some researchers perform histological analysis on multiple single frame images taken from the same muscle section (fields of view) and do not analyze the entire cross sectional area. However, this method assumes that histological features are homogenous throughout the muscle cross section, which may not always be true in dy^W/dy^W muscles.

Histological features (e.g. fibrosis, myofiber necrosis, central nucleation) are identified manually (draw around or count) by the researcher and quantified using image analysis software (e.g. Image Pro Plus, or freeware NIH ImageJ). Data can then be exported directly into Microsoft Excel and a percentage (e.g. % of fibrosis per analyzed area) can be calculated. The data are then subjected to the appropriate statistical analyses.

Analysis of all morphological features at once on whole cross sections can also be performed using the H&E color deconvolution plugin of ImageJ which separates the hematoxylin component and the Eosin component. Normal, undamaged myofibers are represented by the Eosin component and this area can be measured by thresholding. This can distinguish undamaged from damaged/regenerating myofibers and also identify areas of fibrosis. The total area can be determined on the original picture (van Putten et al. 2010).

6 EVALUATION AND INTERPRETATION OF RESULTS

Morphological features to be identified (see also Fig.1, APPENDIX):

- **Healthy muscle tissue:** Normal myofibres have a polygonal shape with peripheral nuclei, intact sarcolemma and non-fragmented sarcoplasm. Intact muscle tissue shows a homogenous fiber size distribution (see wild-type in Fig. 1, APPENDIX).
- **Fibrosis and fatty connective tissue:** With time, muscle tissue in dy^W/dy^W mice is replaced by fibrotic and adipose tissue. Fibrotic tissue stains light pink to “whitish”, because it contains high amount of collagens which is not as eosinophilic (acidophilic) as the cytoplasm. Hydrophobic fat-rich structures (adipocytes, myelin) tend to remain completely clear.
- **Fiber sizes and fiber shape:** Whereas wild-type muscles have homogenously distributed fiber diameters, dy^W/dy^W muscle shows a very high amount of small and mostly rounded fibers as well as some very big fibers. This is a consequence of the muscle undergoing repeated cycles of de-/regeneration.

- **Degeneration:** degenerating fibers stain lighter than an intact muscle fiber and often have irregular shape and few nuclei. Some myofibers are invaded by cells of the immune system whose nuclei are stained by hematoxilin.
- **Secondary inflammation**: inflammation is characterized by many inflammatory cells, that are infiltrating damaged muscle fibers and/or debris of muscle.
- **Regeneration:** Regenerating muscle is identified by activated myoblasts and, 2 -3 days later, small basophilic myotubes. These myotubes subsequently mature into "blueish-stained" (newly formed muscle fibers become basophilic due to their RNA content) myofibers with central nuclei indicating that the muscle fiber is in the process of regeneration.

7 REFERENCES

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8 APPENDIX

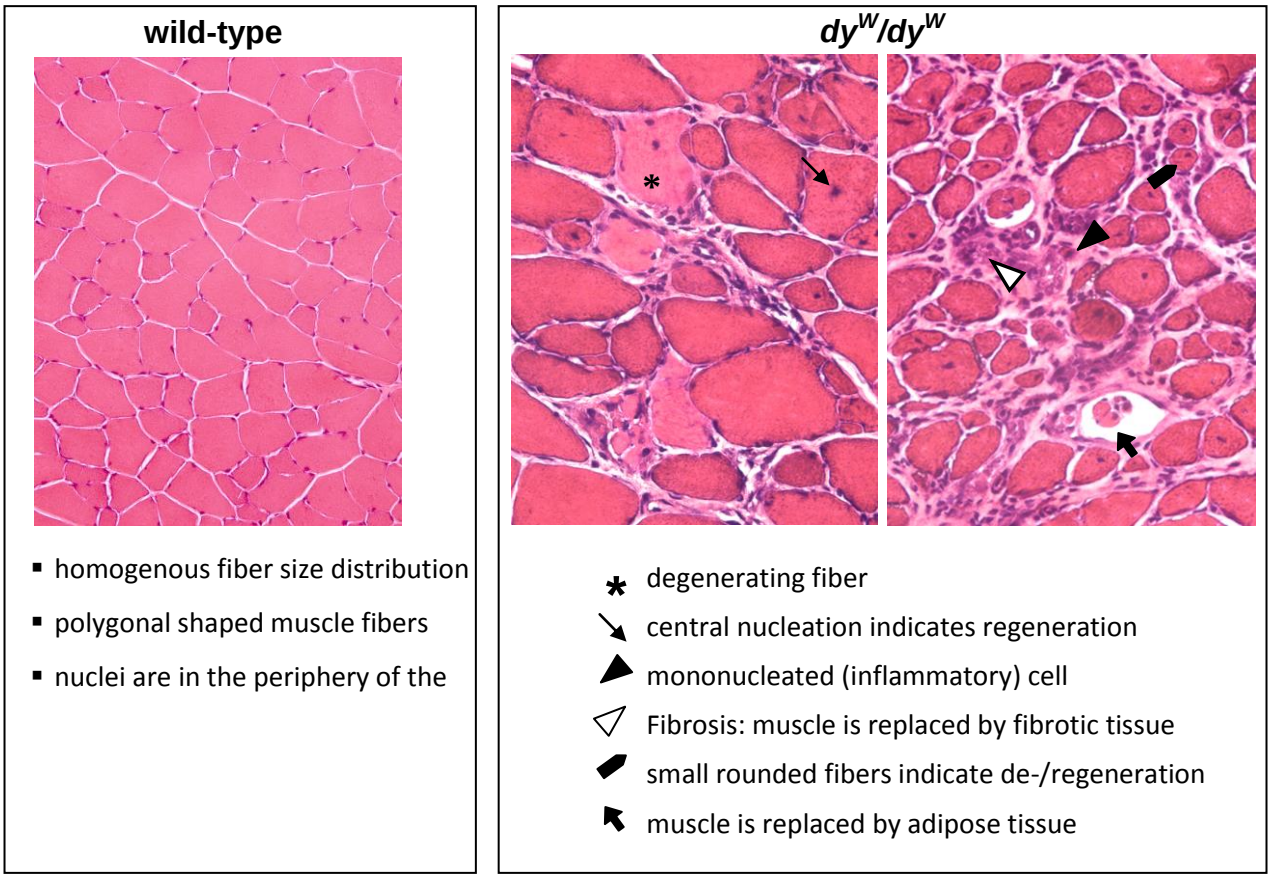

Figure 1. Pathological changes in muscles of dy^W/dy^W mice