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Histopathology in Masson Trichrome stained muscle sections

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1 OBJECTIVE

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

2 SCOPE AND APPLICABILITY

''Trichrome'' stains (Masson) are used for distinguishing collagen from muscle tissue. In general, they consist of nuclear, collagenous and cytoplasmic dyes in mordants such as phosphotungstic or phosphomolybdic acid.

The replacement of muscle with fibrotic tissue is a hallmark of muscles from MDC1A patients and mouse models. Since The Masson Trichrome staining procedure stains the collagen-rich fibrotic regions in blue, it is especially suited to assess and visualize the extent of fibrosis in dystrophic skeletal muscle on transverse muscle sections.

In addition and similar as with the H&E staining, Masson Trichrome stained sections also reveal adipose tissue, the variation in muscle fiber diameters, the presence of small or rounded fibers (de-/regeneration) as well as centralized nuclei (indicative of regeneration).

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)
- Paraformaldehyde (PFA): Fluka (Cat. No. 76240) or 4% Paraformaldehyde (Sigma P6148)
- Bouin's solution (Sigma HT10132)
- Weigert's Iron Hematoxylin Solution Part A & Part B (Sigma-Aldrich, HT107 & HT109)
- Accustain Trichrome Stain (Masson) Kit (Sigma-Aldrich, HT15)
- 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 -20°C.

5.3 Masson Trichrome Staining

Weigert's iron hematoxylin stains the nuclei in black, Biebrich scarlet-acid fuchsin stains cytoplasm & muscle fibers in red and after treatment with phosphotungstic and phosphomolybdic acid, collagen is stained in blue with aniline blue.

Staining procedure:

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. **1° Fixation:** Fix the cryosections in 4% PFA or in 10% Formalin **at RT for 1hr** in the hood.
3. **2° Fixation:** Re-fix sections in Bouin's solution at **RT overnight** to intensify the colors and increase the contrast between the tissue components.
4. **Washing:** Wash slides for **1-2 minutes** under running tap water (18–26°C) to remove yellow color from sections. Then rinse briefly in deionized/distilled water (ddH2O).
5. **Weigert's Hematoxilin Staining:** Mix equal parts of Hematoxylin Solution A and Solution B (stable for 10 days). Incubate the sections for **5 minutes** with Weigert's Iron Hematoxylin Solution to stain the nuclei dark. Discard the Hematoxilin solution.
6. **Blueing:** Put the slides in a glass chamber and wash under warm running tap water for ~10 minutes to remove excess of Hematoxylin and to intensify the black color. Rinse **1 minute** in ddH2O.
7. **Cytoplasm Staining:** Incubate the sections for **5 minutes** with Biebrich Scarlet-Acid Fuchsin Solution to stain the fibers red. Discard the Solution.

8. **Washing:** Wash 3x 1minute with ddH2O

9. **Collagen Staining:** To prepare the uptake of the aniline blue stain, incubate the sections with Phosphotungstic/Phosphomolybdic Acid Solution.

   Preparation of Phosphotungstic/Phosphomolybdic Acid Solution (Discard after use):

   - 1 volume of Phosphotungstic Acid Solution, Catalog No. HT15-2
   - 1 volume Phosphomolybdic Acid Solution, Catalog No. HT15-3
   - 2 volumes ddH2O

   If performed in a glass chamber, incubate the slides for 10 minutes. If performed directly on the slides, then the solution has to be exchanged several times: incubate **3-4x 3 minutes.**

   After incubation, drain (not dry!) the slides on a tissue before proceeding and directly (without rinsing) incubate the slides for **5 minutes** with the Aniline Blue Solution to stain collagen in blue**.**

10. **Washing:** Wash 3x 1 minute with ddH2O

11. **Differentiation:** Incubate the sections for **2 minutes** with **1% of Glacial Acetic Acid** to render the shades of color more delicate and transparent. Discard this solution after use. *If blue staining of connective tissue appears faded, the section has probably been overdifferentiated in the acetic acid solution.*

12. **Washing:** Wash 2x 1minute with ddH2O

13. **Dehydration:** Drain successively in
    - 70% EtOH for **3 minutes**
    - 90% EtOH for **3 minutes**
    - 100% EtOH for **3 minutes**
    - Xylol for **5 minutes**

    Drain the slides on a tissue

14. **Mounting:** Mount the slides with 1-2 drops of a xylene-based mounting media (e.g. Depex) and cover with cover slides, avoid bubbles.

15. Press the slides under heavy weight for 10 minutes at room temperature.

16. 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. 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 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.

The fibrotic area can be evaluated either manually (drawing around) or by separating for the blue color in an appropriate image analysis software (e.g. Image Pro Plus, Photoshop). Moreover, centralized nuclei (indicative of regeneration) can be counted as well. 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. In addition, Masson Trichrome staining gives an overview of the fiber size distribution and the shape of the fibers.

6 EVALUATION AND INTERPRETATION OF RESULTS

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

- **Healthy muscle tissue:** Normal myofibers have a polygonal shape with peripheral nuclei. 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. The collagens present in the fibrotic tissue appear in blue upon Masson Trichrome staining. Hydrophobic fat-rich structures (adipocytes, myelin) tend to remain clear.
- **Fiber size and 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. Necrotic fibers stain darker than healthy fibers.

- **Regeneration:** myofibers with central nuclei indicate that the muscle fiber is in the process of regeneration or has recently been regenerated.

7 REFERENCES

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

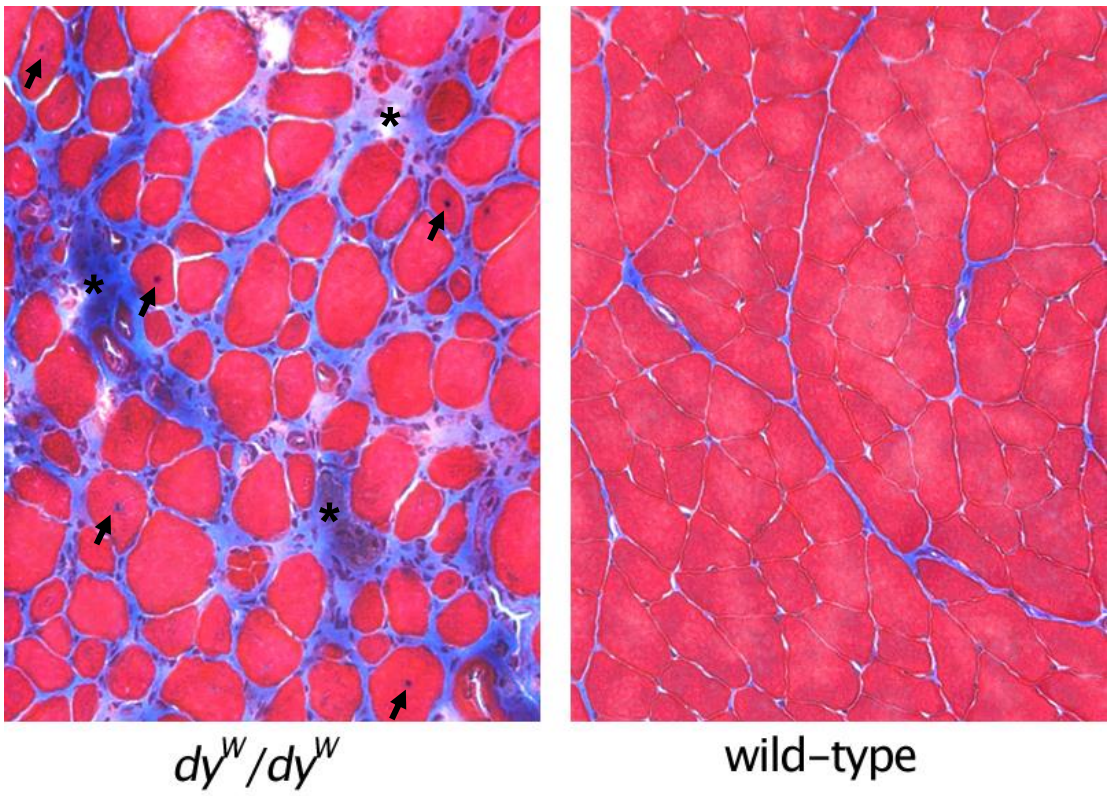

Figure 1. Pathological changes in muscles of *dy*^{W}/*dy*^{W} mice. Fibrotic regions are rich in collagens and therefore appear in blue upon Masson Trichrome staining (asterisks). In addition, centralized nuclei (arrows) as well as the shape and the size distribution of the myofibers are visualized.