Myofiber Damage Evaluation by Evans Blue Dye Injection

Christine I. Wooddell,1 Hannah G. Radley-Crabb,2 Jacob B. Griffin,1 and Guofeng Zhang2

1Roche Madison Inc., Madison, Wisconsin
2School of Anatomy and Human Biology, The University of Western Australia, Crawley, Australia

ABSTRACT

Evans blue dye (EBD) can be used in live mice to study muscle pathology or injury, including exercise-induced muscle damage. EBD is excluded from intact cell membranes but leaks into cells, including muscle fibers, when the cell membrane is ruptured. EBD can be visualized by its autofluorescence under a fluorescence microscope. EBD-stained myofibers can be quantified from microscope images of muscle cross-sections. These myofibers are often in clusters that lend themselves to morphometric analysis. When the damaged myofibers are interspersed among intact myofibers, however, a more suitable approach is to count individual myofibers in the field of view. A much faster approach to measure EBD in muscles from different strains of mice or between treatment groups is to extract the EBD from muscle samples and quantitate it using a spectrophotometric microplate reader. The advantages and disadvantages of using each of these approaches are discussed here. Curr. Protoc. Mouse Biol. 1:463-488 © 2011 by John Wiley & Sons, Inc.

Keywords: Evans blue dye • skeletal muscle • mouse model • mdx mouse

INTRODUCTION

Evans blue dye (EBD) is a cell membrane-impermeable tetrasodium diazo salt (mol. wt. 960.82 g/mol) that has been used as a tracer to study the vasculature of living animals and to evaluate the integrity of cell membranes in vivo (Reeve, 1957). EBD is usually delivered into the vasculature of mice by injecting it into the tail vein or into the peritoneum (Hamer et al., 2002). The injected EBD binds to albumin in the blood and the EBD-albumin conjugate circulates through the vasculature. The EBD-albumin conjugate is excluded from cells by membranes that are intact, but it leaks into and accumulates in myofibers that are damaged by rupture of the plasma membrane (Straub et al., 1997). Thus, EBD can be used for phenotypic characterization of mouse strains that have muscle pathologies such as muscular dystrophy (Straub et al., 1997). Leaky myofibers may also be the result of muscle injury in otherwise healthy animals. EBD is, therefore, useful for studying muscle pathology, muscle injury, and the effects of exercise on myofibers.

The protocols describe procedures for measuring skeletal muscle marked by EBD caused by permeable (damaged) myofiber membranes. Basic Protocol 1 describes two options for delivering EBD into the mouse. Basic Protocols 2 through 4 describe separate approaches for quantitation of EBD staining. More specifically, Basic Protocols 2 and 3 present two different procedures based upon morphometrical analysis for collecting and freezing muscle samples suitable for sectioning. One procedure involves the stabilization and orientation of muscle samples in tragacanth gum, followed by freezing in a slurry of isopentane in liquid nitrogen (see Basic Protocol 2). The other procedure involves muscle stabilization and orientation in embedding molds with O.C.T. (see Basic Protocol 3 and Support Protocols 1 and 2). Both procedures ultimately arrive at the same end point (a muscle sample that can be cut on the cryostat to produce muscle sections) and could be...
interchanged, depending on laboratory facilities. However, for consistency, all muscle samples from an experiment should be prepared the same way. Finally, Basic Protocol 4 details a rapid alternative quantitative method for EBD measurement, which is suitable for analysis when total uptake of EBD is expected to be significant between treatment groups. In the Commentary section, some of the considerations that will help researchers select the most suitable approach for their intended application are discussed.

The protocols presented here focus on evaluation of skeletal muscles of the mouse hind leg as a model system. The hind legs contain the largest muscle mass in the mouse. Another advantage of evaluating the leg muscles is that these can be challenged by exercise protocols. Most of the methods used to evaluate the leg muscles could likely be applied to other muscles.

**BASIC PROTOCOL 1**

**DELIVERY OF EBD**

There are two commonly used approaches to delivering EBD into the mouse vasculature. The EBD solution can be injected intravenously (i.v.) into either the lateral vein of the tail or it can be injected intraperitoneally (i.p.). The advantage of i.v. injection is that the EBD solution is immediately available through the vasculature. Intravenous injection of EBD is the more technically challenging delivery technique, but carries the advantage of immediate availability through the vasculature, which may be desirable for time-critical studies. Intraperitoneal EBD injection, while less demanding of user skill and precision, necessitates a several-hour waiting period to allow for appropriate systemic dye dispersion, before animals can be evaluated. Approximately twice as much EBD is used for the i.p. injection compared to i.v. injection. The mouse should be injected in the lower portion of the abdomen, to avoid damaging the liver, and also away from the midline to avoid the bladder.

**Materials**

- Mice
- Evans blue dye (Sigma-Aldrich, cat. no. E2129) prepared at 5 mg/ml with sterile physiological saline (0.90% NaCl) (see recipe)
- 1% to 2% isoflurane
- Phosphate buffered saline (PBS)
- Heat lamp or 50-ml conical tube containing warm water (50°C)
- 1-ml syringe with 30-G needle for i.v. injection or 500-μl or 1-ml syringe with 27-G needle for i.p. injection
- Anesthesia machine with animal chamber or an animal holder to restrain the mouse during injection

**Injection of EBD solution into the tail vein (i.v.)**

Injecting into the tail vein requires some skill because the needle must puncture the vein in only one spot and remain in the lumen of the vein during the injection. The tail vein is more easily visualized in white mice than in darkly pigmented mice, which may be useful for training purposes.

1a. Weigh each mouse and record its weight along with identifying information, e.g., ear tag number. Calculate how much EBD solution to inject into each animal (50 μl of 5 mg/ml EBD solution per 10 g body weight).

2a. At least 30 min prior to injection, place the cage containing the mouse to be injected under a warming lamp to dilate its blood vessels.

*If a heat lamp is not available, place the tail into a tube of warm water (~50°C) for 1 to 2 min to dilate the blood vessels.*
3a. Using a 1-ml syringe with a 30-G needle, draw the 5 mg/ml EBD solution into the syringe. Remove all air bubbles from the syringe by flicking the sides of the syringe.

4a. Anesthetize mouse with 1% to 2% isoflurane.

   *If an anesthesia machine is not available, a mouse restraining structure (e.g., tube) can be prepared to facilitate the injection. This is made from a 50-ml conical polypropylene centrifuge tube. Cut a breathing hole with a diameter of 0.5 to 1 cm in the bottom of the conical tube. Make a 3-cm long and 0.3-cm wide notch on the upper edge of the tube, perpendicular to the rim. Place the mouse inside the tube with its tail sticking out of the notch. Continue to hold the tail while performing the injection."

5a. Lay the mouse on its side and hold the distal part of the tail with thumb and fourth finger. Position the tail between the index and middle fingers.

6a. Using index and middle fingers, put some pressure on the proximal part of tail to block blood flow in the tail, which causes the tail to brim with blood and allows for easier injection.

7a. Inject the calculated quantity of EBD solution slowly into the tail vein.

8a. Place the mouse back into its cage.

**Injection of EBD solution into the peritoneum (i.p.)**

1b. Weigh each mouse and record its weight along with identifying information, e.g., ear tag number. Calculate how much EBD solution to inject into each animal (100 μl of 5 mg/ml EBD solution per 10 g body weight).

2b. Using a 500-μl or 1-ml syringe with a 27-G needle, draw the EBD solution into the syringe. Remove all air bubbles by flicking the side of the syringe.

3b. Anesthetize mouse as in step 4a.

4b. Holding the abdominal skin away from its body using fingers or forceps, slowly insert the needle into the lower area of the peritoneal cavity from the side and in the direction of the midline, being careful not to damage any organs by inserting the needle too far.

5b. Slowly inject the calculated amount of EBD solution.

6b. Place mouse back into its cage.

**Alternative approach for i.p. injection of EBD solution without anesthesia**

1c. Freshly prepare solution of 1% EBD in PBS.

2c. Weigh each mouse and record its weight along with identifying information, e.g., ear tag number. Calculate how much EBD solution to inject into each animal (100 μl sterile EBD solution per 10 g body weight).

3c. Using a 500-μl syringe with a 27-G needle, draw the sterile EBD solution into the syringe. Remove all air bubbles from the syringe by flicking the sides of the syringe.

4c. Pick up and immobilize the mouse by pinching across the shoulder blades (use thumb and forefinger). Expose the abdomen by holding the tail out of the way (use little finger) and insert the needle slowly into the peritoneal cavity from the side, aiming toward the vicinity of the nipples. If right handed, use left hand to hold mouse and right to hold syringe.

   *If you inadvertently insert the needle into the bladder during i.p. injection of the EBD, blue dye will be evident in the urine within ~5 min.*

5c. Slowly inject the calculated amount of EBD solution.
6c. Return mouse to its cage.

This protocol is appropriate for adult mice when the researcher desires to limit the exposure of mice to anesthesia. Use of gaseous anesthesia is preferable for performing i.p. injections on young mice (≤4 weeks old) as it reduces the risk of injury in very small mice.

MORPHOMETRIC QUANTITATION OF AREAS WITHIN THE MUSCLE

The presence of EBD in skeletal muscle can be formally quantitated in transverse muscle sections. EBD-positive myofibers are visualized using fluorescence microscopy and the percentages quantitated by morphometric analysis (Fig. 1) as per Piers et al. (2011) and Shavlakadze et al. (2004). This procedure utilizes frozen histology methods and thus allows for a relatively quick evaluation by avoiding paraffin processing. Multiple sections can be cut from the same frozen muscle sample, allowing serial sections to be stained with hematoxylin and eosin (H&E) stain to assess muscle morphology or by immunohistochemistry to identify other proteins of interest. One limitation of this technique, as with all frozen histology, is the minor risk of ending up with a freeze fracture in the muscle samples.

Materials

- Tragacanth gum (Sigma Aldrich, cat. no. G1128)
- Isopentane
- Liquid nitrogen
- Isoflurane
- Acetone
- Xylene
- DPX mountant glue (BDH, cat. no. 36029.4H)
- Cork (∼1 cm³ per sample)
- Cryostat
- Glass histology slides (VWR Superfrost Plus Micro slides, cat. no. 48311-703)
- Glass cover slips
- Fluorescence microscope (band pass: excitation 515 to 560 nm; low pass: emission 590 nm) with camera and image capture software (e.g., Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 6.2 software, and Vexta stage movement software)

Figure 1  Morphometric quantitation of EBD-stained myofibers in the tibialis anterior muscle from dystrophic mice (mdx, left; and mdx/IGF.1, right) given 1% EBD in PBS (pH 7.5) by i.p. injection 24 hr prior to sacrifice. Adapted from Shavlakadze et al. (2004). Reprinted with permission from Macmillan Publishers.
Prepare reagents and equipment
1. Mix the tragacanth gum solution 1 day before sampling and refrigerate overnight (prepare as per manufacturer’s instructions).
   
   *Ideally, the tragacanth gum should be the consistency of toothpaste.*

2. Cut cork into ~1-cm³ squares prior to harvesting muscle.

3. Just before harvesting muscles, prepare a slurry of isopentane cooled in liquid nitrogen. Place the small container of isopentane into a larger container of liquid nitrogen, allowing the isopentane to be cooled without mixing with the liquid nitrogen.

Harvest muscle
4. Sacrifice mice as per institutionally approved animal ethics committee protocols (e.g., anesthetize with isoflurane followed by cervical dislocation).

5. Peel away and remove the skin from the limbs to expose the muscles underneath.

6. Dissect out the muscles of interest (e.g., tibialis anterior or quadriceps).

   *This can be done by removing any covering connective tissue, cutting the appropriate tendons, and lifting out the whole muscle, or by sliding a surgical blade under the belly of the muscle and slicing it away from the bone.*

7. Using a surgical scalpel blade, bisect the fresh muscle transversely (in the center and perpendicular to the grain). Cover the top of the cork square with a small layer (8-mm) of tragacanth gum. Mount the pieces of muscle in the tragacanth gum.

   *The tragacanth gum stabilizes the muscle and maintains myofiber orientation. A 1-cm³ piece of cork is large enough to fit both halves of the tibialis anterior muscle side by side, whereas the quadriceps muscle will require two pieces of cork.*

8. Freeze the muscles in a slurry of isopentane cooled in liquid nitrogen by placing the muscles into the isopentane slurry for ~20 sec or until the tragacanth gum turns white.

   *Isopentane reduces surface tension, thus producing a frozen sample that is excellent for histological evaluation with little or no freezing artifacts.*

Process muscle
9. Using a cryostat, cut frozen muscle sections ~8-μm thick directly onto uncoated or silinated glass histology slides.

   *Slides can be stored for ~2 weeks at −20°C until ready to visualize.*

10. Fix muscle sections 1 min using cold acetone (−20°C) and then air dry for 2 to 3 min at ambient temperature (25°C).

11. Dip slides in three changes of xylene, 3 min each time.

12. Cover muscle sections with a small amount of DPX mountant glue (~0.2 ml, depending on size of cover slip) and then mount a glass cover slip over the glue; avoid creating air bubbles.

Collect images
13. View unstained frozen sections by fluorescence microscopy.

   *EBD autofluoresces red under green light (band pass: excitation 515 to 560 nm; low pass: emission 590 nm).*

14. Non-overlapping images of a transverse muscle section can be tiled/stitched together to provide a single digital image of the entire muscle cross-sectional area. To do this, acquire images using a fluorescence microscope, digital camera, and image capture.
software (e.g., Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 6.2 software, and Vexta stage movement software).

More sophisticated digital slide scanners (e.g., Aperio Scanscope) can be used to collect images, although fewer researchers are likely to have access to such machines.

Morphological analysis can be performed on multiple single frame images taken from the same muscle section (multiple fields of view) rather than analyzing the entire cross-sectional area. However, this method assumes that EBD$^+$ myofibers are homogeneous throughout the muscle cross-section, which may not always be true. In the absence of software that allows stitching together images, sequential images can be taken across the diameter of the muscle and evaluated separately.

**Analyze images and quantitate EBD**

15. Identify the EBD$^+$ myofibers manually and then quantify them using image analysis software (e.g., Image Pro Plus). To quantitate the abundance of EBD$^+$ myofibers per muscle cross-sectional area, first open the digital image in the image analysis software (e.g., Image Pro Plus) and measure (draw around) the entire cross-section area of the muscle. This can be done in pixels (sufficient when determining a percentage) or the software can be calibrated and features measured as an absolute value (e.g., $\mu m^2$). Second, identify and manually measure (draw around) all the EBD$^+$ myofibers. The data can be exported directly into Microsoft Excel and the percentage of EBD$^+$ myofibers calculated as:

\[
\text{Percentage of EBD}^+ = \left( \frac{\text{total area of EBD}^+ \text{ myofibers}}{\text{total area of muscle cross-section}} \right) \times 100
\]

Then perform appropriate statistical analyses.

**DETERMINING PERCENTAGES OF EBD-POSITIVE MYOFIBERS**

To determine as accurately as possible the percentages of EBD$^+$ myofibers in the mouse leg, an investigator will need to select a sufficient number of muscle sections to evaluate. These should be representative of the whole. Many of the individual muscles in the mouse leg are small. Rather than attempting to evaluate each individual muscle, the mouse leg can be divided into five large groups of muscles: muscles in the anterior, posterior, and

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**Figure 2** Dissection of the mouse leg into groups of muscles. Muscle groups 1 to 5 are defined by their anatomical location as shown in this color-coded schematic.
medial regions of the upper leg and the anterior and posterior regions of the lower leg (Fig. 2). Multiple cross-sections (specimens) are then cut from each group of muscles and microscope images captured along the entire diameter of the specimen. Dividing the leg into five muscle groups allows sufficient sample to be evaluated by multiple methods. For example, it may be preferred to evaluate the leg muscles by counting how many muscle fibers are positive for EBD and how many muscle fibers are positive for a protein of interest as determined by immunohistochemistry (IHC) or to determine the concentration of a protein of interest by immunoblotting, e.g., dystrophin (Fig. 3).

Preparation of multiple cross-sections is described in this protocol, while determining the percentage of EBD-positive fibers within these sections is detailed in Support Protocol 1. Due to the difficulty of visualizing individual fibers that are not stained by EBD, immunohistochemistry is used to outline fibers. For this purpose, staining for dystrophin is implemented, and the method for generating anti-dystrophin antibodies is described in Support Protocol 2. While success has been achieved using anti-dystrophin antibodies generated by this method, it is expected that polyclonal anti-dystrophin antibodies obtained by other methods and possibly mouse monoclonal antibodies to mouse dystrophin would serve equally well.

**Figure 3** Overlay of EBD staining and dystrophin immunohistochemistry. (A, B) The mdx mouse was given six hydrodynamic limb vein injections with dystrophin-expressing pDNA into the hind legs. When it was 18 months old, it was exercised on a treadmill at 12 m/min for 10 min, injected with EBD the next day, and sacrificed for evaluation 24 hr after this. Consecutive muscle sections were stained for dystrophin (FITC), left panels, or stained with hematoxylin and eosin for evaluation of the histology, right panels. The FITC images were overlapped with images of EBD auto-fluorescence (in the CY3 channel) from the same muscle sections. The FITC-labeled anti-mouse IgG secondary antibody labeled dystrophin expressing myofibers (arrows). This secondary antibody also labeled the endogenous IgG antibodies that accumulated within damaged myofibers (*) in A. Myofibers staining intensely with EBD (*) and those staining only lightly (L) are shown in the merged images and corresponding histology sections from the medial thigh muscles of the same mouse. This figure was adapted from Zhang et al. (2010).
**Materials**

- Tissue-Tek O.C.T. compound (Sakura Finetek, cat. no. 4583)
- Mice
- 3% to 5% isoflurane
- Liquid nitrogen
- Styrofoam container for holding liquid nitrogen and Styrofoam float for holding embedding molds while freezing in liquid nitrogen (Fig. 4)
- Peel-A-Way disposable embedding molds, truncated (8 × 8 × 20–mm; PolySciences, cat. no. 18985)

1. Produce a Styrofoam float rack by cutting off the top of a Styrofoam rack that comes with 50-ml conical polypropylene tubes (Fig. 4), making the Styrofoam float between 2.0- and 2.5-cm thick to ensure that the embedding molds come into contact with the liquid nitrogen and the muscle freezes quickly.

2. Prepare embedding molds before harvesting muscles as follows.
   a. Label an embedding mold for each muscle sample that will be harvested (one group of muscles), including animal number, muscle group number, and left (L) or right (R) leg.
   b. Right before embedding, fill the embedding molds 5- to 7-mm deep with O.C.T. freezing compound.

   *Blocks of O.C.T. containing specimens frozen in embedding molds that are not truncated at the end (such as those shown in Fig. 4) can be truncated by hand to resemble blocks frozen in the truncated molds.*

3. Anesthetize the mouse with 3% to 5% isoflurane, then euthanize by cervical dislocation (or per approved institutional animal ethics protocols).

4. Remove the skin from the hind legs to expose the muscles.

5. Divide the muscles into five groups as indicated by the color coding in Figure 2:

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**Figure 4** Preparation for embedding muscle in freezing medium. Cut out Styrofoam float racks from the Styrofoam tube-holding racks that come with 50-ml conical polypropylene tubes. Draw a line on the Styrofoam rack as shown so that the float rack will be 2.0 to 2.5 cm in height. Embedding molds fit into the holes in the float rack, allowing liquid nitrogen to contact the embedding mold containing O.C.T. freezing compound when the float rack is placed into a Styrofoam box containing liquid nitrogen. Photograph provided by Julia Hegge and Tracie Milarch (Roche Madison).
a. Muscle group no. 1: the anterior group of muscles of the upper leg. This group includes the quadriceps. It comprises \( \sim 20\% \) of the leg muscle mass.

b. Muscle group no. 2: the posterior group of muscles of the upper leg. This group includes the biceps femoris and comprises \( \sim 25\% \) of leg.

c. Muscle group no. 3: the medial group of muscles of the upper leg. This group includes the gracilis and adductor muscles. It comprises \( \sim 25\% \) of the leg muscle mass.

d. Muscle group no. 4: the posterior group of muscles of the lower leg. This group includes the gastrocnemius and soleus. It comprises \( \sim 20\% \) of the leg muscle mass.

e. Muscle group no. 5: the anterior group of muscles of the lower leg that is comprised primarily of the tibialis anterior and extensor digitorum longus. This group includes \( \sim 10\% \) of the leg muscle mass.

6. Freeze the muscle immediately after harvesting in Tissue-Tek O.C.T. compound. The integrity of muscle samples is preserved by rapidly freezing them in tissue freezing medium. The muscle pieces must be oriented in a consistent manner to obtain high-quality cross-sections. This can be achieved by freezing each muscle sample immediately after adding it to the freezing medium in an embedding cup.

a. Embed each muscle with the grain of the muscle in a vertical position within the embedding cup, taking care not to trap air bubbles around the muscle.

b. Add O.C.T. to cover the muscle sample by 3 to 5 mm.

c. Fill the Styrofoam container with enough liquid nitrogen that the Styrofoam float rack will float.

d. Place each embedding mold into the Styrofoam float rack, and then set the float rack into the liquid nitrogen. Take care to keep the muscle piece in a vertical orientation while it is freezing to allow for high-quality cross-sectioning.

e. Wait until the O.C.T. turns hard and solid white. Then, move the embedding molds to a container of dry ice. Place muscle samples from each animal into a separate plastic storage bag. Store the frozen O.C.T. blocks preserved up to 1 year at \(-80^\circ C\).

7. Section the frozen tissue when ready to proceed with immunostaining (see Support Protocol 1).

COUNTING PERCENTAGE OF EBD\(^+\) MYOFIBERS

Determining the percentage of EBD\(^+\) myofibers requires counting the total number of myofibers in the muscle cross-sections. The EBD autofluoresces, so EBD\(^+\) myofibers can easily be counted when they are interspersed among the non-stained myofibers. They are more difficult to distinguish when groups of them are clustered together as can be seen in Figure 5. Individual myofibers within the specimen that are not EBD-stained can also be difficult to distinguish unless a method to outline individual myofibers is used, e.g., an immunohistochemistry staining method utilizing antibodies to label the dystrophin that is associated with the cell membrane, which results in a crisp outline of each myofiber.

In mice such as \textit{mdx} that do not produce dystrophin, anti-mouse IgG antibodies can be used instead to outline the myofiber as shown in Figure 6B. Antibodies to other blood proteins serve the same function (see Fig. 6C.D), although the myofiber membrane is generally not outlined as distinctly when targeting blood proteins as it is when labeling the dystrophin in mice that have the wild-type dystrophin protein.

\textbf{Materials}

Frozen tissue (see Basic Protocol 3)
2\% to 4\% formalin in PBS
PBS
Mouse anti-dystrophin polyclonal antibodies diluted as needed in PBS (see Support Protocol 2)
FITC-conjugated goat anti-mouse IgG (FAB-specific; Sigma-Aldrich, cat. no. F8771) diluted 1:400 in PBS

Cryostat
Glass histology slides (VWR Superfrost Plus Micro slides, cat. no. 48311-703)
Paraffin pen
Microscope, excitation filters (FITC and CY3), camera

Prepare sections and fix
1. Using a cryostat, begin cutting 8- to 10-μm cross-sections from the frozen tissue. Section approximately one-third off the end of each large muscle piece frozen in O.C.T. and less off small muscle pieces prior to collecting specimens.

2. Cut two adjacent sections and place these sections onto a slide. Discard the subsequent ten sections. Cut two more adjacent sections and place on same slide. Repeat this procedure until four to six sections from one muscle sample (a group of muscles) is placed onto the slide. Repeat this process for each muscle sample.

3. Fix tissue slices in 2% to 4% formalin in PBS for 5 to 10 min.

4. Rinse sections three times with PBS for 2 min each time.

Immunostain samples
5. Circle each specimen on the slide with a paraffin pen.

6. Pipet enough polyclonal anti-dystrophin antibodies in PBS onto the specimen to cover the sample (the paraffin circle made around the sample contains the fluid).

   The proper dilution of the mouse anti-dystrophin polyclonal antibodies in PBS is the one that provides clear staining without excessive background.

7. Incubate 40 to 60 min at 25°C.

Figure 5
Dark and light EBD staining compared to histological staining. Myofibers containing dark (intense) or light EBD fluorescence were compared by histological H&E staining on sections of hind leg muscle from a 2-month-old mdx mouse that had been exercised on a treadmill. Consecutive frozen muscle sections from the gastrocnemius were evaluated for EBD fluorescence (A), or stained with H&E for histological evaluation (B). A few of the dark (D) and light (L) EBD-stained myofibers are indicated. Scale bar indicates 100 μm. This figure is from Wooddell et al. (2010).
8. Rinse the sections three times with PBS for 2 min each time.

9. Incubate the sections in FITC-conjugated goat anti-mouse IgG in PBS for 40 min at 25°C.

10. Rinse sections three times with PBS, 2 min each time.

11. Keep sections in the dark until ready to image.

   Typically, images are collected from the specimen covered with some PBS to keep specimen moist, but without adding a cover slip. If images will not be collected immediately, then place a cover slip over the specimen to prevent the sample from drying, which would result in high background, and store for up to 2 days at 4°C.

**Count myofibers**

12. Take a series of images from one representative specimen from each slide that corresponds to one muscle group from one leg. At each microscope position take two images by using two different excitation filters, one for FITC (appears green) and one for CY3 (appears red). When looking at the specimen on the slide under the microscope, begin taking images at the edge farthest from you (or closest to the top of the image). Take the next image right below the first. Continue taking sequential images across the diameter of the muscle specimen until the bottom of the specimen is reached.
13. Merge the two images taken at each microscope position, those taken with the FITC and CY3 filters.

14. Print out the merged images from each microscope position. Count the total number of myofibers in each image.

15. Count EBD+ myofibers on the computer screen. The bright yellow-green myofibers are those that stain intensely with EBD and contain IgG that leaked in through the damaged cell membrane. The myofibers that are only lightly EBD+ appear to be red. See Figure 3 as an example. Decide whether to count all EBD+ myofibers together, only the intensely EBD+ myofibers, or to separately count the intensely and lightly stained myofibers.

16. Combine in data tables for each muscle group the total number of myofibers, the total number of intensely stained EBD+ myofibers, and the total number of lightly EBD+ myofibers (if desired). For each muscle group, the percentage of EBD+ myofibers is determined by dividing the total EBD+ myofibers by the total number of myofibers counted.

**Analyze**

17. Determine the weighted percentages of EBD+ myofibers in the entire hind limb using the formula: \[
\left(\sum \frac{A_1 M_1 + A_2 M_2 + A_3 M_3 + A_4 M_4 + A_5 M_5}{M_t}\right)/M_t,
\] where \(A_i\) is the average number of EBD+ myofibers counted in muscle group \(i\) and \(M_i\) is the weight of that muscle group, and \(M_t\) is the total weight of all groups of muscles in the leg. These averages can be used to compare groups of mice or different treatments.

**Alternative approaches**

The amount of labor involved in comparing the number of EBD+ myofibers between groups of animals and treatments can be reduced by the following approach. Instead of counting the total myofibers in each microscope view, count only the EBD+ myofibers

![Graphs showing EBD+ myofibers in unexercised and exercised limbs](image)

**Figure 7** Counting EBD+ myofibers. (A) Total EBD+ myofibers (dark and light) were counted in ten to eleven microscope views across the diameter of muscle specimens from each of the 5 groups of muscles from the hind limbs of 13- to 19-month-old mdx mice that were either unexercised or had been exercised by running on a treadmill or a rotarod. A weighted average of EBD+ myofibers/view in the entire mouse limb was determined by taking into account the weight of each muscle group. (B) The total numbers of EBD+ myofibers in each of the 5 groups of muscles of the hind limbs of young (4- to 6-month-old) and 13- to 19-month-old mdx mice are evaluated as collectives of muscle samples. Young and old mice were unexercised (E−) or exercised (E+). \(p = 0.434\) for young adult and \(p < 0.0001\) for old animals. Each point represents the average number of EBD+ myofibers per microscope view for one group of muscles (one sample). Figure adapted from Wooddell et al. (2010).
Figure 8  Use of EBD for a gene therapy experiment. (A, B) In a gene therapy study, *mdx* mice were given 6 hydrodynamic limb vein injections with a dystrophin-expressing plasmid (Dys) in one leg and a control plasmid in the other leg. At the end of the study, the mice were exercised on a treadmill for 15 to 30 min at 12 m/min and then evaluated for EBD$^+$ myofibers in each muscle group of the legs. (A) The percentages of EBD$^+$ myofibers in the control leg muscles were arbitrarily set at 100% to determine the relative percentages of EBD$^+$ myofibers in the contra-lateral Dys-injected leg muscles. The average normalized EBD levels are shown for each group of muscles. (B) Each data point represents the weighted average percentage of EBD$^+$ myofibers in one leg. Statistical analysis comparing contra-lateral pairs of muscles was performed with a two-tailed, Wilcoxon signed rank test ($** p < 0.01$). These data are from Zhang et al. (2010).

When the variability in EBD staining is high within the animals that are being compared, as is the case with *mdx* mice, the power of analysis can be improved by comparing samples as collective groups of muscles. See Figure 7B; in this case, the weight of the samples is not taken into account. All samples contribute equally to the resulting average. If one particular group of muscles skews the results, perform the analysis with and without that group of muscles. For example, the tibialis anterior of muscle group no. 5 may be much more affected by an exercise protocol than the rest of the leg, although all of muscle group no. 5 comprises only 10% of the whole leg.

When one leg of the mouse can be used as a control for a treatment given to the other leg, then pairs of muscle groups from the left and right legs can be compared using a two-tailed, Wilcoxon signed rank test for statistical analysis using GraphPad Prism software (e.g., Fig. 8).

**PRODUCTION OF MOUSE POLYCLONAL ANTIBODIES TO DYSTROPHIN**

Naked plasmid DNA expressing a protein of interest can be delivered to mouse muscle to generate polyclonal antibodies against the expressed gene product, as described in Bates et al. (2006). Hydrodynamic limb vein (HLV) injection delivers the pDNA to skeletal muscles (Hagstrom et al., 2004). This procedure is referred to as genetic immunization. The reader is referred to Bates et al. (2006) for details regarding this method of antibody generation. Here, specific details for generating anti-dystrophin antibodies by HLV injection of dystrophin-expressing pDNA into the saphenous vein of an *mdx* mouse are described. The dystrophin protein is large (∼500 kD) and has conserved epitopes between mouse and human dystrophin. Therefore, pDNA expressing either human or mouse
dystrophin can be used to generate antibodies in a dystrophin-deficient mouse that will cross-react with mouse dystrophin. Anti-dystrophin antibodies can also be produced in rats using the same procedure (Bates et al., 2006).

Anti-dystrophin antibodies are usually produced after two HLV injections of the dystrophin pDNA. Sometimes a single injection is sufficient. Antibody production is confirmed using either an enzyme-linked immunosorbent assay (ELISA) or by immunohistochemistry (IHC), as described in Bates et al. (2006). The IHC test is described below.

**Materials**

- pDNA that produces human or mouse dystrophin (prepare or order endotoxin-free plasmid DNA)
- Sterile, physiological saline solution
- C57Bl/10ScSn-\textit{Dmd}\textsuperscript{mdx/J} (\textit{mdx}-10ScSn) mice (Jackson Laboratories)
- 1% to 2% isoflurane anesthesia
- Ketoprofen
- Serum tubes with gel and clot activator
- 50-ml polypropylene tubes
- Heating pad at 37°C
- Scalpel
- Latex tourniquet
- 4-0 absorbable sutures
- 1-ml syringes
- Additional reagents and equipment for muscle harvesting, processing, and immunostaining (see Basic Protocol 3 and Support Protocol 1) and HLV injection (Hagstrom et al., 2004)

**Immunize mice**

1. In a 50-ml tube, prepare 50 to 100 μg of dystrophin expression pDNA in a physiological saline solution at 25°C such that the final volume is 1 ml per injection. *The commonly used cytomegalovirus (CMV) promoter works well for driving expression of this protein from pDNA in muscle (Bates et al., 2006; Zhang et al., 2010).*

2. Anesthetize the mouse with 1% to 2% isoflurane and then place mouse on a 37°C heating pad for surgery.

3. Using a scalpel, make a small incision near the ankle of the C57Bl/10ScSn-\textit{Dmd}\textsuperscript{mdx/J} (\textit{mdx}-10ScSn) mouse to visualize the saphenous vein. *Other strains of \textit{mdx} mice can be used as well, but these are less readily available.*

4. Using a latex tourniquet around the upper part of the mouse hind limb to block blood flow as described (Bates et al., 2006), perform an HLV injection with 1 ml of pDNA/saline solution into the saphenous vein of the \textit{mdx} mouse hind limb at a rate of 8 ml/min. For the HLV injection, refer to Hagstrom et al. (2004) for a detailed description.

5. Close the incision with a 4-0 suture. Give the mouse a subcutaneous injection of ketoprofen (5.0 mg/kg) as analgesia while it is still anesthetized. Keep the mouse on the heating pad until it awakens.

6. Repeat the HLV injection 2 weeks later.

7. Ten days after the second pDNA injection, collect blood from the mouse by retro-orbital bleed. Isolate serum using serum tubes with gel and clot activator according to the manufacturer’s instructions.
**Immunohistochemistry test for antibody production**

8. Use a wild-type mouse for IHC testing of the antibody. Sacrifice the mouse, harvest hind leg muscle, freeze muscle in O.C.T., and cut 8-μm thick cross-sections of the muscle as described for muscle sample preparation for myofiber counting.

9. As a negative control, use wild-type mouse serum.

   *This control will demonstrate the background labeling from the secondary antibody.*

10. Prepare the following dilutions of serum from the pDNA-injected mouse in PBS: 1:20, 1:40, 1:100, 1:200, 1:400, and 1:800.

11. Follow the steps described for dystrophin immunostaining in Support Protocol 1, including a 1:400 dilution of FITC-goat anti-mouse IgG.

12. Use the dilution that shows clear outlines around each myofiber with a minimum of background fluorescence for immunostaining.

   *If the antibodies do not give adequate immunostaining when diluted at 1:100, then give the mouse an additional HLV injection of the dystrophin pDNA.*

13. When the antibody titer is adequate for use at a 1:100 or greater dilution, then collect additional serum from the mouse as follows:

   a. Anesthetize the mouse with 3% to 5% isoflurane until it is fully unconscious.
   b. Open the chest cavity of the mouse by making an incision through the abdominal wall and extending it to the upper chest. Avoid cutting the diaphragm.
   c. Hold the right lung and cut it off through the root of the lung. Blood will then flow into the chest cavity.
   d. Collect the blood by aspirating slowly into a 1-ml syringe.
   e. Sacrifice the mouse promptly by cervical dislocation. Do not allow it to regain consciousness.
   f. Allow 15 min for blood to clot. Separate the serum using serum tubes with gel and clot activator. Centrifuge 2 min at 8000 × g, 4°C.
   g. Dispense the serum into 5- to 10-μl aliquots per tube and freeze for up to at least 3 years at −80°C.

**SPECTROPHOTOMETRIC QUANTITATION**

Muscle samples from which EBD will be extracted may be fresh, snap frozen without freezing medium, or frozen in O.C.T. freezing compound. If muscles are frozen in O.C.T., rinse under cold running water just until the frozen O.C.T. melts off, taking care to keep the muscle specimen frozen as much as possible. Muscle samples must be frozen prior to grinding to generate a fine powder from which the EBD is extracted using N,N-dimethyl formamide (DMF).

The extracted EBD can be quantitated using either a spectrophotometer or a fluorimeter. Using a spectrophotometric microplate reader is straightforward, fast, and convenient. In this case, absorbance is measured at 630 nm. Standards should be prepared such that the samples will fall within range of the standards. This protocol describes the range that is convenient for studies with leg muscle samples from *mdx* and wild-type mice.

**Materials**

- EBD (see recipe)
- N,N-dimethyl formamide (DMF)
- Liquid nitrogen
- Muscle samples
Prepare for EBD extraction
1. Generate a spreadsheet with all sample numbers and spaces to record the whole muscle sample weight as well as the quantity of powdered muscle used for the extraction.

2. Prepare dilutions of EBD in DMF for a standard curve as shown in Figure 9, beginning with 8.0 μg/ml EBD. Prepare serial two-fold dilutions down to 0.061 μg/ml. Prepare additional standards in 0.5-μg/ml increments to cover the range from 8.0 μg/ml down to 3.0 μg/ml.

   If absorbance from samples falls outside the range of standards used, then prepare additional concentrations of EBD as needed.

3. Set up a mortar and pestle with a container of liquid nitrogen that can be poured into the chilling chamber under the mortar as needed to keep tissue samples extremely cold.

4. Pre-chill 1.5-ml microcentrifuge tubes on dry ice.

Perform EBD extraction
5. Weigh and record each muscle sample as fast as possible to keep cold.

6. Freeze muscle samples in liquid nitrogen or at −80°C.

   When a tissue sample is placed into a tube prior to freezing, it often sticks to the side of the tube, making it difficult to remove. Dropping the piece of tissue directly into liquid nitrogen allows it to freeze without sticking to the tube and then remove from liquid nitrogen with forceps.

7. Grind the frozen muscle sample into powder using a mortar and pestle. Place ground sample into a pre-chilled 1.5-ml microcentrifuge tube (or larger if needed), keeping tissue as cold as possible.

Figure 9  Standard curve prepared with EBD. Dilutions of EBD in DMF were used to generate a standard curve. A graph and standard curve formula can be generated by plotting the data using Microsoft Excel, although this graph was generated by GraphPad Prism.
8. Weigh 50 mg of this muscle powder as quickly as possible to keep cold and place into a clean pre-chilled 1.5-ml microcentrifuge tube. Tare the tube before adding muscle powder and weigh afterward to determine the actual weight.

9. Add 1 ml DMF to each tube of muscle powder and mix well using a vortexer.

10. Incubate with continual rotational mixing (on a rotator) for 24 hr at 25°C.

11. Centrifuge tube 10 min at 6500 × g, 25°C.

12. Transfer the blue supernatant containing the EBD to a fresh 1.5-ml microcentrifuge tube.

Quantitate EBD in muscle samples
13. Prepare a 96-well UV-transparent microplate with duplicate samples by pipetting 200 μl sample to each well. Be sure to include the standard curve dilutions on the plate as well as a blank with DMF only.

14. Use a spectrophotometric microplate reader to measure the absorbance at 630 nm ($A_{\text{max}}$).

15. Use Excel to determine the concentration of EBD in each sample using the formula generated from the standard curve (see Fig. 9).

Analyze EBD in muscle samples
16. Compare whole limbs (see example in Fig. 10A) as follows:

a. Determine the weighted percentages of EBD in the entire hind limb using the formula: ($\sum A_1M_1 + A_2M_2 + A_3M_3 + A_4M_4 + A_5M_5)/M_t$, where $A_i$ is the amount of EBD extracted per mass of muscle from muscle group i and $M_i$ is the original weight of that muscle group, and $M_t$ is the total weight of all limb muscles.

b. Calculate the weighted average for each leg and then calculate the average value for the treatment group (all the legs in the group).

Even if some of the muscle was first used for immunohistochemistry or some other procedure, make sure to use the original muscle sample weight when calculating the weighted average of EBD in the leg.

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**Figure 10** Extracted EBD from hind leg muscles. The 15- to 17-month-old mdx mice were either unexercised or were exercised by running on a treadmill 30 min at a speed of 12 m/min, $n = 12$ legs for each group of mice. (A) The weighted average amount of EBD in the whole leg is shown for each group of mice. EBD extracted from the exercised legs was 26% higher than from the unexercised legs ($p = 0.0026$). (B) Each data point indicates the amount of EBD in one muscle sample. The average amount of EBD extracted from muscle samples of the exercised mice was 39% higher than from those of the unexercised mice ($p < 0.0001$). Averages are indicated by a bar ± SD. See Wooddell et al. (2010) for additional details.
17. Compare muscle samples as collectives (Fig. 10B).

As described for analysis of EBD⁺ myofibers, the power of analysis can be improved by comparing samples as collective groups of muscles. In this case, the weight of the samples is not taken into account and all samples contribute equally to the resulting average.

18. In cases where one leg of the mouse can be used as a control for a treatment given to the other leg, then pairs of muscle groups from the left and right legs can be compared using a two-tailed, Wilcoxon signed rank test for statistical analysis.

**REAGENTS AND SOLUTIONS**

Use deionized, distilled water in all recipes and protocol steps.

**Evans blue dye (EBD) in PBS (1% weight/volume)**

Dissolve 1 g Evans blue dye (EBD; Sigma-Aldrich, cat. no. E2129) in 100 ml sterile, filtered, phosphate buffered saline (PBS), pH 7.5, and use fresh.

**Evans blue dye (EBD) in saline (5 mg/ml)**

Dissolve 200 mg Evans blue dye (EBD; Sigma-Aldrich, cat. no. E2129) in 40 ml sterile, commercially-prepared physiological saline (0.90% NaCl). Filter through a 0.22-μm filter. Store at 4°C.

**COMMENTARY**

**Background Information**

Evans blue dye is an exclusion dye that is widely used to assess cellular integrity, due to its inability to enter (and, therefore, stain) intact cells. The propensity of the dye to bind to serum albumin following injection makes it a useful tool for assessing a number of physiological parameters, and the spectral characteristics of the dye facilitate tissue- and cellular-level visualization. In this manuscript, several methods for injecting and quantitatively measuring uptake of Evans blue dye in mouse hindlimb skeletal muscle are described to provide the researcher with a flexible set of tools for assessment of skeletal muscle damage. The different quantitation protocols balance workload with specificity and resolution, and further discussion of these qualities is presented in the Critical Parameters and Troubleshooting section.

Damaged muscles containing EBD can be observed macroscopically due to the bright blue stain (Fig. 11) or microscopically by the red auto-fluorescence of the dye in muscle sections (Fig. 12). Myofibers with histologically distinct necrosis (cell death) always stain positive for EBD and myofibers with an intact sarcolemma do not stain (Matsuda et al., 1995; Brussee et al., 1997; Straub et al., 1997; Archer et al., 2006). Blood proteins can be detected in spaces occupied by the necrotic myofibers (Fig. 6). EBD can also be present within myofibers that appear morphologically normal in hematoxylin and eosin (H&E)-stained sections (Brussee et al., 1997; Hamer et al., 2002) and EBD uptake into leaky myofibers does not always reflect severe myofiber damage (necrosis) that will provoke regeneration and require myogenesis (reviewed in Grounds et al., 2008). Temporary lesions do not necessarily cause myofiber death because the membrane of the myofiber can reseal (Doherty and McNally, 2003; McNeil and Kirchhausen, 2005). Most EBD-stained myofibers in mdx mice are intensely stained and clearly necrotic, but those that resemble normal (non-necrotic) myofibers when viewed by H&E stain only lightly with EBD (Figs. 5 and 6).

EBD is also used as an end-point to demonstrate an efficacious therapy in dystrophic mdx mice (Matsuda et al., 1995; Straub et al., 1997; Grounds et al., 2008; Zhang et al., 2010). However, demonstration of a therapeutic effect requires that one first determines the natural variation between animals, which is extremely high in dystrophic mdx mice (for an example, see Fig. 11; Straub et al., 1997; Grounds et al., 2008). The therapeutic effect must be significantly greater than the natural variation between animals in order to detect and quantify efficacy (Wooddell et al., 2010). If one leg of the animal receives a therapeutic treatment and the other leg receives a control treatment, then the variation between the left and right legs of individual mice must also be determined.
**Figure 11** Macroscopic evaluation of two 9-week-old dystrophic (mdx) litter-mates given i.v. injections of EBD (mouse 1, a and b; mouse 2, c and d). Mice were sacrificed 6 hr after EBD injection and fixed in 8% formaldehyde solution. See original article for additional information (Straub et al., 1997; Rockefeller University Press. Originally published in J. Cell Biol. 139:375-385. doi: 10.1083/jcb.139.2.375).

**Figure 12** Tibialis anterior muscle in cross section showing positive staining for EBD in dystrophic (mdx) myofibers 48 hr after an exercise-induced muscle damage protocol. EBD was administered via i.p. injection 24 hr prior to sacrifice.
Critical Parameters and Troubleshooting

Timing of muscle evaluation relative to EBD delivery

In published reports of EBD studies, dystrophic mice have been sacrificed at varying times between 3 and 96 hr after injection of the EBD, most commonly at 12 to 24 hr (Straub et al., 1997; Richard et al., 2000; Hamer et al., 2002; Kobinger et al., 2003; Chakkalakal et al., 2004; Sokolow et al., 2004; Sher et al., 2006). In addition to the EBD that leaks into muscle fibers with permeable cell membranes and can be visualized for at least 96 hr, some EBD can also be visualized in the interstitial space for just as long (Fig. 13). In the authors’ studies, very little EBD circulating in the blood was detected 24 hr after it was intravenously injected (Wooddell et al., 2010).

In muscle sections taken during the first few hours after EBD delivery, up to ∼6 hr, EBD appears to have incompletely penetrated into the myofibers (Straub et al., 1997; Wooddell et al., unpub. observ.). Even with this characteristic, EBD-positive (EBD+) myofibers can be distinguished as early as 1 hr after i.v. injection of EBD (Wooddell et al., unpub. observ.).

Exercise protocols to challenge the muscles

Muscle contractions can induce injury in the fragile myofiber plasma membranes of dystrophic mdx mice. When subjected to sustained exercise and then injected with EBD, the muscles of mdx mice accumulate the dye much more than those of normal mice (Brussee et al., 1997; Tinsley et al., 1998; Vilquin et al., 1998; Hamer et al., 2002).

Some mouse strains with muscle defects do not exhibit a particularly strong myopathy when the mice are sedentary. This is the case for mdx mice. In these mice, dystrophopathy can be exacerbated by subjecting the mice to an exercise protocol. The most commonly used exercise protocols are treadmill running under a number of conditions and balancing on a rotating rod (rotarod) (Grounds et al., 2008; Wooddell et al., 2010; Marcaletti et al., 2011). Exercise protocols are beyond the scope of this paper and are described elsewhere; here, treadmill running is used in some examples. Additional protocols (SOPs) for exercising mdx mice are available on the TREAT-NMD Neuromuscular Network Website http://www.treat-nmd.eu/research/preclinical/dmd-sops/.

The mdx mice may be reluctant to participate in exercise protocols, such as treadmill running, after being injected with EBD (Wooddell et al., unpub. observ.). For this reason it is more suitable to perform the exercise regimen prior to EBD injection, generally followed 20 to 30 min later by an EBD injection. If mdx mice must be injected with EBD prior to an exercise regimen, allowing them at least a 30-min rest may make them better able to perform the exercise.

Quantitative methods for assessment of EBD uptake

As demonstrated by Straub et al. (1997), macroscopic evaluation of EBD in the whole

![Figure 13](image-url) Strength of EBD fluorescent signal in skeletal myofibers and interstitium of mdx mice given an i.p. injection of 1% EBD in PBS at various times before sacrifice and muscle harvest. Adapted from Hamer et al. (2002).
mouse is highly useful for a qualitative assessment of phenotypes and to visualize the variation between animals in a group (Fig. 11). Some other approaches that allow quantitation of EBD in the muscles are based on microscopic evaluation or spectrophotometric detection of EBD that is extracted from the muscles.

**Microscope-based methods of analysis**

Muscle damage in dystrophic mdx mice tends to be in regions composed of multiple damaged muscle fibers. For example, see the myofibers labeled as darkly (intensely) EBD-stained in the sectioned muscle specimen shown in Figure 5A. Such regions in images captured by fluorescence microscopy can be quantitated by morphometric analysis (Fig. 1).

Another option for quantitating EBD-stained muscle is to count EBD+ myofibers in cross-sections of muscle samples. This method is suitable for determining a therapeutic effect from a treatment that affects some but not all muscle fibers within the leg, e.g., following gene therapy (Fig. 3). In this example, the muscle fibers that received a dystrophin-expressing plasmid DNA (pDNA) were protected from exercise-induced muscle damage. The dystrophin-positive muscle fibers excluded EBD while neighboring muscle fibers that did not receive the therapeutic plasmid were found to be leaky and EBD-stained. This method of visualizing the EBD+ myofibers also allows one to distinguish between those that stain intensely with EBD (generally necrotic) and those that stain only lightly.

Microscopic analyses are labor intensive. Multiple representative muscle specimens from throughout the limb need to be examined to assess EBD staining in the whole limb. For mice in which morphological features appear to be equally divided throughout the muscle from tendon to tendon, such as reported for unexercised mdx mice, it is sufficient to take specimens from one location within the muscle, i.e., the middle (van Putten et al., 2010). Nevertheless, the leg is composed of many muscles. A researcher may choose to use microscopic techniques to visualize specific EBD-stained muscles in detail rather than evaluating muscle specimens sampled from the whole leg.

Myofibers are long, thin muscle cells. Determining the percentages of them that are EBD-positive requires the researcher to quantitate a representative number in cross-sections of muscle specimens. The sections should be cut perpendicular to the muscle fibers to obtain high-quality cross-sectional images. The easiest way to accomplish this is by taking great care to keep the muscle specimens properly oriented while freezing them.

EBD staining of the myofibers is better preserved when muscle specimens are frozen in a freezing compound such as O.C.T. than when they are embedded in paraffin and processed for histological evaluation (Hamer et al., 2002). EBD causes high background fluorescence throughout paraffin-embedded muscle specimens (Wooddell et al., unpub. observ.). Muscle specimens frozen in O.C.T. and then sectioned are ideal for immunohistochemistry (Figs. 3 and 6). Additionally, sections of muscle can be cut from the frozen blocks of O.C.T. and then stained for histological evaluation with H&E (Fig. 5). Although the quality of the H&E staining is not as sharp as it is when sections are cut from paraffin blocks, this approach allows adjacent sections to be evaluated by H&E as well as by EBD fluorescence and IHC, even with multiple antibodies in sequential sections (Fig. 6).

**Immunohistochemistry**

EBD staining is commonly used to evaluate mouse models for myopathies, which may be caused by defects in myofiber cell membrane proteins such as dystrophin. Immunostaining of such membrane proteins in wild-type mice allows the researcher to distinguish the individual myofibers. However, genetic defects that result in muscle myopathies often also cause disruption of the dystrophin-glycoprotein complex. As a consequence, proteins that are localized to the myofiber membrane in wild-type mice may not be similarly located in mice that have myopathies. In this case, immunostaining of blood proteins can serve the purpose of outlining individual myofibers.

Use of an antibody to detect a blood protein serves another function in conjunction with EBD staining. Some of the EBD+ myofibers are clearly damaged and stain intensely (Figs. 5 and 6). Blood proteins can be found in these myofibers (Fig. 6). Overlaying the signals from the red-fluorescing EBD with the green signal from fluorescein isothiocyanate (FITC)-labeled antibodies results in a bright yellow-green signal in the severely damaged myofibers (Fig. 3A). The myofibers that stain lightly with EBD exclude the blood proteins. They appear red in the microscope images rather than yellow-green (Fig. 3B).

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**Myofiber Damage**

**Evaluation by Evans Blue Dye Injection**

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Figure 14  EBD extracted from the whole leg of unexercised and exercised young and old mdx mice and wild-type mice. Groups of mice were unexercised or exercised by running on a treadmill for 10 to 30 min at 12 m/min. The average amount of EBD extracted per gram muscle in each leg is shown for unexercised (E−) and exercised (E+) mice: male wild-type C57Bl/6 mice (C57, n = 4 mice, 8 legs); male and female young adult mdx4Cv mice, 3 to 7 months old (y mdx, n = 5 mice, 10 legs); and male and female 15 to 17 months old mdx-10ScSn mice (o mdx, n = 6 mice, 12 legs). Figure taken from Wooddell et al. (2010).

The researcher must decide whether to count only the intensely-stained EBD+ myofibers or to include all EBD+ myofibers, the intensely and lightly stained ones. It was observed that the intensely-stained myofibers increased following exercise in old mdx mice, but the lightly stained ones did not (Wooddell et al., 2010).

**Spectrophotometric quantitation**

An alternative, faster, simpler, and highly quantitative method of evaluating EBD in the muscles is to grind up the muscle samples, extract EBD from them, and quantitate the EBD spectrophotometrically using a microplate reader. This approach reduces the sampling variation inherent in cutting sections from specimens.

This spectrophotometric method may be employed when the amount of EBD taken up by muscles in one group of mice differs significantly from that in another, as shown in Figure 14. The method is not suitable for small differences between experimental groups because EBD in the vasculature and especially that in the interstitium between muscle fibers causes high background signal in all animals. Care should be taken to evaluate the muscles at a time when the EBD signal in the myofibers is sufficiently high and the background of EBD in the interstitial space is sufficiently low (Fig. 13). The timing of muscle harvest relative to the time of EBD injection affects this background. Sacrificing mice 24 hr after EBD injection is recommended when EBD is to be extracted from the muscles and evaluated by the spectrophotometric method.

A fluorimeter can also be used to quantitate the extracted EBD, but the conditions would need to be worked out for individual instruments. Only a cuvette-reading fluorimeter (VARIAN model Cary Eclipse) was used by the authors, a much slower process than using a microplate reader. With this fluorimeter, excitation was set at 640 nm; emission at 680 nm; slit width at 5 nm and the voltage at 600 V. DMF will dissolve some plastic cuvettes, so plastic ware must be tested prior to adding samples containing DMF. Diluting the DMF-extracted EBD samples in water resulted in an opaque liquid that caused scatter during fluorimeter reading, so this option for decreasing the DMF concentration is not recommended. DMF can be handled in polypropylene tubes and in the microplates described in Basic Protocol 4 for spectrophotometric microplate reading.

When an experiment contains a small number of animals and the variability between them in terms of muscle uptake of EBD is expected to be broad, the sample size must be large enough to determine if there are any statistically significant differences between treatment groups. This is a common dilemma when using mdx mice. The statistical power of analysis can be increased by increasing the sample size, either by using more animals or by evaluating more samples within animals. In Figure 10, for example, all of the five groups of muscles from all of the mice in one treatment group...
group were compared as collectives to all the muscle samples of all the mice in another treatment group.

**Anticipated Results**

EBD has been used to characterize mice with muscle defects and to evaluate therapies, frequently with quantitation performed by the morphometric method (Brussee et al., 1997; Straub et al., 1997; Richard et al., 2000; Kobinger et al., 2003; Chakkalakal et al., 2004; Shavlakadze et al., 2004; Sokolow et al., 2004; Archer et al., 2006; Minetti et al., 2006; Li et al., 2008; Miura et al., 2009; Piers et al., 2011). See Figure 1 for an example. EBD is also used without quantitation for qualitative examination of muscle. In this case, it is often used to visualize co-localization or lack thereof with other cellular proteins or with histological features (Straub et al., 1997; Cifuentes-Diaz et al., 2001; Hamer et al., 2002; Durbeej et al., 2003; Goyenvalle et al., 2004; Sher et al., 2006; Fougerousse et al., 2007; Wang et al., 2008). When EBD is used as an assay for therapy studies, the evaluation can be quantitative and/or qualitative (Shavlakadze et al., 2004; Wang et al., 2008; Zhang et al., 2010; Figs. 3 and 8).

The hind limb muscles are commonly evaluated, but EBD can also be used to evaluate other mouse muscles such as those in the diaphragm (Voisin et al., 2005) or the heart (Bostick et al., 2009). In some cases, macroscopic visualization of the blue stain can be used to characterize a mouse strain or evaluate a therapeutic (Straub et al., 1997; Voisin et al., 2005). See Figure 11 for example.

Use wild-type naïve mice as a control when working with other mouse strains or experimental conditions expected to cause muscle damage. Particularly when using the spectrophotometric method to quantitate EBD in muscles, it is important to remember that much of the EBD is in the interstitial space. The consequence of this is that a substantial amount of EBD can be extracted from wild-type mouse muscle, as shown in Figure 14. Approximately 10 μg EBD/g leg muscle was extracted from wild-type mice, whereas >30 μg EBD/g muscle was extracted from 3- to 7-month-old mdx mice. The amount of EBD in the interstitial space decreases over time as Hamer et al. (2002) showed (Fig. 13), but the EBD within the damaged myofibers also decreases over time. Damaged myofibers are replaced by newly formed myofibers in the course of a few days.

**Mdx** mice are notorious for exhibiting high levels of variation in the extent of dystrophy. This will be reflected in the analysis of EBD+ myofibers (percent cross-sectional area or percent counted myofibers). It must also be noted that if analyzing myofiber necrosis and EBD+ myofibers in the same muscle section, results for EBD+ myofibers will often be higher, as not all leaky myofibers (which will stain positive for EBD) will undergo necrosis. See Table 1 for examples of anticipated results in mdx mice.

Mouse age and their spontaneous activity can complicate experimental design. The physiology of dystrophic mdx mouse muscle, for example, changes as the mice age (Coulton et al., 1988; McGeeachie et al., 1993; Lefaucheur et al., 1995). Measuring a significant difference in muscles of the whole legs of exercised compared to unexercised mdx mice can be more challenging in 2- to 6-month-old compared to 13- to 19-month-old mice (Fig. 7 and Wooddell et al., 2010). The younger mice have much more variable levels of EBD staining than the older mice, even from one leg to the other. This appears to be due to the higher spontaneous activity level of young mice compared to sedate older mice.

**Time Considerations**

As with any animal experiment, the greatest time consideration is that of breeding or otherwise obtaining the desired number of mice of the appropriate age and gender. If antibodies need to be produced for immunohistochemistry, allow at least 4 weeks for this process.

Injection of EBD (see Basic Protocol 1) is a quick procedure, but allow ~30 min prior to injection for mice to be placed under a heat lamp when performing the intravenous injection. Anesthetizing the mouse takes only 2 to 3 min.

The amount of time required to produce skeletal muscle sections on glass slides suitable for morphometric EBD imaging without immunohistochemistry (see Basic Protocol 2) is fairly short, depending on the number of samples. Immediately after freezing the muscle samples, they can be cut on a cryostat and tissue sections produced. Preparing frozen muscle samples avoids waiting for paraformaldehyde fixation and overnight paraffin processing. The speed of cryostat cutting depends on the individual researcher, but generally a group of eight muscle samples can easily be cut in a 3-hr cryostat session. EBD is autofluorescent and, thus, no additional
staining is needed. Once cut, muscle sections are simply fixed with acetone and covered with glass cover slips. Image capture and image analysis, however, are two rather time-consuming steps. The time taken to capture an image of a whole muscle in cross section depends on the size of the muscle, the capabilities of the image capture software, and the microscopic magnification. Capturing an entire mdx quadriceps muscle section in cross section at 10× magnification requires ∼20 min. Image analysis is also a time-consuming step and must be done accurately. This step also depends on the size of the muscle and the amount of muscle damage. Analyzing an entire mdx quadriceps muscle generally takes 20 to 30 min, whereas a smaller tibialis anterior muscle may take 10 to 15 min.

Basic Protocol 3 is fairly quick once the researcher has a feel for the dissection. Mice are anesthetized and sacrificed. Then the leg muscles are cut into pieces, weighed, and frozen. This is a convenient stopping point before proceeding with Support Protocol 1.

Support Protocol 1 is the most labor-intensive of these protocols, especially counting of the myofibers. In addition to the time required for cryostat sectioning, ∼2 hr are required for immunostaining a batch of slides. Then allow 2 to 3 hr per leg to take ten images across the diameters of each of the five groups of muscles using two different filters. Image capture should be performed within 2 days of immunostaining. Once the images are acquired, positive myofibers are counted on the computer screen. This takes more time when there are many EBD+ myofibers, if the researcher wishes to distinguish the lightly from the intensely stained myofibers, or if specifically immunostained myofibers are to be counted. The total number of myofibers can be counted from printed images for determination of the percentage of EBD+ myofibers. Allow 1 day per leg for counting all myofibers in the cross-sectional images for EBD and specific immunostaining staining. The time can be reduced if only the positive myofibers in each image are counted and these are reported as EBD+ myofibers/view (Fig. 7) rather than as a percentage of the total. The time can be further reduced by selecting just a subset of the muscle pieces for evaluation.

Support Protocol 2 is for production of antibodies. After a suitable plasmid and animals are acquired, allow at least 4 weeks for the animals to develop antibodies. Hands-on time is minimal. The plasmid injection procedure requires some skill, but each injection typically takes <10 min. Immunohistochemistry testing can be performed in 1 day. It does not involve myofiber counting.

Tissue grinding accounts for the majority of hands-on time required to perform Basic Protocol 4. After samples are ground, EBD is extracted over 24 hr. The microplate reader assay is quick. Overall, nine samples can be processed in an average of 4 hr hands-on time.

**Literature Cited**


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