An introductory biology lab that uses enzyme histochemistry to teach students about skeletal muscle fiber types

Lauren J. Sweeney, Peter D. Brodfuehrer, and Beth L. Raughley
Department of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania 19010

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Sweeney, Lauren J., Peter D. Brodfuehrer, and Beth L. Raughley. An introductory biology lab that uses enzyme histochemistry to teach students about skeletal muscle fiber types. Adv Physiol Educ 28: 23–28, 2004; 10.1152/advan.00019.2003.—One important goal of introductory biology laboratory experiences is to engage students directly in all steps in the process of scientific discovery. Even when laboratory experiences are built on principles discussed in the classroom, students often do not adequately apply this background to interpretation of results they obtain in lab. This disconnect has been described at the level of medical education (4), so it should not be surprising that educators have struggled with this same phenomenon at the undergraduate level. We describe a new introductory biology lab that challenges students to make these connections. The lab utilizes enzyme histochemistry and morphological observations to draw conclusions about the composition of functionally different types of muscle fibers present in skeletal muscle. We report that students were not only successful at making these observations on a specific skeletal muscle, the gastrocnemius of the frog Rana pipiens, but that they were able to connect their results to the principles of fiber type differences that exist in skeletal muscles in all vertebrates.

mitochondrial enzymes; succinic dehydrogenase; fast-twitch fibers; slow-twitch fibers

Skeletal Muscles Contain Different Fiber Types

Fiber types differ in their contractile characteristics, including the speed of contraction and their resistance to fatigue. The speed of contraction is determined in part by the pattern of innervation received by each muscle fiber. Contractile characteristics correlate with differences in morphological and biochemical properties, such as oxidative vs. glycolytic metabolism. Two of the key morphological features are the numbers of mitochondria present and the cross-sectional diameter of fibers. Oxidative fibers contain many mitochondria and have relatively small diameters to maximize oxygen diffusion to the mitochondria in the cell’s interior. Glycolytic fibers contain few mitochondria and so can have larger diameters. Because dependence on oxygen for metabolism cannot sustain a fast rate of contraction, fibers that contract rapidly (fast-twitch fibers) are large-diameter glycolytic fibers. The small-diameter aerobic fibers are slow-contracting fibers (slow-twitch fibers). Slow-twitch fibers fatigue slowly and so have the advantage of being able to sustain contraction for a long period of time. They are found in great numbers in muscles that are important for endurance activities, like maintaining posture. In vertebrates, these muscles include most of the small muscles of the back (such as the erector spinae) that extend or straighten the vertebral column. Fast-contracting fibers develop maximum contractile velocity quickly but fatigue rapidly and so are used mainly for short but intense bursts of activity. These fibers predominate in many muscles that move the limbs, like the pectoralis, which flexes the forelimb in many organisms. Most muscles contain an intermediate fiber type that is (in most organisms) a fast-contracting fiber with a hybrid metabolic and morphological profile. These fibers, called fast oxidative-glycolytic, have both aerobic and glycolytic metabolic capacities and an intermediate fiber diameter. A combination of these three fiber types is found in most muscles, where the mix provides for a combination of rapid and sustained activity as demands change.

The muscle used in this experiment, the gastrocnemius of the frog R. pipiens, is a major calf muscle used in all vertebrates to propel the legs during motion, as well as to maintain standing posture. The gastrocnemius contains a combination of fiber types in most animals. It shows considerable variability in

Address for reprint requests and other correspondence: P. D. Brodfuehrer, Dept. of Biology, Bryn Mawr College, 101 N. Merion Ave., Bryn Mawr, PA 19010 (E-mail: pbrodfue@brynmawr.edu)

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fiber type composition in different animals, depending on whether that species uses its hindlegs mostly for brief but fast, powerful bursts of activity (like short runs or hops, as is the case in this frog), or for sustained but slower movements, like walking (3). Technically, it is a good muscle to use because it is one of the larger leg muscles and easily accessible.

Enzyme Histochemistry Techniques Test for Presence of Specific Enzymes

The test used in this lab detects the presence of the enzyme succinic dehydrogenase (SDH) in mitochondria, one of the enzymes in the TCA or citric acid cycle. Tissue is incubated with a reaction solution containing succinate, the substrate for this enzyme, and a reaction detector molecule, nitroblue tetrazolium. As the enzyme-substrate reaction proceeds, nitroblue is reduced and precipitates out of solution as a blue compound, thus marking the mitochondria. Although mitochondria are too small to be seen as individual organelles under the light microscope, the greater their concentration within a cell, the darker the stain will be with this reaction.

Students used this enzyme histochemistry test as the basis for determining qualitatively whether individual muscle fibers contained relatively large numbers of mitochondria (dark stain) or relatively small numbers of mitochondria (lighter stain). They then combined these histochemistry results with morphological measurements of fiber diameters to draw conclusions about which fibers in their experiment were fast twitch and which were slow twitch.

EXPERIMENTAL PROCEDURES

Preparation for Lab

Tissue preparation. Gastrocnemius muscles were dissected from R. pipiens after euthanasia by injection of 1% Tricaine or MS 222 (Sigma), following procedures approved by our institutional animal care and use committee (Fig. 1). To preserve enzyme activity, each muscle was rapidly frozen on dry ice and stored frozen at –20°C until use. Although we used all muscle within 24 h of freezing, tissue can be prepared weeks ahead of time if stored frozen in sealed containers (such as 50-ml centrifuge tubes). Either at the time of preparation or before lab, each muscle should be cut in half across its width to 1) double the number of blocks of muscle available to students, and 2) provide students with a sharp cross-sectional face from which to cut sections.

Reaction solution preparation. The SDH reaction medium consisted of the following (in mM): 7 succinic acid, 1.2 nitroblue tetrazolium, 0.85 NADH, and 200 Trizma buffer at pH 7.4 (3). All ingredients except the Trizma buffer were prepared fresh for each lab, because NADH in solution can form enzyme inhibitors. It is also very important to keep the solution out of the light until use for it to remain fully effective.

Lab set-up. A few simple materials were all that was needed to scale up this lab for multiple sections. Students were supplied with sharp scalpels and forceps, slides subbed with Histo Grip (Zymed), coverslips, and a coverslipping solution of 50% glycerol in distilled water. They used a “PAP” pen (Zymed) to draw a coverslip-size, water-tight, rectangular barrier on their slides to contain the reaction solution. At the start of the lab, each pair of students was supplied with a block of frozen muscle on a bed of crushed dry ice in a small plastic petri dish. To keep the muscle blocks frozen until sectioning began, each small dish was stored in a second, larger dish containing more dry ice. Reaction solution was provided in small containers for groups of four to six students.

Student Instructions in Lab and Their Success/Problems

Students used scalpels to cut 3–4 slices as thin as possible from the end of the frozen muscle and transferred them to their prepared slides (Fig. 1). We estimated section thickness at 100 μm or more. One slide was sufficient to produce usable experimental results. Students were advised to repeatedly cool their instrument tips in dry ice to make sectioning easier and to keep sections from sticking to the instruments. The sections expanded and flattened out on contact with the warm (room temperature) slide. Students were successful in cutting usable sections with this relatively crude technique. The only technical problem that we encountered at this step was that some muscle blocks...
started to thaw before students finished their sectioning. A few extra muscle blocks kept in reserve for each lab will solve any thawing problems.

Before being labeled, sections were left at room temperature for 15–30 min to allow them to thaw and for water to evaporate from the tissue, which helped to anchor the sections to the slides [published protocols suggest that sections can be left for 1 h without diminishing enzyme activity (3)]. This time permitted instructors to help students experiencing problems and to check sections to ensure they were true cross sections. Even without the contrast provided by stains, cross sections can be easily distinguished from tangential or longitudinal sections on examination with a dissecting microscope.

Students applied the reaction solution to their sections with Pasteur pipets. We determined in our preparation for this lab that a 15-min incubation period produced the best results [in contrast to published protocols, which suggest 1 h (3)]. This was verified by our student results, although we did not observe noticeably different results in slides that students incubated for several minutes to either side of the 15-min target.

The reaction was stopped and the solution removed by rinsing with Ringer solution or distilled water. If sections began to float off the slide, students were instructed to remove the rinse solution by gentle pipetting rather than pouring. Any loosened sections could be floated apart from each other and flattened out during this process. A 50% solution of glycerol in distilled water was pipetted over the sections, and a coverslip was applied.

Students analyzed their results using compound microscopes with eyepiece reticles (to measure diameters of muscle fibers). Slides could be saved for later photographic documentation or comparison with results from other labs, as we observed no diminution in staining intensity over a month-long period even when slides were kept in the light. However, coverslips must be sealed to the slide for storage longer than a few days, or the tissue begins to dehydrate.

**Types of Data and Observations Students Were Asked to Collect and Analyze**

First, students were asked to observe whether there was any pattern of distribution of fiber types within the muscle that would prevent them from randomly selecting a small region for analysis. In preparation for this lab, we had found that different fiber types showed no apparent concentration into groups within the gastrocnemius muscle, which is consistent with findings in many mixed fiber muscles (2). Because students did not find any concentration of fiber types, they were able to use random regions for their observations and measurements. They were next instructed to record the staining intensity in 60 adjacent fibers in a region chosen for its good tissue preservation. They were directed to classify staining intensities into a maximum of three categories (strong, intermediate, or weak) on the basis of the well-established categories that have been detected in research. It was up to them to decide whether two or three categories existed in their particular muscle sections. They were then asked to measure the cross-sectional diameter of 20 fibers in each of these staining categories to determine whether the different staining intensities correlated with differences in fiber diameter. Students were easily able to assess 60 fibers and assign them to a staining intensity category and to measure the cross-sectional diameter of 20 cells in each category. Despite the great thickness of their sections (estimated at \( \geq 100 \mu m \)), clear results were obtained because of a unique feature of skeletal muscle cells: each runs the length of the whole muscle. Therefore, any cross section will go through just one cell at each point in the section. Furthermore, multiple sections from the same muscle should demonstrate the same fiber type profile.

Students were asked to do several calculations to derive the total percentage of cross-sectional area occupied by each of the fiber types observed. They performed these calculations on data collected from their whole lab section. First, they calculated the percentage frequency of each fiber type. They then calculated the cross-sectional area of the average fiber in each category from their diameter measurements by using a circle as an approximation of the cross-sectional shape of the muscle fibers. They then multiplied the average cross-sectional areas for each fiber type by the percentage of each fiber type present in the muscle.

**RESULTS AND DISCUSSION**

**Fiber Type Composition**

All students got positive results that produced three fiber type categories uniformly distributed within the muscle (Fig. 2). First, the lab visually demonstrated that staining intensity for mitochondrial enzyme activity can be used to document the relative concentration of mitochondria within cells. By utilizing a protocol that depended on one of the many reactions in the TCA cycle, students were given a concrete example of the importance of understanding the oxidation-reduction steps in this cycle. The consistency of staining within and between these student labs demonstrated that the SDH enzyme activity test is not a technically finicky assay.

Second, the measure of mitochondrial content correlated qualitatively with fiber cross-sectional size: the dark-staining fibers (those with many mitochondria) were always the narrowest fibers and the lightest staining fibers (those with fewest mitochondria) the widest fibers. A third, intermediate fiber category was also found, which had both intermediate diameter and staining intensity. These results are consistent with published findings for the frog gastrocnemius muscle in particular and for most limb skeletal muscles in most species in general (1, 2, 3). The specific percentages of fiber types, as well as their distributions within muscles, have been shown to have characteristic profiles in individual muscles in a number of species that have been examined, from amphibians to birds to mammals (3).

**Quantification of Fiber Type Characteristics**

Students provided individual measurements to a group data table for their whole lab section. Each lab contained between 21 and 25 students. Figure 3 contains a graphic summary of these results from each of the five lab groups in this past year’s Introductory Biology course, as well as the combined means for all five labs. In all labs, a mixed-fiber population was observed. In every lab, fast glycolytic fibers (FG) were the most frequent (overall mean of 46.4%), with slow oxidative (SO) and fast oxidative-glycolytic (FOG) much less frequent (overall mean of 28.4% for SO and 25.2% for FOG). In every lab, the FG fibers were the largest in cross-sectional area (overall mean of 21,135 \( \mu m^2 \)), the FOG were intermediate in size (overall mean of 10,133 \( \mu m^2 \)), and the SO much smaller (overall mean of 3,922 \( \mu m^2 \)). Thus the mean fiber cross-sectional size of the FG fibers was over five times that of the smallest SO fibers and over twice the size of the intermediate FOG fibers. If one applies these measurements to the muscle as a whole, as we did for this lab, one can calculate the total cross-sectional area of the muscle occupied by each fiber type. FG fibers would therefore occupy 71.1% of the total cross-sectional area of the gastrocnemius muscle, whereas FOG and SO fibers would occupy only 20.1 and 8.8%, respectively. This last calculation demonstrated to students the importance of how data are presented and interpreted. If fiber type frequency alone
is discussed, FG fibers are the most common (overall mean of 46.4%). However, when their larger cross-sectional area is factored in, it becomes clear that FG fibers make up >70% of the total mass of the muscle (overall mean). Conversely, the small-diameter SO fibers are >28% of the number of fibers in the muscle, but their small size means that they make up <10% of the total muscle mass. Both numbers are relevant. The relative number of fibers of each type present in any muscle is relevant because each fiber is innervated and recruited separately. However, the overall contractile properties of the whole muscle are determined by the total mass of each fiber type present in the muscle.

Interpretation of Variations in Lab Group Data

As demonstrated in Fig. 3, modest variations were observed in the data from one student to another within each lab. Nevertheless, the data for individual labs told a clear story in each lab. However, we have found that, all too often, students view any variability as a sign of defective experimental design or data collection rather than a reflection of intrinsic or natural biological differences. The results of this lab can be used to engage students in a discussion about factors that contribute to both types of variability. Obviously, technical factors contributed to variations in results, as they will in any teaching lab in which inexperienced students perform experiments with little or no repetition. When challenged, students usually can think of changes they would make to minimize these sources of variability. However, intrinsic differences still remain, and many of them provide valuable insights into the nature of fiber type diversity. Students can use their own experiences in this lab to understand the major sources of intrinsic variation. First, intrinsic variations exist in fiber cross-sectional diameter within each fiber category. Second, small but important differences in percentage composition of fiber types are likely to exist in different regions of the same muscle even when it appears from a qualitative examination that they are uniformly distributed. Finally, variations in percentage composition of fiber types will exist in gastrocnemius muscles from different individuals of the same species. Using their own lab findings, students can draw conclusions about the types of changes they would make to the lab protocol to get a more accurate picture of these intrinsic variations, rather than viewing the objective as designing a way to eliminate all variability to obtain a single “correct” answer.

Ways in Which This Lab Could Be Expanded

This lab could be expanded in a number of different ways in either an introductory biology course or an upper-level course. We intend to add some of the following possibilities in future years, with an emphasis on adding variations for students to test that will make this lab more investigative. For example, the fiber type composition of two different muscles from the same species can be compared, as can the fiber type composition of the same muscle from two different organisms. Muscles that can be used for these comparisons include the soleus (largely SO) and extensor digitorum longus (largely FG) hindlimb muscles in rat and mouse (2, 5); the iliofibularis (almost entirely FOG) and the iliotibialis (equal mix of SO, FOG, and FG) hindlimb muscles in frog, salamander, and toad (1, 3); and the anterior and posterior latissimus dorsi shoulder girdle muscles, largely SO and FO, respectively, in chicken (6). It is advisable for investigators to verify these distinctions for themselves before proceeding with a lab. Alternatively, this can become part of the experimental design of a teaching lab, with students being asked to determine the distinctions between muscles in fiber type composition.

Analysis of results could also be made much more rigorous by asking students to analyze all fibers within a whole muscle...
cross section. More sophisticated and accurate measurements of fiber cross-sectional area can be obtained using digital photography and image analysis software to measure areas. Serial sections could be used to compare several different measurements of fiber type, such as mitochondrial cytochrome-c oxidase (5), myosin ATPase levels, glycogen content, or myosin isoform distribution (6). Students could do these comparisons themselves, but this would most likely require using cryostat sections of whole muscles. Alternatively, one or more of these comparisons could be presented to students as prepared slides for comparative analysis with their own slides.

Evaluation of Student Learning Experience and the Role This Lab Plays in a Multi-week Module on Skeletal Muscle Biology

Student comprehension of the concepts covered in this lab was evaluated qualitatively by 1) discussions during the lab and 2) evaluation of individual reports written by each student. Most students grasped the basic concepts that the lab was designed to illustrate. Many suggested good alterations or additions to the existing protocol that would provide more information about intrinsic variations within muscles or other questions that could be asked when this protocol is used. For example, some students suggested comparing the fiber type composition of the pectoralis muscle of birds that fly with those of birds that do not and even provided hypotheses about anticipated differences. Most importantly, a majority of students were able to apply their new knowledge to a subsequent set of investigative labs that explored skeletal muscle physiology. These physiology labs are still being modified, but they have a dual set of objectives. First, students develop an understanding of major concepts in muscle physiology by utilizing recordings of electrical activity of their own skeletal muscles under a set program of activities. These include summation of individual muscle twitches to sustained contraction, recruitment of new muscle fibers, and development of fatigue after prolonged activity. The students then use this new understanding to design simple experiments to test these functional parameters by utilizing the same types of recordings. As these physiology labs are further developed, we will expand our assessment criteria to examine more closely how each lab contributes to student understanding of implementing and designing the next lab in the module.

In summary, this lab demonstrates to students key concepts underlying the functional and structural differences in skeletal muscle fiber types. The results challenged students to think further about the correlations that were demonstrated in this lab: why oxidative fibers are the smallest; why glycolytic fibers are the largest; and what the purpose is of having mixed fiber types within a single muscle like the gastrocnemius. The relevance of this lab’s basic histochemical protocol to ongoing...
research on both biochemical and electrical functioning of skeletal muscle fiber types can be directly documented for students by having them read recent research (1).

On the technical level, the lab demonstrated to students that they have the skills necessary to interpret visual observations and categorize them into groupings (here, on the basis of intensity level of staining of cells). The range of individual measurements should demonstrate graphically to students that a certain amount of variability in measurement is part of the process of doing science but that, with sufficient numbers of individual measurements, trends will emerge that may represent intrinsic, naturally occurring variations. The fact that we saw such consistency from lab to lab for this experiment should provide students with some support for that concept.

This lab could be expanded to serve as one in an upper-level course in a number of different ways, depending on the purpose of the course. The fiber type composition of two different muscles from the same species can be compared, as can the fiber type composition of the same muscle from two different organisms. Analysis of results could be made much more rigorous by asking students to analyze all fibers within a whole muscle cross section. Serial sections could be used to compare several different measurements of fiber type, such as mitochondrial cytochrome-c oxidase activity, myosin ATPase levels, glycogen content, or myosin isoform distribution.

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