**1. Myosin heavy chain expression studies in whole muscles**

1.1. Myosin heavy chain expression studies by immunofluorescence

1.1.1. Embed the whole muscles in optimal cutting temperature (OCT) compound frozen in nitrogen-cooled isopentane, as published 1.

1.1.2. Cut five serial cryosections (8 µm) and mount them on separate glass, charged slides.

1.1.3. Fix the preparations with freezer-cooled acetone for 30 min at room temperature.

1.1.4. Wash gently 3x with a phosphate buffered solution (PBS) supplemented with 0.2% bovine serum albumin (BSA) and 0.04% Triton X-100, and subsequently block with PBS with 2% BSA, 2% goat serum and 0.4% Triton X-100 for 30 min at room temperature.

1.1.5. Wash gently 3x with PBS supplemented with 0.2% BSA and 0.04% Triton X-100 and incubate with the primary antibodies as follows:

1.1.5.1. Dilute each anti-myosin heavy chain (MHC) primary antibody in a separate vial in PBS with 1% BSA and 0.04% Triton X-100: anti-I (1:1,500), anti-II (1:600), anti-IIA (use entire conditioned media from the hybridoma), and anti-IIB (1:500).

1.1.5.2. Incubate each slide with one antibody and the remaining slide with PBS as a control for 12–16 h at 4 °C.

NOTE: In this protocol, fibers type IIX remained unlabeled in all samples.

1.1.6. Wash gently 3x with PBS and incubate all slides with the secondary antibody (1:800) coupled to a fluorescent green molecule for 1–2 h at room temperature.

1.1.7. Wash gently 3x with PBS, carefully add 20–40 µL of mounting medium and place a coverslide.

NOTE: Gentle, but repetitive washing-outs ensure good-quality, low background results.

1.1.8. Acquire fluorescence images using a 20x objective suitable for fluorescence and a filter set with the following wavelengths for excitation/dichroic/emission: 450–490/510/515 nm. Use a camera of at least 5 megapixels mounted on an inverted fluorescence microscope and store the images in .TIFF format for offline analyses.

1.1.9. Perform the quantitative semiautomated analyses as follows:

1.1.9.1. Duplicate all images to preserve the original intact.

1.1.9.2. Enhance contrast (5—10 %) and subtract background (800-1000).

1.1.9.3. Split the image in its color components and convert the green one to an 8 bits, black and white photo.

1.1.9.4. Convert the black and white photo to a binary image using a threshold of ~25 %, which must be optimized for each antibody.

1.1.9.5. Fill holes and separate bound fibers with the help of the watershed tool.

1.1.9.6. Count the number of positive and negative fibers in the image using the multipoint tool. Count hybrid I/IIA fibers by identifying those cells positive for the anti-I and the anti-IIA antibodies. Manually record the results in a database.

1.1.9.7. Subtract hybrid I/IIA fibers from the total number of I and IIA fibers to have the number of pure type I and IIA fibers. Calculate the percentage of each fiber type (I, IIA, IIB and total II) based on the total number of pure fibers present in the corresponding cryosection. Calculate the percentage of IIX fibers by subtracting the sum of IIA+IIB+hybrid from the percentage of total II fibers.

1.2. Myosin heavy chain expression studies by sodium dodecyl sulfate polyacrylamide gel electrophoresis

1.2.1. Purify myofibrils from the whole muscles 2, 3, as follows:

NOTE: Perform all work at 4–6 °C.

1.2.1.1. Homogenize each muscle in 1 mL of buffer A (in mM: 50 KCl, 10 K2HPO4, 2 MgCl2, 0.5 EDTA, 2 dithiothreitol (DTT), pH 7.0) and then centrifuge at 15800 *g* for 15 min at 4 °C.

1.2.1.2. Resuspend the pellets in buffer A plus 1 % Triton X-100 and centrifuge again as above.

1.2.1.3. Resuspend the pellets in a solution with (in mM): 60 KCl, 30 imidazole, 2 MgCl2, 1 DTT, pH 7.0 and measure the amount of total proteins with the Bradford method 4. Store the samples at −80 °C.

1.2.2. Separate the samples through a sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) 5 in a vertical chamber, as follows:

1.2.2.1. Prepare the stacking gel with 4.8 % polyacrylamide, 30% glycerol and 4 mM EDTA. Prepare the separating gel with 9 % polyacrylamide and 30% glycerol.

1.2.2.2. Load each well of the gels with 4-5 µg of total proteins of the myofibrils samples in loading buffer (62.5 mM Tris, 1 % sodium dodecyl sulfate, 0.01 % bromophenol blue, 5 % mercaptoethanol, 15.2 % glycerol).

1.2.2.3. Run at 72 V for 27–28 h at 6-8 °C.

NOTE: Adding 10 mM 2-mercaptoethanol to the interior running buffer improves the separation and resolution of the bands 6.

1.2.3. Stain the gels with Coomassie blue for 1 h.

1.2.4. Decolorize the gels for ~30 h.

1.2.5. Acquire images of the gels with a camera of 12 megapixels.

1.2.6. Load the images in a software for electrophoreses analyses:

1.2.6.1. Place a rectangular region of interest (ROI) over the bands of the first lane of the gel. Activate the Gaussian mode and the simplex fitting algorithm to obtain the band density profiles and the area under the curve of each band.

1.2.6.2. Calculate the percentage of each MHC isoform according to the summatory of the area under the curve of all bands present in the image.

1.2.6.3. Repeat the procedure for the remaining lanes of the gels.

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