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SUPPLEMENTARY METHODS AND DATA

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Damaged muscle fibers might masquerade as hybrid fibers – a cautionary note on immunophenotyping mouse muscle with mouse monoclonal antibodies

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HISTOLOGICAL METHODS

Sectioning and fixation

We made 5 µM, frozen, serial, cross sections, at the mid-belly of the QF and TA, with an HM 525 NX cryostat (ThermoFisher Scientific, Waltham, MA, USA). We collected the sections onto microscope slides (15-188-48, Tissue Path Superfrost™ Plus Gold Slides, ThermoFisher Scientific, Waltham, MA), fixed the sections with cold acetone (-20°C), and allowed them to air-dry.

Blocking non-specific labeling

Following fixation with acetone, we washed the sections three times with phosphate-buffered saline (PBS). Since we followed a labeled streptavidin biotin (LSAB) method to label MyHC

and mouse IgG, we blocked endogenous biotin by incubating the sections with 1 μg/mL avidin in PBS for 10 min. We then washed the sections three times with PBS, and applied a blocking agent containing 3% bovine serum albumin (BSA), 0.01% Triton X-100 and 0.02% sodium azide in PBS, for 30 min.[1,2] The same blocking agent served as a primary antibody diluent.

Applying primary antibodies

We diluted primary antibodies in primary antibody diluent, applied the antibodies on tissue sections at their working dilutions, and incubated the sections overnight at 4°C. To label MyHC in QF sections, we applied antibodies specific to sMyHC or fMyHC, on separate serial sections (mouse monoclonal antibodies; sMyHC, M8421, or, fMyHC, M4276, 1:1000, MilliporeSigma, St. Louis, MO, USA). Since we wanted to confirm whether muscle fibers are damaged or not, we additionally labeled desmin on the same sections, by simultaneously including antibodies to desmin along with anti-MyHC antibodies (rabbit polyclonal, RB-9014-P, 1:200, ThermoFisher Scientific).

To label MyHC in TA sections, we applied antibodies to sMyHC or fMyHC, on separate serial sections, as above. However, we did not concurrently label desmin on these sections. Rather, on separate serial sections, we labeled desmin (as above) or dystrophin (rabbit polyclonal, RB-9024-P, 1:200, ThermoFisher Scientific).

Applying secondary antibodies and streptavidin

We washed off the primary antibodies with a first wash buffer (0.1 % Triton X-100 in PBS) for 10 min, performed three additional 5-min washes with PBS, and then incubated the sections at room temperature for 60 min, with secondary antibodies diluted in PBS containing 0.01 % Triton X-100 (secondary antibody diluent).

To label MyHC in QF sections, we applied goat-anti-mouse secondary antibodies (biotin-conjugated goat-anti-mouse IgG, B2763, 1:200, ThermoFisher Scientific), to bind to the mouse monoclonal antibodies against MyHC. To colabel desmin in QF sections, we concurrently applied goat-anti-rabbit secondary antibodies, to bind to the rabbit polyclonal antibodies against desmin (goat-anti-rabbit F(ab')2 fragments, A-11070, 1:200, ThermoFisher Scientific). Following incubation with secondary antibodies, we washed the sections once with first wash buffer (10 min), and then washed them three times with PBS (5 min per wash). After washing off unbound secondary antibodies, we incubated the sections with streptavidin conjugated to Alexa 568 for 15 min (S11226, 1:200 in secondary antibody diluent, ThermoFisher Scientific).

To label MyHC in TA sections, we followed the same steps as above, but because we did not colabel desmin, we applied biotin-conjugated goat-anti-mouse IgG to bind mouse IgG, followed by streptavidin conjugated to Alexa 568.

To label desmin and mouse IgG in TA sections, we labeled desmin as described, above for QF. Since we wanted to assess on the same sections, whether or not muscle fibers were permeable to IgG, we included goat-anti-rabbit secondary antibodies conjugated to Alexa 488 and goat-anti-mouse IgG secondary antibodies conjugated to biotin. We then labeled sections with streptavidin conjugated to Alexa 568 to visualize mouse IgG under fluorescence optics. With this technique, damaged muscle fibers can easily be identified based on the loss of desmin and the inclusion of IgG in the myoplasm.[1,3,4]

To label dystrophin in TA sections, we followed a method similar to desmin labeling albeit with primary antibodies against dystrophin.

Negative control labeling

We performed suitable negative control immunolabeling experiments, by replacing specific primary antibodies with non-specific host-species IgG – example: replacing mouse monoclonal antibodies against MyHC with a similar concentration of non-specific mouse IgG, and replacing rabbit polyclonal antibodies against desmin with non-specific rabbit IgG. For IgG labeling to detect damage in the TA, we omitted goat-anti-mouse IgG, but did apply streptavidin conjugated to Alexa 568. Negative labeling results are summarized under Supplementary Data, Fig. S1.

Mounting sections for fluorescence microscopy

After incubations with secondary antibodies and streptavidin, we washed the sections as done following incubation with primary antibodies. We mounted the immunofluorescently-labeled sections in Vectashield anti-fade medium (H-1000, Vector Laboratories Inc., Burlingame, CA) under a coverslip.

Fluorescence microscopy

We captured digital images, under fluorescence optics, with a 10x (for tiled images of QF) or 20x (for all other images) objective lens on a Zeiss Axio Scope.A1 microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). To obtain tiled images of the entire cross section of the QF, we combined individual overlapping images with the Photomerge function (File Menu → Automate → Photomerge) in Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA). For the TA, we captured 12 digital images, moving in a grid-like fashion, across the cross section of the muscle.

Hematoxylin and eosin staining

We collected 5 µm serial cross sections of the QF, while making sections for the immunofluorescence experiments described, above.

We fixed the sections with cold acetone (-20°C), and allowed the sections to air-dry at room temperature. We then immersed the sections in hematoxylin (GHS332-1L, MilliporeSigma) for 5 min, washed off the unbound hematoxylin in tap water (with a gentle stream), immersed the sections in bluing agent (R6697000-1A, Ricca Chemical Co. Arlington, TX, USA) for 2 min, and then rinsed the sections again in tap water. Following hematoxylin staining, we immersed the sections in eosin (HT110132-1L, MilliporeSigma) for 2 min, washed off the unbound eosin in tap water, aspirated the excess water with a suction pipette, and performed 10 quick dips of the sections in 95% ethanol. These methods have been slightly modified since earlier publications.[2,5] Digital images were captured with a 20X objective lens under brightfield optics with the same microscope, as above.

References

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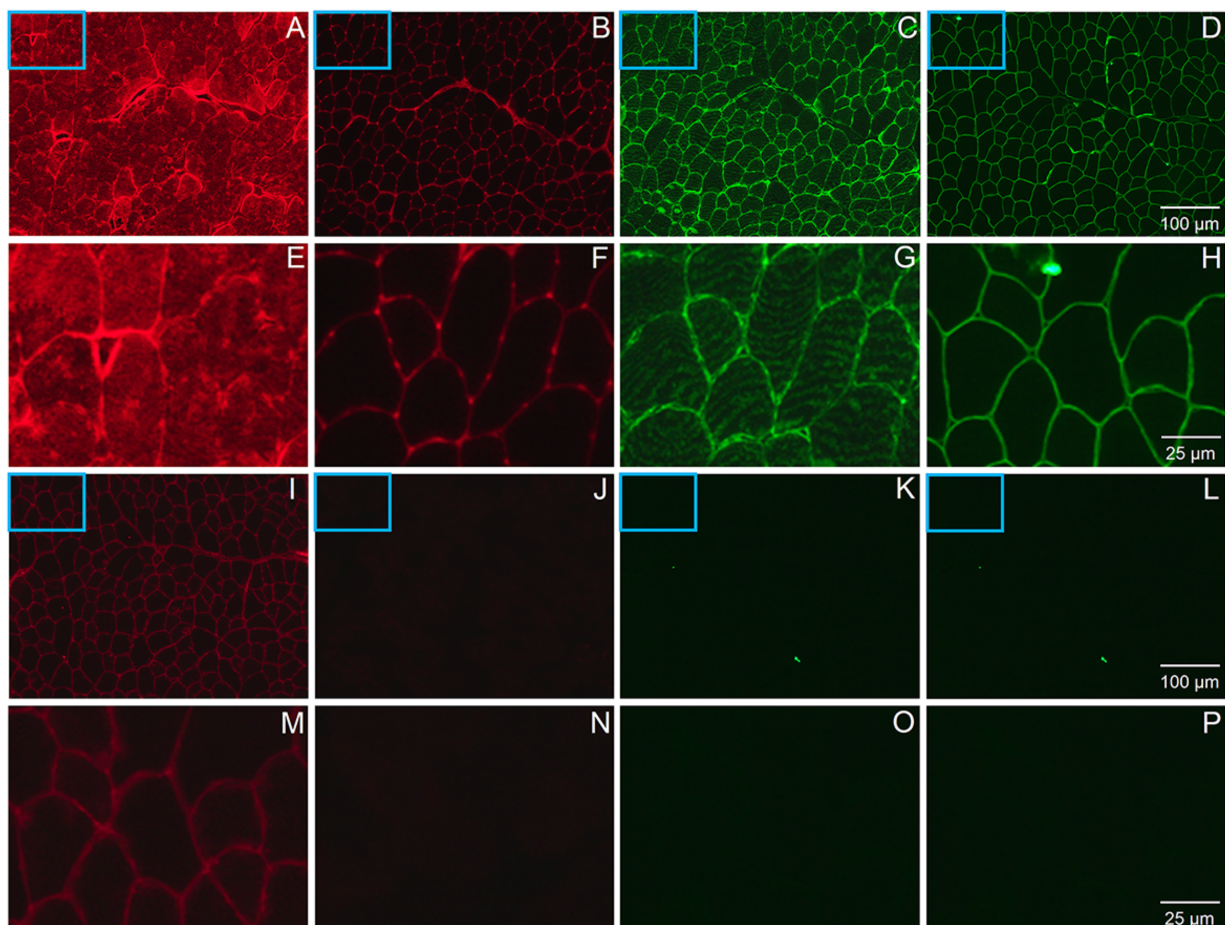

Figure S1.

Positive- and negative-control labeling of MyHC, IgG, desmin and dystrophin in control TA muscle.