**Day 1**

1. Section muscle tissue at 7 µm and allow to dry for 3-4 hrs at room temperature (RT)
2. Circle sections with Pap pen (ImmEdge, Vector Laboratories, H-4000) and allow to dry for an additional 20-30 minutes at RT
3. Fix sections in ice cold (-20 ºC) acetone for 3 min (this step is done in the -20 ºC freezer)
4. Wash 3x 3 min in 1x PBS in a coplin jar
5. Remove slides from the coplin jar, flick the excess PBS from the slide, wipe the back with a paper towel to remove PBS droplets and transfer to a humidifying slide chamber with ~¼ inch of water in the bottom (VWR, 10 slide staining tray with black lid, 102097-500).
6. Block endogenous peroxidases by pipetting 3% hydrogen peroxide in 1x PBS onto your sections (into your Pap pen area) and incubate for 7 min at RT (VWR, BDH7690-1).
* Bubbles should form on your sections during hydrogen peroxide incubation. The absence of bubbling will not affect staining of the tissue but is indicative of poor quality tissue/sections.
1. Wash 3x 3 min in 1x PBS by dumping the PBS off of the slide, flicking excess PBS, wiping the back of the slide and pipetting fresh PBS onto your section/into the Pap pen area (\*all washes from here on out are done this way)
2. Block 1hr in 2.5% normal horse serum (NHS) at RT (Vector Laboratories, S-2012)
* 2.5% NHS is fairly viscous and can be advantageous to saving the volume needed for primary antibody incubation. Once the sections are covered in 2.5% NHS it is possible to gently reduce the volume on the section by pipeptting, being careful not to touch the section with the pipette tip. Often, I only need ~100 µl to cover each slide.
1. Following the 1 hr block, gently tap the NHS off the slide. If you look at the Pap pen you will see parts where the NHS clings to the Pap pen – this disrupts your hydrophobic barrier. Gently dab these areas of the Pap pen with a kimwipe to remove the NHS and re-establish the hydrophobic barrier.
2. Pipette enough primary antibody to just cover the section and incubate sections in primary antibody (1° Ab) in **2.5% NHS** overnight (ON) at 4 °C, rocking
	* Pax7 mouse (Ms) IgG1 (1:100) (Concentrate from DSHB)
	* MyHC Type 1: BA.D5 IgG2b (1:75) (Concentrate from DSHB Iowa)
	* Rabbit (Rb) anti(α)-Laminin (1:100) (Sigma, cat# L9393) or Rb α-Dystrophin (1:100)(Abcam, ab15277)

**Day 2**

1. Wash 4x 5 min in 1x PBS
2. ****Incubate for 90 min with goat (Gt) α-Ms IgG1 biotin–SP-conjugated (1:1000) in **2.5% NHS** (Jackson Immuno Research, 115-065-205)
3. Wash 3x 5 min in 1x PBS
4. Incubate 1hr in the following antibodies in 1x PBS at RT
	* SA-HRP (1:500) (Invitrogen, S-911; stock solution = 2.5µg/µl)
	* Gt α-Rb IgG (H+L) AF488 (1:250) (for laminin, Invitrogen, cat# A-11034)
	* Gt α-Ms IgG2b AF647 (1:250) (for MyHC Type1, Invitrogen, cat# A-21242)
5. Wash 3x 5 min in 1x PBS
6. Incubate 20 min in Superboost TSA Alexa flour 594 (1:500) in 1x PBS (ThermoFisher, B40957).
7. Wash 3x 5 min in 1x PBS.
8. Incubate 10min in DAPI (1:10,000 of stock) in 1x PBS at RT (Thermo Fisher Scientific, Molecular Probes, D1306).
	* Alternatively, you can mount and coverslip with Vectashield with DAPI, but you will need to give the DAPI 10 minutes or so to really intercalate into the nuclei (Vector Laboratories, H-1200)
9. Wash 3x 5 min in 1x PBS.
10. Mount with PBS/glycerol or Vectashield (Vector Laboratories, H-1000) and coverslip. For higher resolution imaging, use a hardset mounting medium with better optical clarity like Prolong Gold.
* Following mounting with aqueous medium (PBS/glycerol or Vectashield), drain excess mounting from underneath coverslip by standing slides vertical on a paper towel for ~5 minutes. This adheres the coverslip to the slide and prevents the formation of air pockets underneath your coverslip over time. Slides are sufficiently drained when no more medium leaks out from under the coverslip.
1. Once coverslipped, slides can be imaged or stored at 4 ºC. For long-term storage, coverslip edges can be sealed with clear nail polish to prevent air from getting under the coverslip over time. If stored correctly, in the dark, staining will last for several months.

**Recipes:**

1. 3% Hydrogen peroxide – dilute 30% hydrogen peroxide 1:10 in 1x PBS
2. 1x PBS
	* + 1. Mix 69.68 g NaCl , 17.36 g Na2HPO4־7 H2O, 2.08 g KH2PO4
			2. Stir to dissolve in DI water
			3. ****Dilute 10N NaOH 1:5 with DI water to make a 2N solution; dilute 6N HCl 1:3 with DI water to make a 2N solution
			4. pH PBS with 2N NaOH or HCl
			5. Bring to a final volume of 8 L
			6. 1x PBS can be kept at room temperature for up to 3 months
3. Gt α-Ms IgG1 biotin stock
	* + 1. Dissolve ~1 mg/ml (lot specific) in 250 µl of distilled water and transfer to a 1 ml eppendorf tube
			2. Add 250 µl of glycerol and vortex well to mix
			3. Aliquot and store at -20 ºC
			4. Resuspension volume can be adjusted for a total of 1 ml vs 500 µl, then use at a dilution of 1:500 instead of 1:1000
4. Streptavidin-horseradish peroxidase (SA-HRP) stock – add 400 µl 1x PBS to 1 mg of lyophilized SA-HRP and vortex well to dissolve. Aliquot and store at -20 ºC.
5. Superboost TSA AF594 stock – resuspend in 150 µl of DMSO, vortex well to dissolve completely, aliquot and store at -20 ºC
6. DAPI for staining cell nuclei
	* + 1. Prepare a 5mg/ml stock solution by diluting in 1x PBS
			2. Aliquot and store at -20ºC
			3. A working dilution of 1:10,000 in 1x PBS is used for labelling nuclei

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| **Channel Combinations with Filter Sets** |
| **Antibody** | **Secondary/Channel** |
| Pax7 | AF594/TRITC/TxRed |
| Type 1 | AF647/Cy5 |
| Laminin or Dystrophin | AF488/FITC/GFP |
| Nuclei | DAPI/AMCA |