**Day 1**

1. Section muscle tissue at 7 µm and allow to dry for 3-4 hours (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 then proceed with staining or store at -20 ºC until ready to stain
	* If sections have been stored at -20 ºC, pull them from the freezer and allow to dry on the bench for 15-20 min prior to staining
3. Fix sections with 4% paraformaldyhyde (PFA) for 7 min in a chemical hood (see Recipes below)
	* \*PFA if harmful and should be handle with appropriate PPE (gloves, lab coat and a mask when appropriate)
4. Wash 3x 3 min in 1x PBS in a coplin jar (see Recipes below)
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
* Bubbles may form on your sections during hydrogen peroxide incubation indicating the peroxide is working. The absence of bubbling will not affect staining but may indicate poor tissue quality.
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 out are done this way)
2. Rinse sections with RT Sodium Citrate buffer (see Recipes below) for 2 minutes then perform antigen retrieval as follows:
	* + Pre-warm Sodium Citrate buffer to 65°C in water bath (15-20 minutes)
		+ After rinsing slides for 2 min in RT Sodium Citrate buffer, add slides to warmed Sodium Citrate now in the water bath
		+ Re-set the bath temperature to 92°C and allow the Sodium Citrate buffer to warm with the bath
		+ Once the bath temperature reaches 92°C, incubate the slides for an additional 11 minutes
		+ Following the 11 minute incubation, turn the water bath off, remove the lid and allow the slide chamber and bath to cool to roughly 50°C
		+ Once cooled to 50°C remove from the water bath and cool on the bench until roughly RT (can be luke warm) (\*proper cooling is important for protein re-folding and proper antibody labelling)
		+ Wash 3x3 min with PBS then proceed with the staining
3. Block sections for 45 min at RT with mouse on mouse, anti-IgG blocking (M.O.M), 1 drop/1 ml of PBS (Vector laboratories, MKB-2213)
4. Wash 3x 3 min with 1x PBS
5. Block 1hr in **1% BSA blocking**
	* ****Dissolve 10mg powdered BSA per 1 mL of 1x PBS (will last for a long time so we usually make 15 mL). Powdered BSA will dissolve more readily if rocked at 4 ºC
6. Pipette enough primary antibody (1° Ab) to just cover the section and incubate overnight (ON) at 4 °C, rocking (primary antibody is diluted in **1% BSA blocking** solution)
	* Pax7 mouse (Ms) IgG1 (1:100) (Concentrate from DSHB)
	* MyHC Type 1: BA.D5 IgG2b (1:75) (Concentrate from DSHB)
	* 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 **1% BSA blocking** (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 10 min 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 (1:1) 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. ****4% PFA
	1. Place 800 mL of 1x PBS in a glass beaker and heat to 60 ºC on a stirring hot plate
	2. While stirring, add 20 g of paraformaldehyde powder (VWR, 200058-220), cover and maintain at 60 ºC (\*\*DO NOT heat above 70 ºC or PFA will break down)
	3. Add 5 drops of 2N NaOH (1 drop/100 mL). The solution should clear within a couple of minutes (some fine particles will remain and will be filtered out at the end).
	4. Remove from heat, allow to cool and add 195 mL of 1x PBS
	5. Adjust pH to 7.2 and fill if needed to a final volume of 1 L with 1x PBS
	6. Filter with Whatman paper (#1), aliquot, foil wrap to protect from light and store at -20 ºC
		* When aliquots are thawed, PFA may fall out of suspension. If this happens, simply heat gently (~40 ºC) and vortex vigorously to resuspend
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. 3% Hydrogen peroxide – dilute 30% hydrogen peroxide 1:10 in 1x PBS (VWR, BDH7690-1)
4. 10 mM Sodium Citrate buffer, pH 6
	1. Add 2.94 g Tri-sodium citrate, dehydrate (VWR, ) to 800 mL distilled water
	2. Stir to dissolve fill to 1 L with distilled water and pH to 6
	3. Store at 4 ºC
5. 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
6. 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.
7. Superboost TSA AF594 stock – resuspend in 150 µl of DMSO, vortex well to dissolve completely, aliquot and store at -20 ºC
8. 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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| **Possible Staining/Channel Combinations with our Antibodies and Filter Sets** |
| **Antibody** | **Secondary/Channel** | **Secondary/Channel** | **Secondary/Channel** | **Secondary/Channel** |
| Pax7 | AF488/FITC.GFP | AF594/TRITC.TxRed | AF488/FITC.GFP | AF647/Cy5 |
| Type 1 | AF647/Cy5 | AF647/Cy5 |  |  |
| Laminin or Dystrophin | AF594/TRITC.TxRed | AF488/FITC.GFP | AF647/Cy5 | AF488/FITC.GFP |
| AF594/TRITC.TxRed | AF594/TRITC.TxRed |
| Nuclei | DAPI | DAPI | DAPI | DAPI |