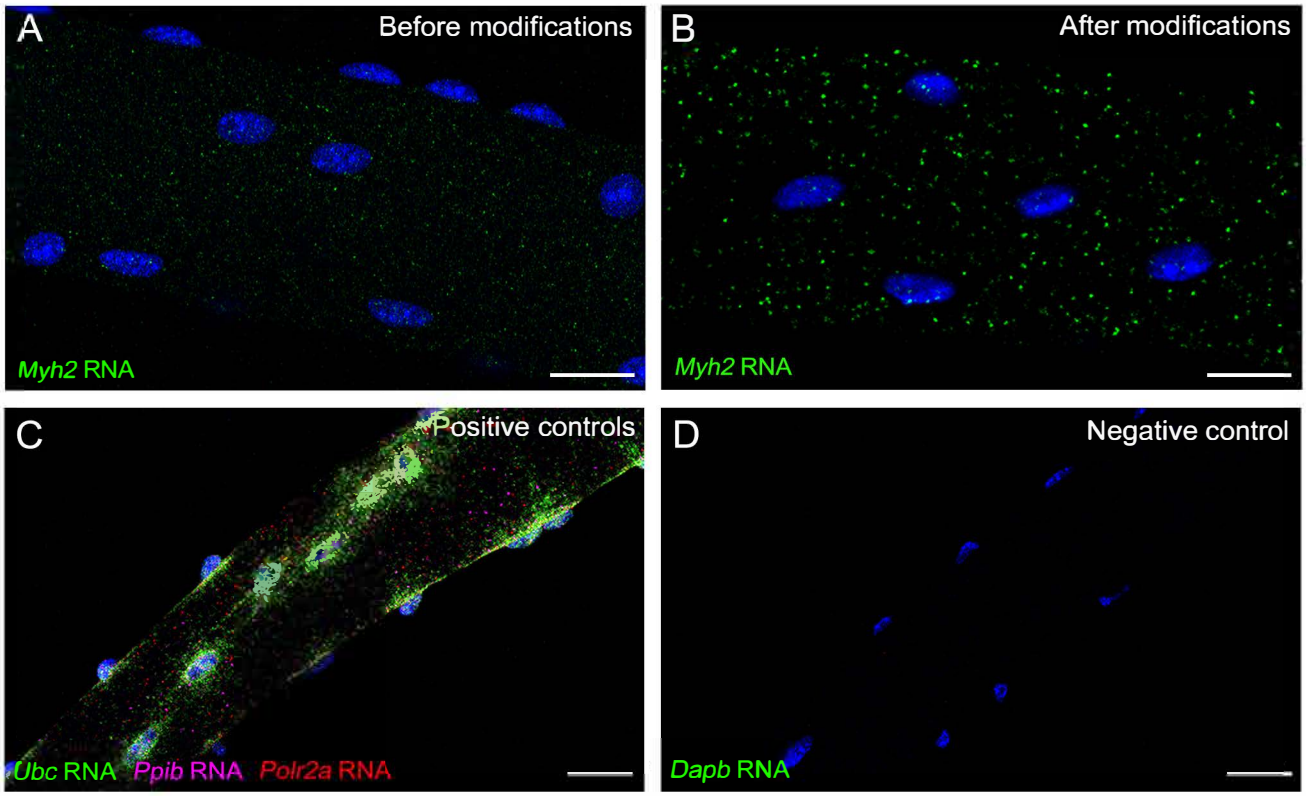


Figure S1. MF-RNAscope allows sensitive detection of single transcripts in whole-mount muscle fibers.

(**A,B**) MF-RNAscope of freshly isolated EDL fibers probed for *Myh2* RNA, shown (A) before and (B) after modifications to the manufacturer's V2 system protocol, as presented in the paper.

(**C,D**) MF-RNAscope of isolated EDL fibers probed for (C) manufacturer-provided positive control genes *Ubc, Ppib,* and *Polr2a* and (D) negative control bacterial gene *Dapb*.

Scale bars: (A,B) 20μm; (C,D) 25μm. Nuclei are identified with DAPI.

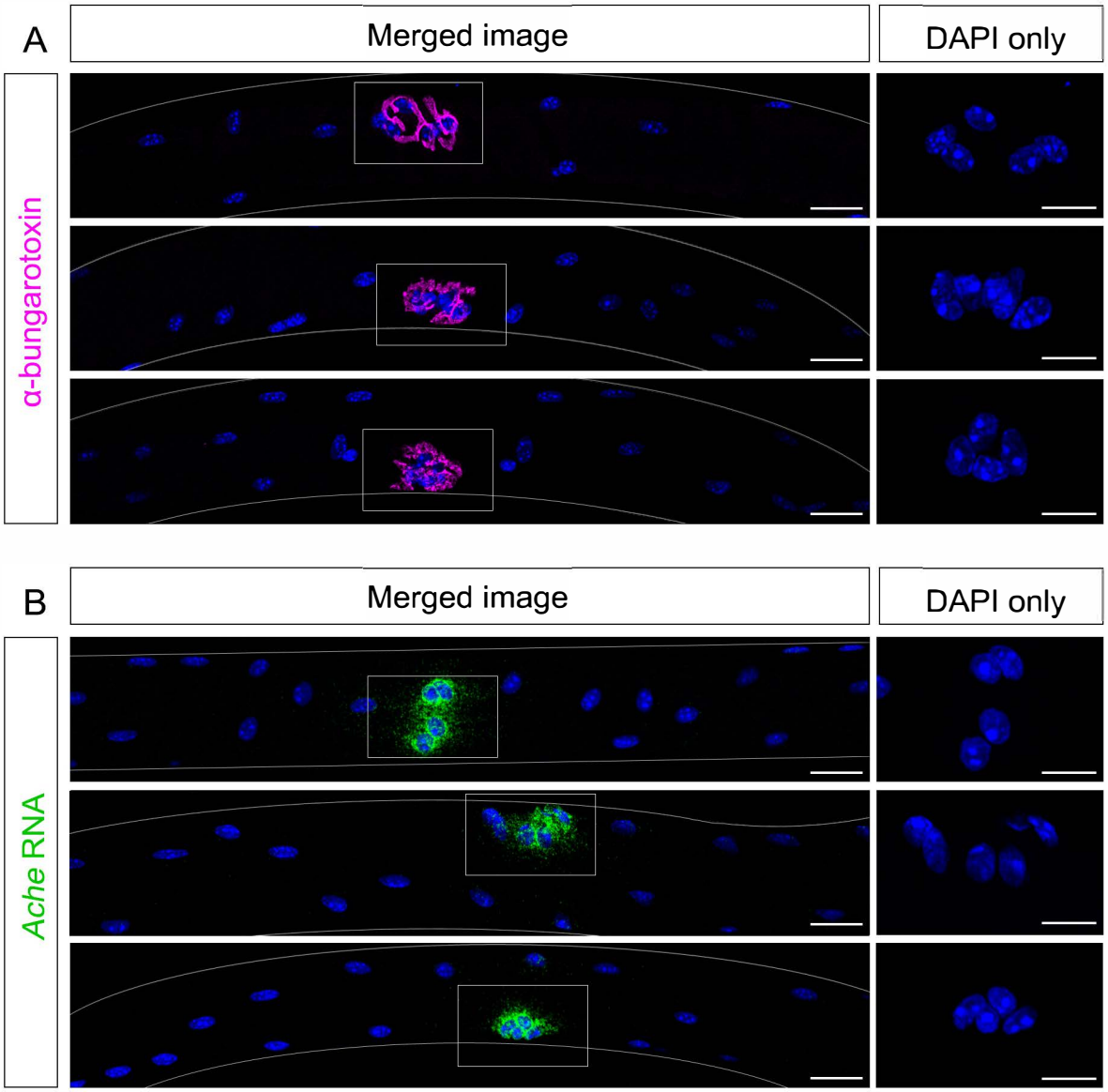


Figure S2. *Ache* transcripts are specifically localized around the NMJ.

(**A,B**) Synaptic myonuclei organize in distinctive clusters on myofibers and can be labeled by (A) α-bungarotoxin (magenta) or (B) *Ache* RNA (green). Insets on the right show DAPI-stained clusters of synaptic myonuclei. We note that these clusters of myonuclei are unique to the postsynaptic side of the NMJ (therefore only one per myofiber is observed), and DAPI staining alone is sufficient for their identification.

Scale bars: (A,B) 25μm (A,B insets) 20μm.

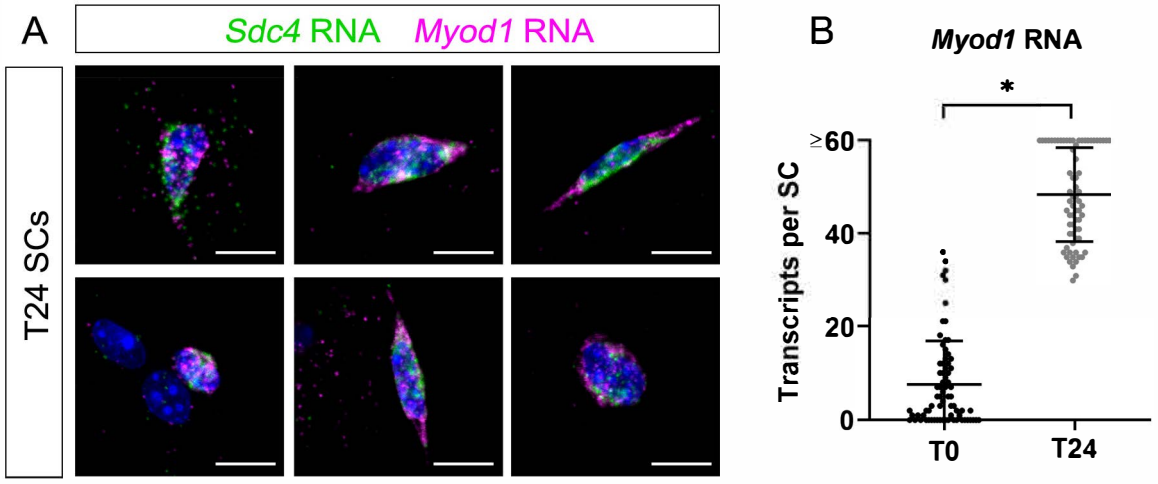


Figure S3. *Myod1* transcripts in SCs are upregulated upon activation.

(**A**) MF-RNAscope of EDL fibers cultured with CEE for 24 hours, probed for *Sdc4* (green) and *Myod1* (magenta) RNAs. Images are maximum intensity projections of confocal images throughout each SC. (**B**) Quantification of *Myod1* transcripts at T0 (data from Figure 2B) and T24. Mean ± SD. n=73 (T0) or n=70 (T24) SCs from 3 mice each. * = p<0.0001 using a two-tailed unpaired *t*-test.

Scale bars: (all) 10μm. All nuclei are identified with DAPI.

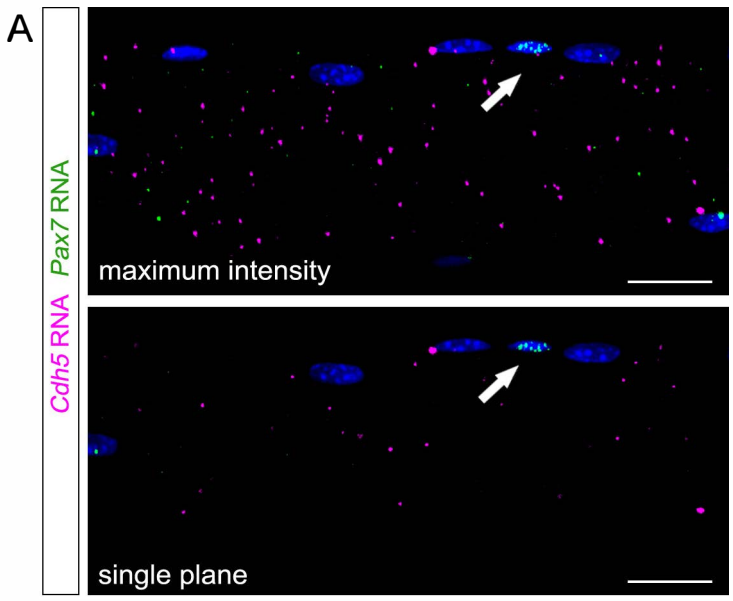


Figure S4. *Cdh5* transcripts are distributed evenly throughout the length and depth of myofibers.

(**A**) MF-RNAscope of single EDL fibers probed for *Pax7* (green) and *Cdh5* (magenta) RNAs. Top image shows a maximum intensity projection of confocal images throughout a myofiber section (40x magnification); z-stack distance = 0.5μm. Bottom image shows a single confocal plane. Arrows indicate a $Pax7^{+}$ SC.

Scale bars: (all) 25μm. Nuclei are identified with DAPI.

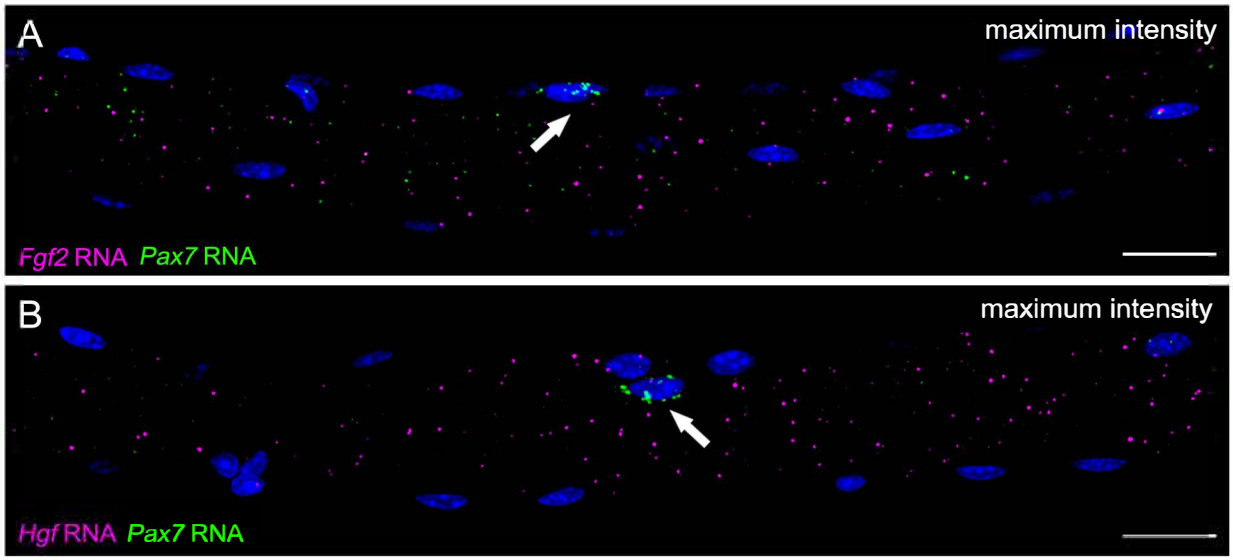


Figure S5. *Fgf2* and *Hgf* transcripts are distributed throughout myofibers.

(**A,B**) MF-RNAscope of single EDL fibers probed for *Pax7* (green) and either (A) *Fgf2* or (B) *Hgf* (magenta). Images are maximum intensity projections of confocal images throughout each myofiber section (40x magnification); z-stack distance = 1μm. Arrows indicate $Pax7^{+}$ SCs. Scale bars: (all) 25μm. Nuclei are identified with DAPI.

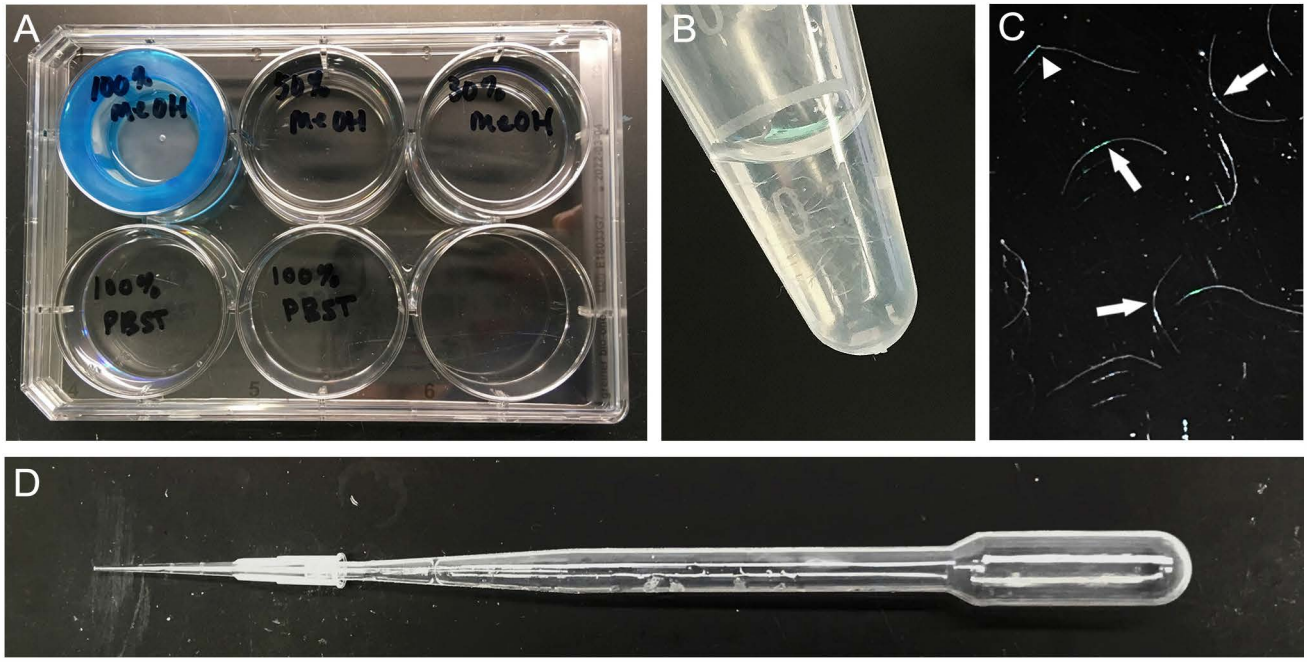


Figure S6. Tools used during the MF-RNAscope protocol.

(**A**) Rehydration set-up showing a 40µm nylon filter in a 6-well untreated tissue culture plate containing 100% MeOH, 50% MeOH/50% PBST, 30% MeOH/70% PBST, and 100% PBST. (**B**) Visibility of myofibers in Axygen 1.7mL tubes. (**C**) Examples of rehydrated myofibers. Arrows indicate healthy intact myofibers, arrowhead indicates a kinked myofiber; the former perform well with MF-RNAscope, the latter do not. (**D**) Transfer apparatus comprised of a 10µl pipette tip on the end of a transfer pipette.

Table S1: Reagents used

Antibodies:

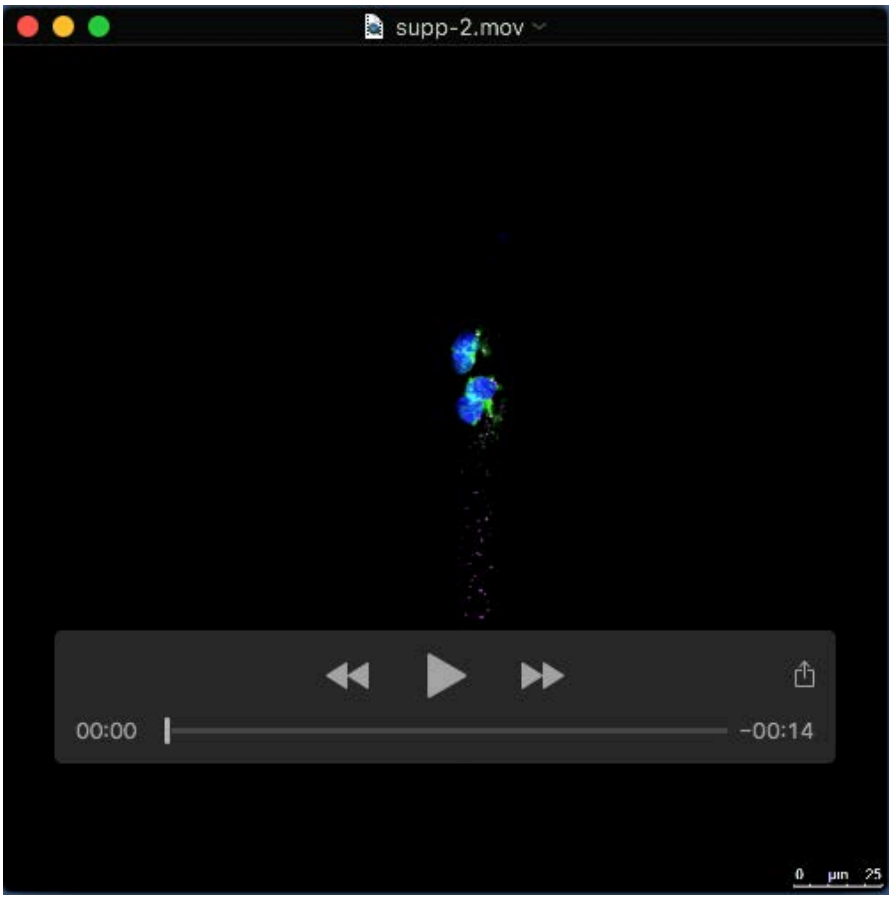
Antibody	Host	Isotype	Concentration	Manufacturer	Product #
Pax7	Mouse	IgG1	1:100	DSHB	PAX7c
Caveolin-1	Rabbit	IgG	1:750	Abcam	ab2910
M-cadherin	Mouse	IgG1	1:50	Santa Cruz	12G4
Dll-4	Rabbit	IgG	1:500	Abcam	ab7280
GM130	Mouse	IgG1k	1:50	BD Biosciences	610822
α-Bungarotoxin, Alexa594-conjugated			1:100	ThermoFisher	B13423
goat Alexa488-conjugated anti-mouse IgG1			1:300	Thermofisher	A-21121
goat Alexa568-conjugated anti-rabbit IgG			1:300	Thermofisher	A-11011
goat Alexa647-conjugated anti-mouse IgG1			1:300	Invitrogen	A21240

Benchtop reagents:

Reagent	Composition
4% PFA	Filtered 1X PBS + Electron Microscopy Sciences Paraformaldehyde
PBTX	RNase-free 1X PBS + 0.2% Triton-X-100 (filter before use)
PBST	RNase-free 1X PBS + 0.01% Tween-20 (filter before use)
SSCT	RNase-free 0.2X saline-sodium citrate buffer + 0.01% Tween-20 (filter before use)

RNAscope reagents:

Reagent	Reference #
RNAscope Multiplex Fluorescent Detection Reagents v2	323110
RNAscope Protease III	322381
RNAscope Probe – Mm-Cdh2 (C1)	489571
RNAscope Probe – Mm-Cdh5 (C1)	312531
RNAscope Probe – Mm-Dll4 (C1)	319971
RNAscope Probe – Mm-Myod1 (C1)	316081
RNAscope Probe – Mm-Myh2 (C1)	401401
RNAscope Probe – Mm-Myf5 (C1)	492911
RNAscope Probe – Mm-Fgf2 (C1)	316851
RNAscope Probe – Mm-Hgf (C1)	315631
RNAscope Probe – Mm-Sdc4 (C1)	473591
RNAscope Probe – Mm-Cdh15 (C2)	473711-C2
RNAscope Probe – Mm-Ache (C2)	490021-C2
RNAscope Probe – Mm-Myod1 (C2)	316081-C2
RNAscope Probe – Mm-Vcam1 (C2)	438641-C2
RNAscope Probe – Mm-Cd34 (C2)	319161-C2
RNAscope Probe – Mm-Pax7 (C3)	314181-C3
RNAscope Probe Diluent	300041
RNAscope TSA Buffer	322809
PerkinElmer TSA Plus Fluorescein System	NEL741001
PerkinElmer TSA Plus Cyanine 5 System	NEL745001



Movie 1. MF-RNAscope permeates throughout the entire depth of myofibers.

Detection of *Ache* (green) and *Myh2* (magenta) RNAs throughout a myofiber using MF-RNAscope. Video is a confocal z-stack of the NMJ taken at 120x magnification; z-step distance between images = 0.5µm.