High Resolution Genome Wide Expression Analysis of Single Myofibers Using SMART-Seq

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Running title: *Single myofiber RNA-Seq*

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Abstract

Skeletal muscle is a heterogeneous tissue. Individual myofibers that make up the contractile muscle tissue exhibit variation in their metabolic and contractile properties. Although there are biochemical and histological assays to study myofiber heterogeneity, efficient methods to analyze the whole transcriptome of individual myofibers are lacking. We have developed single myofiber RNA-Seq (smfRNA-Seq) to analyze the whole transcriptome of individual myofibers by combining single fiber isolation with Switching Mechanisms at 5' end of RNA Template (SMART) technology. Our method provides high-resolution genome wide expression profiles of single myofibers. This method will be useful to study developmental and age-related dynamics in the composition of skeletal muscle.

Introduction

Skeletal muscle is composed not only of myofibers but of a variety of different cell types (e.g. endothelial cells, fibro/adipogenic progenitors, adipocytes, among others)(1). Not only is there a variety of different cells, but there is also heterogeneity in the myofibers. Muscles are formed from multiple fiber types(2). Fiber types are often categorized based on their contractile properties, giving two categories: fast twitch muscles and slow twitch muscles(2,3). These types can be further subcategorized based on metabolic properties and myosin heavy chain (MyHC) isoforms(2,4).

Skeletal muscle has a great plasticity in its fiber type composition. The tissue can respond to a wide range of stimuli, adapting to the needs of its environment(2). Changes in fiber type have been shown to occur due to exercise(5,6), molecular signaling(7), ageing(8-11), and disease(12).

Standard RNA sequencing captures the entirety of the muscle tissue, along with all other cell types present, and cannot distinguish between the different fiber types. Methods of investigating changes in fiber type in response to different stimuli rely on staining and biochemical analyses of individual fibers, biochemical analyses of the whole muscle or sequencing of entire muscles(13). There is a need for a method to effectively sequence the entire transcriptome of a single myofiber.

The emergence of single cell technology provides ample opportunity for further investigation into the heterogeneity of single muscle fibers at the transcriptome level. Here, we combine single myofiber isolation of the Extensor Digitorum Longus (EDL) in *Mus musculus* with Switching Mechanisms at 5' end of RNA Template (SMART) to analyze whole transcriptome of individual myofibers (Figure 1). This combination allows us to perform Single MyoFiber RNA-Seq (smfRNA-Seq). By isolating single fibers from whole muscle and extracting their RNA for sequencing, we can now see how the

transcriptome varies among individual fibers and changes throughout development, aging, and in disease contexts.

Here we describe a robust method to extract RNA from a single myofiber followed by generation of sequencing ready libraries and whole transcriptome analysis.

Results and Discussion

Common methods aiming to study the transcriptome of myofibers involve sequencing RNA extracted from the entire muscle. This results in non-myogenic cells being sequenced, and the transcriptome of all the various fiber types cannot be distinguished. The intention of our novel method is to give researchers the ability to sequence RNA from a single myofiber without the confounding presence of other cell types and to see how the transcriptome varies between different fiber types throughout development and ageing.

Single fiber isolation

From the EDL, we can isolate a single myofiber using collagenase digestion (Figure 2A). The key to a successful isolation is to ensure that when dissecting the EDL to only cut the tendon. Cutting into the muscle instead will result in the loss of the majority of the myofibers. If the EDL is isolated properly one can obtain over 200 myofibers.

Removal of Satellite Cells

Myofibers have a population of muscle stem cells, known as satellite cells, associated with them that are few in number, but may still provide an unwanted signal. The satellite cells associated with the myofibers can be almost completely removed with the addition of trypsin to the digestion buffer at a final concentration of 0.25% (Figure 2B-D). This process will strip the myofibers of their satellite cells without damaging the fiber itself.

RNA Extraction from a Single Myofiber

Extracting the RNA from a single myofiber can prove challenging. The fiber is very tough and does not readily breakdown under normal lysing conditions. With a whole muscle, a method to overcome this is by freezing the muscle in liquid nitrogen and grinding it into a powder with mortar and pestle(14). However, this method cannot realistically be done to a single fiber and still collect all of the RNA. Therefore, we lysed the fiber with lysis buffer in RNAse free water, utilizing osmotic pressure and gentle pipetting to breakdown the fiber and retrieve the intact RNA. This method proved effective as more than an adequate amount of RNA was recovered, even from a single myofiber, for use in the SMART technology cDNA synthesis and amplification (Table 1).

Generation of Sequencing Ready Libraries

From the extracted RNA, we successfully generated cDNA libraries using the DNA SMART-Seq HT kit (Takara Biosciences). After the amplification of the libraries, we obtained sufficient quantity of cDNA for downstream sequencing (Table 1).

To generate cDNA libraries for sequencing the cDNA needs to be tagmented and have adaptors incorporated. To accomplish this, we used the NexteraXT DNA Library Preparation Kit. For the single fiber samples, we performed tagmentation with Tn5 enzyme on 250 pg of cDNA. The cDNA is amplified once more and then size selected using AMPure beads at 0.85x of the sample volume. This amount of beads will remove DNA that is smaller than 200 bp. After size selection, we visualized the cDNA on an agarose gel and see that the fragments are an acceptable size in both the whole muscle and in the single fiber, implying the initial RNA was of good quality(Figure 2 E,F). The ideal size for sequencing is approximately 300-400 bp.

Sequencing Results

Single fiber RNA sequencing had a depth that was comparable to the whole muscle RNA sequencing (Figure 3A). The overall expression profile of the single myofibers is very similar to that of the whole muscle, indicating that we are capable of performing RNA sequencing of a single myofiber, at a high resolution similar to what is seen in whole muscle RNA-Seq (Figure 3A). However, when looking at the PCA plot we see clustering of the single fibers together on both axes, but away from the whole muscle on one of the axes (Figure 3B). This indicates that although the single fibers are similar to the whole muscle in regards to muscle specific genes, the lack of other cell types allows the single fibers to form a distinct cluster away from the whole muscle. In this paper, we also extracted RNA from five and twenty myofibers to compare the efficiency of single cell technologies with increasing input. When the number of fibers is increased we see less similarities between them and the whole muscle and even with the single myofiber (Figure 3A,B). This is most likely due to the increased quantity of sample and could be resolved by scaling up the protocol.

When looking more in depth at individual genes we see many similarities between the single myofiber and the whole muscle, but also crucial differences. Of particular note, we see that muscle specific genes have similar reads between the single fibers and the whole muscle. Here we show several example genes that are expressed in skeletal muscle. The *Myh* cluster codes for a variety of myosin heavy chain proteins (MyHC), which are the motor proteins of muscle whose various isoforms are the basis of the different fiber types(4,15). Here we show a part of the *Myh* gene cluster on chromosome 11. The similar expression between the single fiber and the whole muscle conclusively shows that the RNA sequenced

came from a myofiber (Figure 4A). For further confirmation, we also display *Ckm*, the muscle specific creatine kinase(16) and *ACTA1*, which codes for skeletal muscle alpha actin(17). These genes have the same pattern as is seen with *Myh* (Figure 4B,C).

Skeletal muscle is composed of various non-muscle cells that are captured during whole muscle RNA sequencing. Using smfRNA-seq we are capable of completely removing these unwanted cell types and sequence only the myofiber transcriptome.

To demonstrate the removal of non-myogenic cell populations we looked at cell specific genes of a variety of different cell types that are present in the whole muscle. Particularly, we demonstrate that satellite cells, fibroblasts, endothelial cells, macrophages, adipocytes, fibro/adipogenic progenitors (FAPs) and hematopoietic cells are not captured using the smfRNA-seq method.

In addition to the removal of satellite cells by trypsin, we looked at the amount of reads for *Pax7,* a gene that is expressed in muscle stem cells, and we see that there is no expression of *Pax7* in the single fiber transcriptome (Figure 4D). We also analyzed markers for fibroblasts, particularly *Col1a1* and *Thy1(18,19)*. As expected for these genes, there is expression in the whole muscle, but no expression in the single fiber (Figure 5A,B). With regards to endothelial cells, we used the markers *Kdr* and *PECAM1(20,21)*. Once more, there is expression in the whole muscle and none in the single fiber (Figure 5C,D). The other genes used were *Retn* to identify the presence of adipocytes(22), *Cd34* for hematopoietic cells(23), *Ly6a* as a marker for FAPs(24), and *ADGRE1* for macrophages(25) (Figure 5E-J). As expected, in the single fiber transcriptome we see no expression for any of these genes, all of which were present in the whole muscle. These results clearly show that the RNA that was sequenced from a single myofiber came from only that fiber and not from any other resident cell types present in the muscle.

Conclusion

smfRNA-Seq is a powerful new technique that allows high resolution whole transcriptome sequencing of a single myofiber. Here, we have demonstrated that RNA can be successfully isolated from a single myofiber at a high enough concentration and quality to be used with SMART-Seq technology, generating a high-quality cDNA library for sequencing. Our data from smfRNA-seq is of a comparable depth and resolution to what is seen in whole muscle RNA (Fig 3). The RNA sequenced from a single fiber does not display enrichment of genes specific to various non-myogenic cells, clearly indicating that only the myofiber RNA was sequenced. This provides a novel tool for researchers interested in the change in transcriptome between fiber types during development, aging and disease, without the added confounding factors of other cell types and various fibers.

Experimental Procedures

All procedures that were performed on animals were approved by the McGill University Animal Care Committee (UACC)

A detailed step by step protocol is provided with the supplemental materials

Commercial Kits

The following commercial kits were used in this experiment.

SMART-Seq HT Kit (Takara Cat# 634437)

Nextera XT DNA Library Preparation Kit (Illumina Cat# FC-131-1024)

Nextera XT Index Kit (Illumina Cat# FC-131-1001)

Buffers

Myofiber digestion buffer was prepared using 1000 U/mL of Collagenase from Clostridium histolyticum (Sigma Cat# C0130) in unsupplemented DMEM (Invitrogen Cat# 11995073)

Myofiber immunofluorescence blocking buffer is composed of 5% horse serum (Wisent Cat# 065-250), 2% Bovine Serum Albumin (Sigma Cat# A8022), 1% Triton-X_{100} (Sigma Cat# T9284), in PBS (Wisent Cat# 311-425-CL)

RNA extraction buffer is made using 19 μL of the 10X lysis buffer plus 1 μL of RNAse inhibitor from the SMART-Seq HT Kit. 1μL of the previously composed 10X lysis buffer is added to 9 μL of RNAse free water to make 1X lysis buffer

Myofiber Isolation

Briefly, individual myofibers were isolated from the Extensor Digitorum Longus (EDL) in the following manner. To expose the EDL, the Tibialis Anterior (TA) must first be removed. The EDL was cut at each tendon and then placed it in the myofiber digestion buffer. Trypsin was added to a final concentration of 0.25% to remove the associated satellite cells. Incubated the EDL at 37°C and 5% CO_2 for at least one hour.

To disassociate the myofibers, we transferred the EDL to a 6-well plate with 2 mL of unsupplemented DMEM, that had previously been coated with 10% horse serum (HS) in DMEM for at least 30 minutes. The EDL was gently pipetted up and down with a large bore pipette coated in HS until no more myofibers could be retrieved.

Immunofluorescence of Myofibers

Briefly, fixed freshly isolated myofibers at T_0 using 4% paraformaldehyde in PBS for 5 minutes. Washed 3 times with 0.1% Triton-X_{100} in PBS. Permeabilized with 0.1% Triton-X_{100} and 0.1M glycine in PBS for 15 minutes. Washed 3 times with 0.1% Triton-X_{100} in PBS. Blocked for 1 hour with blocking buffer. Incubated with a Pax7 hybridoma primary antibody (DSHB #AB_528428) at 1:100 dilution in blocking buffer overnight at 4ºC. Washed 3 times with 0.1% Triton-X_{100} in PBS. Incubated the Alexa Fluor 488 goat anti-mouse IgG1 secondary antibody (Invitrogen Cat# A21121) at a 1:400 dilution in blocking buffer for 1 hour. Fibers were washed 3 times with 0.1% Triton-X_{100} in PBS and mounted on a microscope slide with Prolong Gold Antifade Reagent with DAPI (Invitrogen Cat# P3695)

Myofiber RNA Extraction

Myofibers were transferred to a 6-well plate with 2 mL of PBS to wash the fibers. A single myofiber was transferred to a 0.2 mL PCR tube and excess PBS was removed. Next, we added 10 μL of lysis buffer, and gently pipetted the myofiber up and down for 3 minutes and then incubated the fiber on ice for 5 minutes while periodically vortexing and spinning down the sample.

The residual fiber pieces were removed by spinning down the sample and transferring the supernatant to a fresh PCR tube.

Whole Muscle RNA Extraction

The whole muscle from the hindlimb of a mouse was dissected, and frozen in liquid nitrogen and ground into a powder using a mortar and pestle. RNA from whole muscle was extracted using TRIzol reagents.

cDNA Library Preparation

cDNA was constructed using DNA SMART-Seq HT kit, following the manufacturers recommendations. For a single fiber, we used 12 cycles of PCR amplification on the cDNA. The cDNA was then purified using AMPure XP beads at a 1:1 ratio.

Preparing Sequencing Ready Libraries

For a single fiber, we used as a starting material 250 pg of cDNA in 1.25 μL of water. Followed the directions provided with the Nextera XT DNA Library Preparation Kit, but reduced all quantities by 4x. The libraries were size selected using AMPure beads at 0.85X of the sample volume, to remove all fragments below 200 bp.

Sequencing and Analysis

The Illumina NextSeq 500 High Output Flow Cell was used for sequencing. The sequenced reads were then mapped to the mouse mm10 genome by using HISAT2(26), using an index downloaded from the HISAT2 website that jointly indexes the mm10 genome and the ENSEMBL transcriptome definition. FeatureCounts was used in order to quantify gene expression using GENCODE gene definitions(27).

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Disclosures

The authors have no conflict of interest to disclose

Table Legends

Table 1: RNA concentration after SMART cDNA synthesis and amplification, and after incorporation of Illumina adaptors and size selection.

Figure captions

Figure 1: **SMART technology and incorporation of Illumina adaptors to the fiber mRNA.** Schematic displaying the steps and biochemical reactions involved in the generation of sequence ready cDNA fragments

Figure 2: **Isolation of total RNA from a single myofiber.** (A) Isolated myofibers after a properly performed dissection and digestion. (B) Counts of the number of satellite cells per fiber in myofibers treated with and without trypsin. (C) Representative picture of a myofiber stained for PAX7 and counterstained with DAPI after no treatment with trypsin. (D) Representative picture of a myofiber stained for PAX7 and counterstained with DAPI after treatment with trypsin. (E) Representative image of cDNA from 20 fibers (20F), 5 fibers (5F), and single fiber (1F) on an agarose gel after library preparation and size selection. (F) Representative picture of cDNA from whole muscle on an agarose gel after library preparation and size selection.

Figure 3: **Comparative analysis of whole transcriptome from single myofibers and whole muscle.** (A) Heatmap of gene expression in single fibers, groups of five fibers, groups of twenty fibers, and whole muscle, each with three replicates. Colors represent mean gene expression within each sample, from highest expression (yellow), to lowest expression (dark blue). Genes are ordered from top to bottom by their average expression across all samples. (B) Projection of samples along first two principal components found by PCA applied to log reads-per-million gene expression.

Figure 4: **UCSC snapshots showing expression of myogenic genes in single myofibers and whole muscle.** (A) Part of the myosin heavy chain (*Myh*) gene cluster located on chromosome 11. (B) The muscle creatine kinase (*Ckm*). (C) Actin alpha 1 (*Acta1*) gene. (D) Paired Box 7 (*Pax7*) gene expressed in the associated satellite cells.

Figure 5: **UCSC snapshots showing expression of non-myogenic genes between single myofibers and whole muscle.** (A) Genes collagen type 1 alpha 1 chain (*Col1a1*) and (B) CD90 (*Thy1*) expressed in fibroblasts. (C) The genes kinase insert domain receptor (*Kdr*) and (D) CD31 (*PECAM1*) as markers for endothelial cells. (E) Resistin (*Retn*) as a marker for adipocytes. (F) *Cd34* as a marker for hematopoietic cells. (G) *Ly6a* to detect the presence of fibro/adipogenic progenitors (FAPs). (H) Adhesion G protein-coupled receptor E1 (*ADGRE1*) gene for macrophages. (I) Housekeeping genes *RPS2* and (J) *Gapdh*

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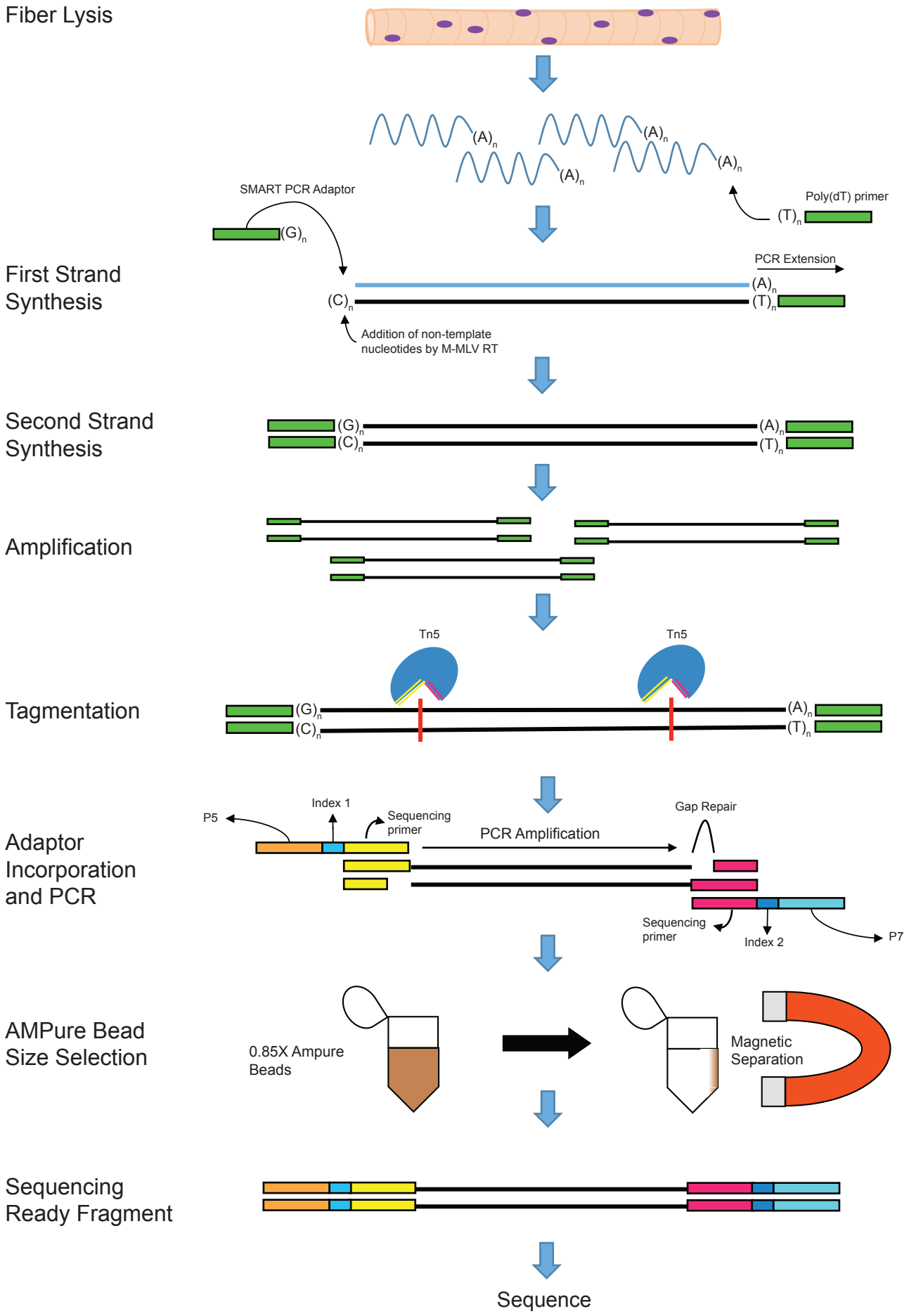
Table 1: RNA concentration after SMART cDNA synthesis and amplification, and after incorporation of Illumina adaptors and size selection.

Samples	**RNA concentration (ng/µL) After SMART reaction and amplification**	**RNA concetration (ng/µL) after adding Illumina adapters and size selection**	**Average fragment size after size selection (bp)**
Single Fiber 1	2.14	6.55	313
Single Fiber 2	7.68	4.46	355
Single Fiber 3	5.28	4.3	329
Five Fibers 1	1.34	4.08	351
Five Fibers 2	2.86	10.79	382
Five Fibers 3	2.41	3.61	382
Twenty Fibers 1	1.45	3.12	486
Twenty Fibers 2	2.17	4.45	483
Twenty Fibers 3	1.71	5.77	484
Whole Muscle 1	0.22	2.31	617
Whole Muscle 2	0.28	4.23	528
Whole Muscle 3	0.3	3.55	456

Figure 1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(A)n
(A)n
(A)n
SMART PCR Adaptor
(G)n
Poly(dT) primer
(T)n
First Strand Synthesis
PCR Extension
(A)n
(T)n
(C)n
Addition of non-template nucleotides by M-MLV RT
Second Strand Synthesis
(G)n
(C)n
(A)n
(T)n
Amplification
Tagmentation
Tn5
Tn5
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(T)n
Adaptor Incorporation and PCR
P5
Index 1
Sequencing primer
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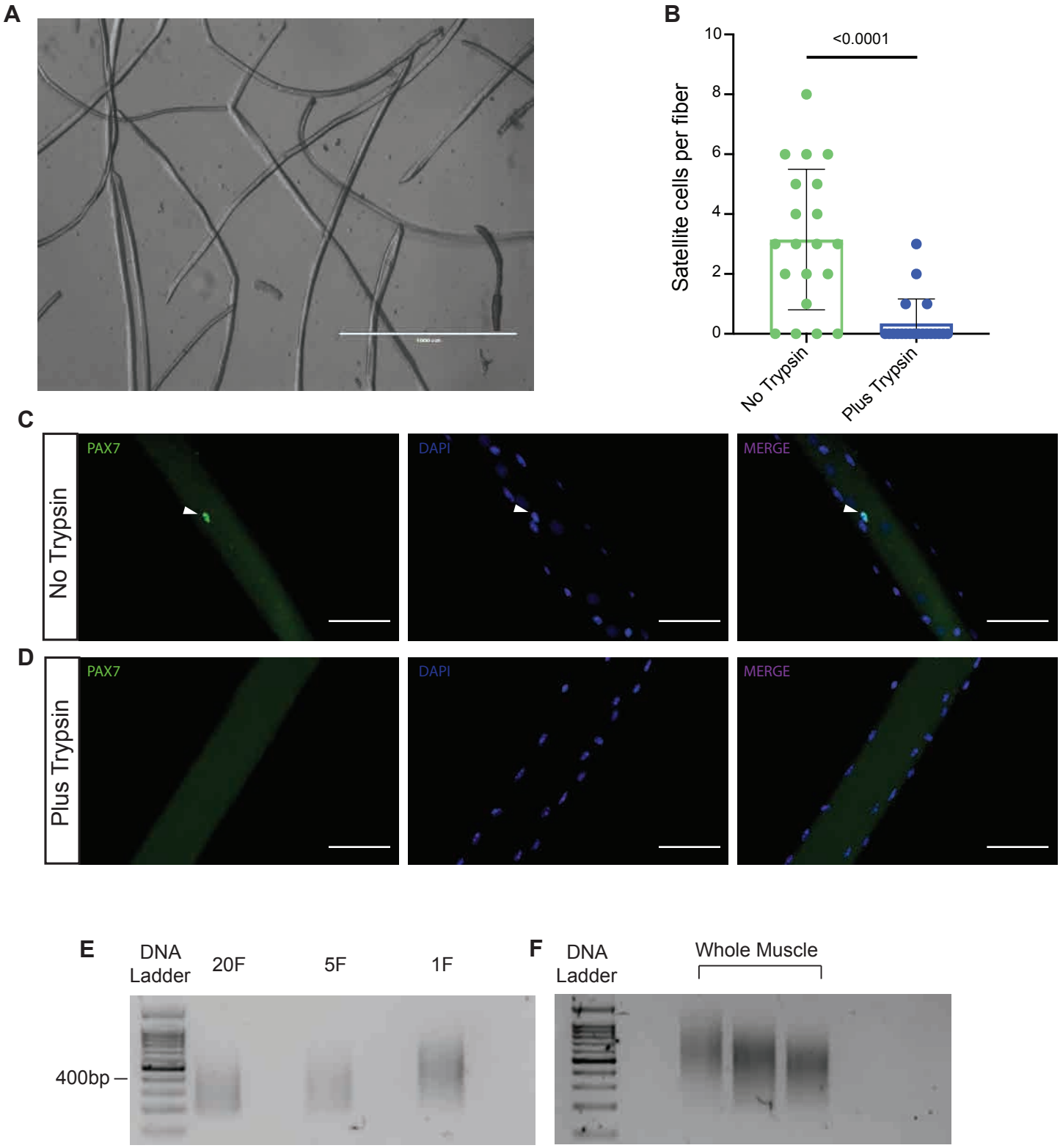
Sequence

Figure 2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B
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6
4
2
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Plus Trypsin
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PAX7
DAPI
MERGE
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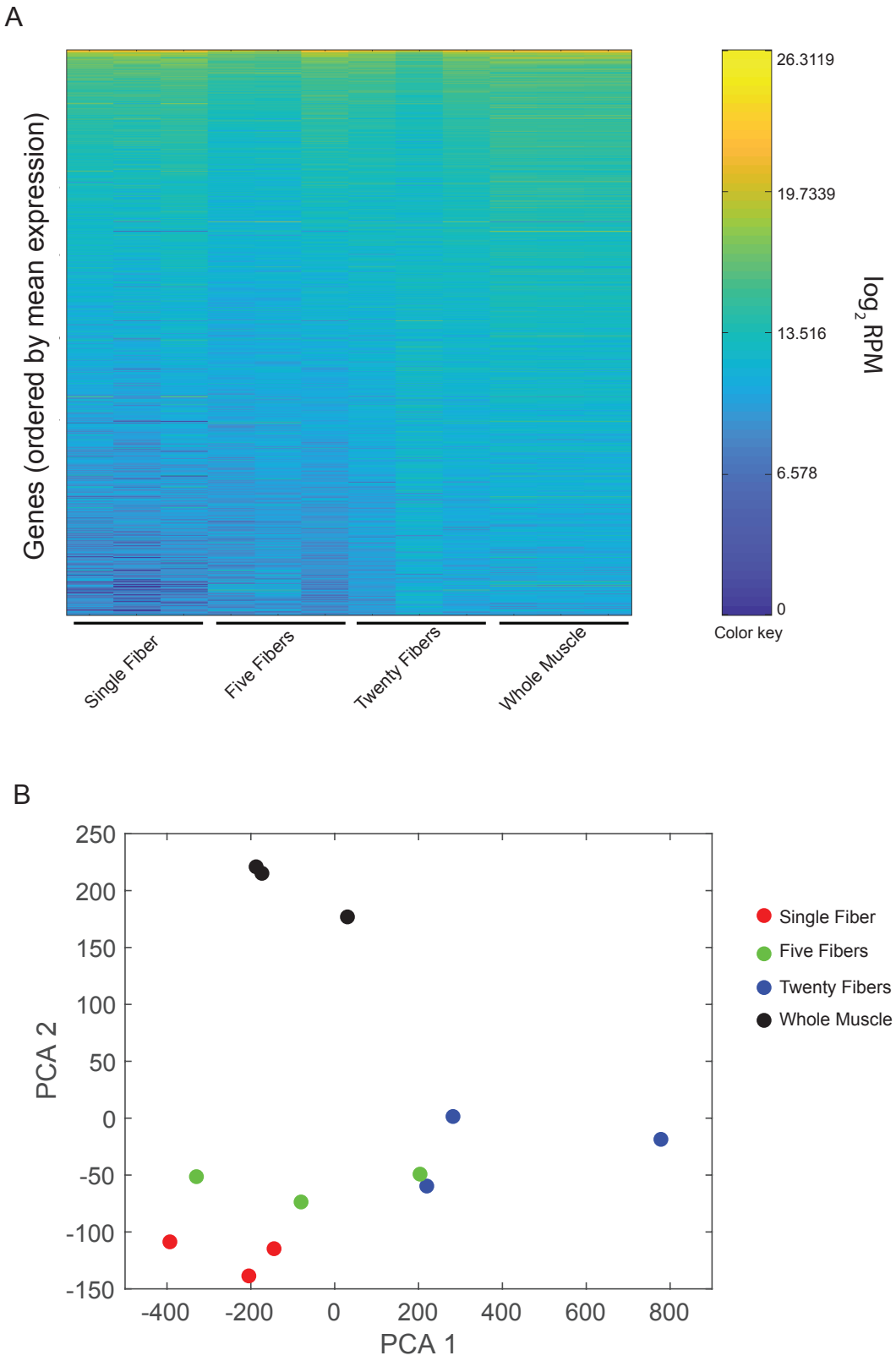
DNA Ladder
Whole Muscle

Figure 3


A
Genes (ordered by mean expression)
Single Fiber
Five Fibers
Twenty Fibers
Whole Muscle
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19.7339
13.516
6.578
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log2 RPM
B
PCA 2
PCA 1
250
200
150
100
50
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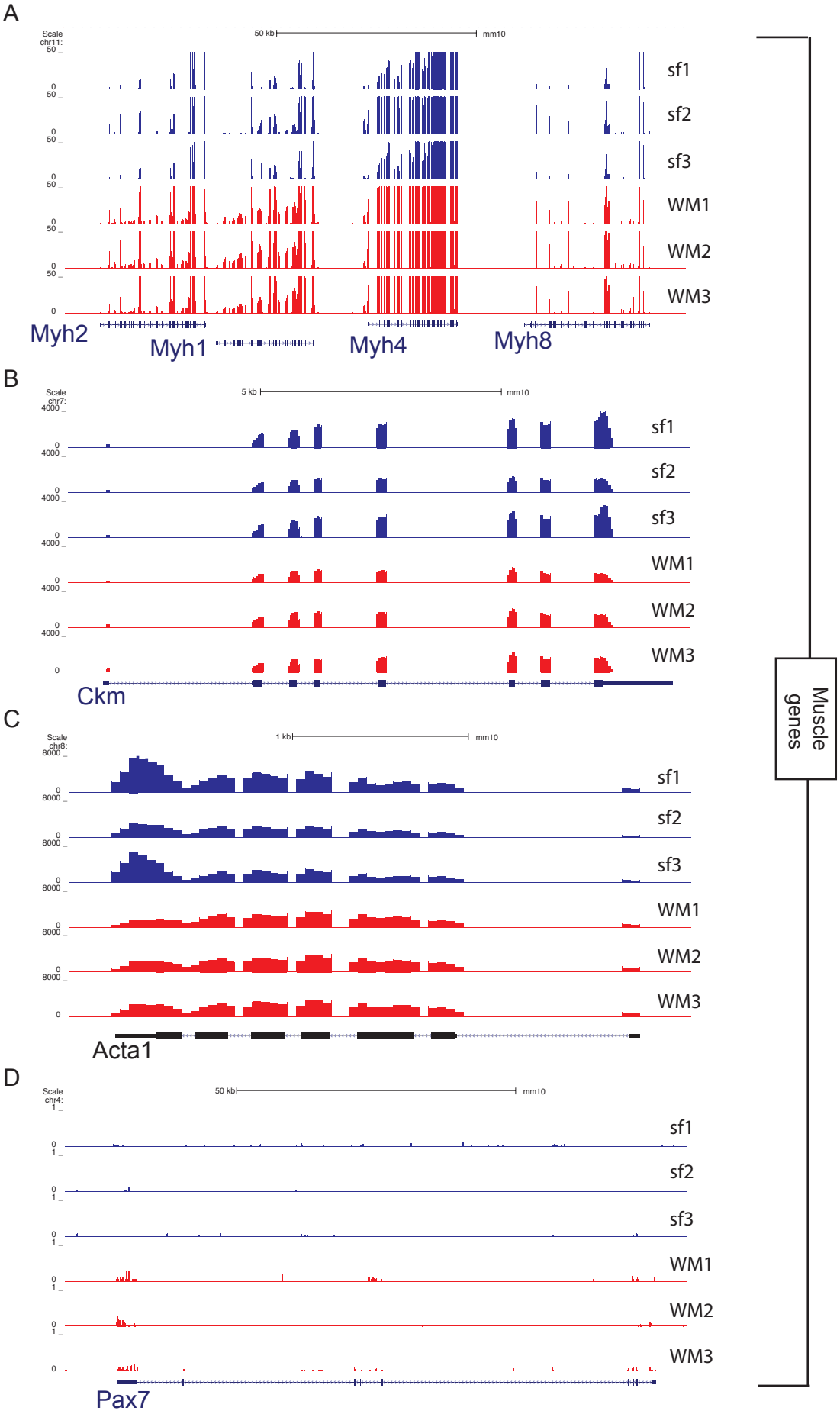
Single Fiber
Five Fibers
Twenty Fibers
Whole Muscle

Figure 4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Ckm
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1 kb
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sf2
sf3
WM1
WM2
WM3
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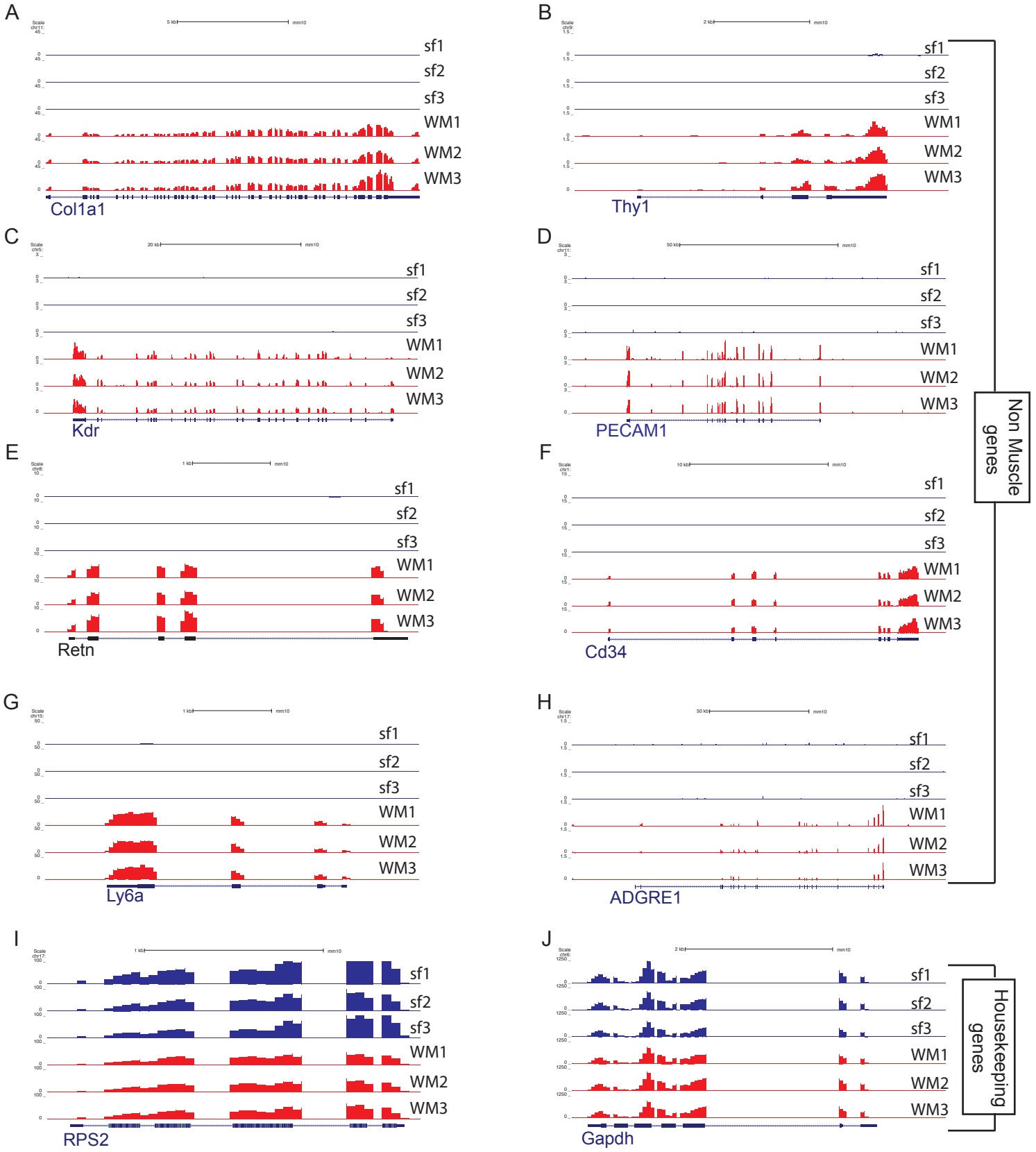
WM3
Pax7
Muscle genes

Figure 5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sf1
sf2
sf3
WM1
WM2
WM3
Col1a1
B
Thy1
C
Kdr
D
PECAM1
E
Retn
F
Cd34
G
Ly6a
H
ADGRE1
I
RPS2
J
Gapdh
Non Muscle genes
Housekeeping genes

Supplement materials: Single Myofiber RNA-Seq Extended Protocol

1 Dissection of Extensor Digitorum Longus (EDL) from *Mus musculus*

1.1 Remove the skin on the hindlimb of the mouse with a pair of scissors by cutting the skin around the ankle and making an incision along the ventral side of the leg.

1.2 Remove the epimysium covering the Tibialis Anterior (TA) muscle.

1.3 Cut the TA tendon at the ankle, being careful to only cut the top tendon as the bottom tendon belongs to the extensor digitorum longus (EDL).

1.4 Using forceps, gently peel off the TA up the leg until you hit the knee, exposing the EDL. Cut off the TA as close to the knee as possible.

1.5 The biceps femoris is covering the proximal EDL tendon at the patella and must be removed to expose the tendon.

1.6 Slide a pair of sharp forceps underneath the EDL and gently run it along its length to ensure that both tendons are exposed and accessible.

1.7 Cut the distal EDL tendon at the ankle. Lift the EDL upright by the tendon with a pair of forceps and, without pulling too hard, cut the proximal tendon as close to the knee as possible.

NOTE: It is very important to cut the EDL at the tendons, cutting into the muscle will result in most of the fibers being lost.

2 Extensor Digitorum Longus Digestion

2.1 Prepare a digestion buffer of 1000 U/mL of collagenase in unsupplemented DMEM and filter the buffer with a 0.22 micron filter.

2.2 Aliquot 800 µL of the digestion buffer to a 1.5 mL tube and place in an incubator at 37 ^{o}C and 5% CO_2 for 30 minutes before dissection to warm up the buffer and infuse it with CO_2.

NOTE: The Myofibers are very sensitive to oxygen, all buffers and media used on them should be first placed in the incubator for at least 30 minutes. When working outside the incubator do not do so for longer than 5 minutes at a time if possible and allow the fibers to recover in the incubator for 10 minutes before reusing them.

2.3 Place the dissected EDL into the digestion buffer and incubate for an hour in the incubator, while periodically inverting the tube to mix the solution. Trypsin can be added to a final concentration of 0.25% at this moment to remove the satellite cells from the fibers.

2.4 Coat a 6 well plate with coating media, composed of 10% horse serum (HS) in DMEM for at least 30 minutes

2.5 Replace the coating media with 2 mL of unsupplemented DMEM and place the plate in the incubator for at least 30 minutes

2.6 Coat a large bore glass pipette with HS and rinse with coating media. Use the large bore pipette to transfer the EDL from the digestion buffer to the 6-well plate, being careful to transfer as little of the digestion buffer as possible with it.

NOTE: A large bore glass pipette can be made by cutting a pasteur pipette with a diamond pen where the area begins to widen and then flame rounding the edges with a Bunsen burner.

2.7 Disassociate the fibers by gently pipetting the EDL up and down. Transfer the EDL to subsequent wells as fibers are isolated to avoid damaging them.

3 Myofiber RNA Extraction

3.1 Coat a 6-well plate with coating media and add 2 mL of PBS. Place the plate in the incubator for at least 30 minutes.

3.2 Coat a small bore glass pipette with HS and rinse with coating media. Use the small bore pipette to transfer fibers to the PBS filled 6-well plate.

NOTE: A small bore glass pipette can be made by using a Bunsen burner and bending the thin portion of a pasteur pipette into a curve. Round off the edges of the opening.

3.3 Transfer a single fiber to a PCR tube and remove the PBS.

3.4 Lyse the fiber with 1 µL of 10X reaction buffer, composed of 19 µL of 10X lysis buffer plus 1 µL of RNAse inhibitor from a DNA SMART-Seq HT kit, in 9 µL of RNAse free water. Pipette the fiber up and down for 3 minutes. Put the tube on ice for 5 minutes and vortex as needed.

NOTE: The fiber will never fully breakdown, but it does so enough for the RNA to be extracted in sufficient quantity.

3.5 To remove the residual fiber pieces, spin down the sample and keep the supernatant.

4 cDNA Library Preparation

4.1 Bring volume of the sample up to 11.5 µL with RNAse free water

4.2 Using the DNA SMART-Seq HT kit perform the following steps: Add 1 µL of 3' SMART-Seq CDS primer II A to the sample. Mix by pipetting or gently vortexing and spin down

4.3 Incubate for 3 minutes at 72 ^{o}C, then place immediately on ice

4.4 Prepare template switching master mix. For one reaction use the following reagents: 0.7 µL nuclease free water, 8 µL one-step buffer, 1 µL SMART-seq HT oligonucleotide, 0.5 µL RNAse inhibitor, 0.3 µL SeqAmp DNA polymerase, 2 µL SMARTscribe reverse transcriptase, total volume 12.5 µL per reaction.

NOTE: add the enzymes last to the master mix and use immediately.

4.5 Add 12.5 µL per reaction. Mix and spin down.

4.6 Place samples in a thermal cycler using the following settings:

42 °C 90 min

95 °C 1 min

98 °C 10 s

65 °C 30 s

68 °C 3 min

X number of cycles

72 °C 10 min

4 °C forever

Here we used 11-12 cycles for a single fiber.

NOTE: This is a stopping point. cDNA can be left at 4 °C overnight or at -20 °C until purification

5 Purification of cDNA

5.1 Add AMPure XP beads directly into the PCR tube at a 1:1 ratio. Mix thoroughly and allow the DNA to bind to the beads at room temperature for 8 minutes.

NOTE: Allow AMPure XP beads to warm to room temperature before pipetting. Vortex AMPure XP beads vigorously before use.

5.2 Place the tubes on a magnetic strip and wait for the solution to clear, approximately 5 minutes.

5.3 Discard the supernatant, being careful to not remove the magnetic beads. Wash the beads twice with 200 µL of 80% ethanol for 20 seconds.

NOTE: On the last wash, make sure to remove all the ethanol and use an aspirator to remove any residual droplets on the side of the tube, if necessary.

5.4 Elute the DNA using 17 µL of elution buffer. Add the buffer to the tube, mix thoroughly until the beads go into solution, and incubate at room temperature for 5 minutes.

NOTE: Be careful not to allow your beads to over dry. When dried the DNA becomes difficult to elute, resulting in low yield. If the beads are dry place them in elution buffer at 45°C and vortex periodically until no clumps are visible and the beads do not settle at the bottom of the tube.

5.5 Place the tubes on a magnetic strip until the solution clears and keep the supernatant.

NOTE: This is a stopping point. cDNA can be kept at 4°C or -20°C

6 Preparing Sequencing ready libraries

6.1 Quantify the samples using Quant-IT PicoGreen dsDNA assay kit.

6.2 Prepare 250 pg of single fiber cDNA in 1.25 µL of water in a microtube

NOTE: It is important to accurately quantify the cDNA concentration. The amount of starting material can alter the size of your fragments. More DNA results in larger fragments while too little gives small fragments that will be lost during size selection.

6.3 Using the Nextera XT DNA Library Preparation kit, add to the mix 2.5 µL of TD buffer, and 1.25 µL of ATM, mix and spin down.

6.4 Incubate at 55 ^{o}C for 5 minutes

6.5 remove from heat and add 1.25 µL of NT, mix and spin down

NOTE: Add NT buffer directly after the tagmentation reaction (step 6.4) to deactivate the Tn5 transposase, since the enzyme is still active at this point.

6.6 Incubate the sample for 5 min at RT.

6.7 To amplify your libraries, add 1.25 µL of an i7 adaptor and 1.25 µL of an i5 adaptor and 3.75 µL of NPM. Mix and spin

6.8 Run your PCR program using the following steps:

72 ^{o}C for 3 min

95 ^{o}C for 30 s

95 ^{o}C for 10 s

55 ^{o}C for 30 s

72 ^{o}C for 30 s

12 cycles

72 ^{o}C for 5 min

10 ^{o}C forever

NOTE: this is a stopping point.

7 Purification and Size Selection

7.1 Add AMPure XP beads directly into the PCR tube at a 0.85x ratio to the volume of sample. Mix thoroughly and incubate at room temperature for 8 minutes.

NOTE: This ratio will remove fragments smaller than 200 bp.

7.2 Place the tubes on a magnetic strip and wait for the solution to clear, approximately 5 minutes.

7.3 Discard the supernatant. Wash the beads twice with 200 µL of 80% ethanol for 20 seconds. On the last wash, make sure to remove all the ethanol and use an aspirator to remove droplets on the side of the tube, if necessary.

7.4 Elute the DNA fragments by adding 20 µL of resuspension buffer and mixing thoroughly until the beads go into solution. Incubate at room temperature for 5 minutes.

7.5 Place the tubes on the magnetic strip until the solution clears and keep the supernatant.

7.6 Quantify your samples using Quant-IT PicoGreen dsDNA assay kit.

7.7 Store at -20 ^{o}C until ready for sequencing.