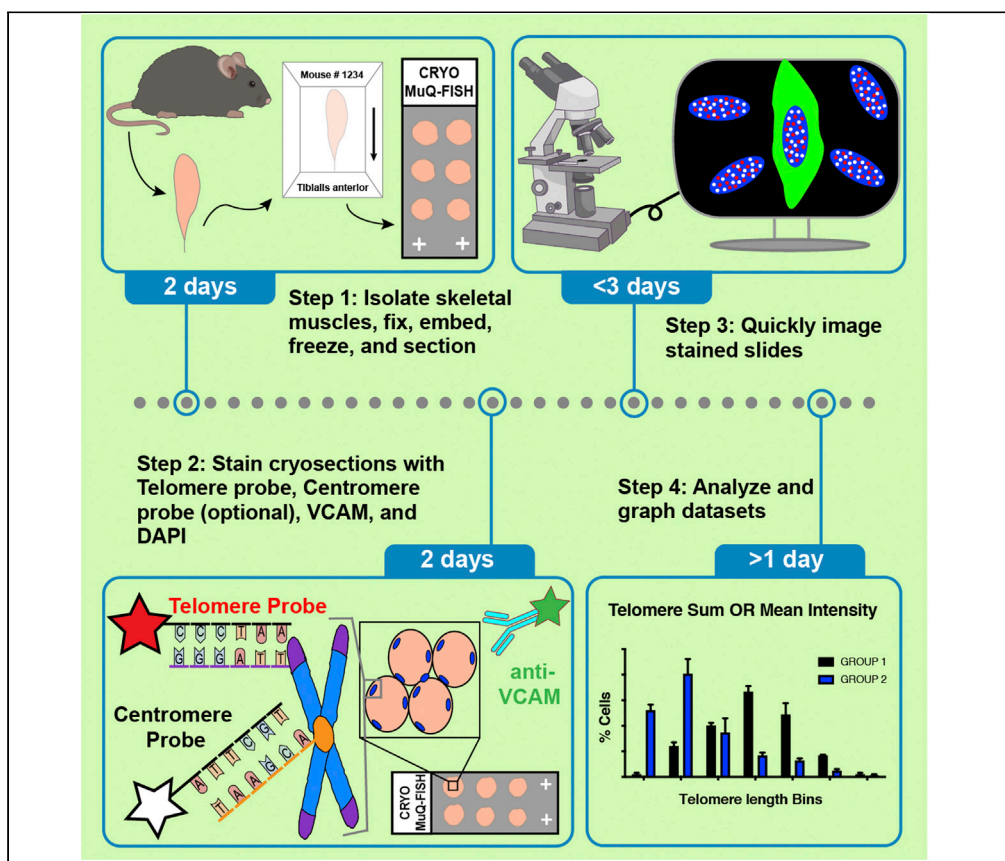


## Protocol

# Telomere length assessments of muscle stem cells in rodent and human skeletal muscle sections



Measurements of telomere length in skeletal muscle stem cells (MuSCs), a rare cell population within muscles, provide insights into cellular dysfunction in diseased conditions. Here, we describe a protocol (cryosection muscle quantitative fluorescent in situ hybridization) using skeletal muscle cryosections for assessments of telomere length in MuSCs, in their native environment. Using a free software, telomere length measurements are assessed on a single-cell level. We also provide methodology to perform data analyses in several ways.

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### Highlights

CRYO MuQ-FISH can measure telomere length on resident skeletal muscle stem cells

This protocol is ideal for archival samples

Telomere length quantitation can be normalized to DAPI or centromere intensity

Telomere outputs include both total sum intensity and mean intensity per nucleus

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## Protocol

## Telomere length assessments of muscle stem cells in rodent and human skeletal muscle sections

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## SUMMARY

Measurements of telomere length in skeletal muscle stem cells (MuSCs), a rare cell population within muscles, provide insights into cellular dysfunction in diseased conditions. Here, we describe a protocol (cryosection muscle quantitative fluorescent *in situ* hybridization) using skeletal muscle cryosections for assessments of telomere length in MuSCs, in their native environment. Using a free software, telomere length measurements are assessed on a single-cell level. We also provide methodology to perform data analyses in several ways.

For complete details on the use and execution of this protocol, please refer to Tichy et al. (2021).

## BEFORE YOU BEGIN

## Experimental design and considerations

Reductions in telomere length in muscle stem cells (MuSCs) have been recently discovered in chronic muscle injury conditions, including Duchenne muscular dystrophy (Tichy et al., 2017, 2021), which results in a more severe disease phenotype (Sacco et al., 2010). Many common methods for conducting telomere length assessments in other cell and tissue types requires the isolation of primary cells, which may disrupt the tissue environment, requires large numbers of cells to obtain enough genomic DNA for appraisal [Telomere Restriction Fragment/TRF analysis (Mender and Shay, 2015)], or only provides the average telomere length over the entirety of the cell population [Telomere: single copy gene QPCR methodology (Cawthon, 2002)]. The quantitative fluorescent *in-situ* hybridization (Q-FISH) technique, which allows for assessment of telomere length based on the fluorescence intensity of a hybridized probe, coupled with semi-automated telomere length assessments using a free software (Telometer), provides robust information that can eliminate such limitations. Here, we provide a protocol where our previously established and validated muscle stem cell-specific Q-FISH protocol for measuring telomeres in FACS-isolated MuSCs [MuQ-FISH (Tichy et al., 2017)], has been altered and adapted for use on cryosections of murine skeletal muscle (CRYO MuQ-FISH), leaving the MuSC niche intact. This method can be beneficial for telomere length quantitation of archival samples and may also be adapted to examine telomere lengths in additional muscle-resident cell types, as well as cell types from other tissues. This protocol is also compatible with skeletal muscle cryosections derived from species other than mice but testing and optimization should be conducted by the end user.



**Note:** This protocol utilizes skeletal muscle tissue from research animals and human cohorts. Ensure that the use of research animals has been approved by the appropriate institutional animal care and use committee (IACUC) or equivalent oversight body. Additionally, procurement and use of tissues derived from human sources should follow the appropriate organizational rules regarding informed consent and be approved by the end user's institutional review board. Sample collection for this protocol was done according to institutional guidelines and regulations of the University of Pennsylvania.

**Note:** For telomere data extraction from images, the following software must be installed on the analysis computer: java se (<https://www.oracle.com/java/technologies/javase-downloads.html>) and the freeware Telometer (<https://demarzolab.pathology.jhmi.edu/telometer/download.html>). Install software according to instructions listed on the websites.

This protocol includes the following steps: 1) Skeletal muscle preparation, embedding, sectioning, 2) CRYO MuQ-FISH, 3) image collection, 4) analysis and statistics

### Skeletal muscle preparation, embedding, and sectioning

⌚ Timing: 2 days

1. Humanely euthanize mice according to an approved IACUC protocol methodology and institutional guidelines.

**Note:** This protocol uses male mice between 2 and 5 months of age. However, this protocol can be applied to samples derived from other ages and both sexes of mice.

2. Spray the lower half of mouse body with 70% ethanol.
3. Lay mouse on its back and completely remove skin from the back legs.
4. Dissect desired hindlimb skeletal muscles for assessment.

**Note:** For downstream assessments, it is ideal to compare the same types of muscles, rather than mixing different anatomical groups. We routinely utilize either the Tibialis anterior or the gastrocnemius muscles for telomere length measurements.

5. Fix and process tissues for embedding.
  - a. Place isolated muscles in 3–4 mL of cold (4°C) 4% paraformaldehyde in PBS per well in a 6-well plate.
  - b. Incubate tissue on a rocker at 4°C for at least 2 h.

**Note:** Tibialis anterior muscles up to 200 mg are fixed for 2 h, but larger muscles, such as the gastrocnemius, require a 3-h incubation for fixative to fully enter the tissue.

- c. Remove muscles and place in pre-chilled 30% sucrose in water (4°C) overnight (16 h) at 4°C.
6. Embed tissues in O.C.T. and freeze.
    - a. Label 15 mm × 15 mm × 5 mm base molds with pertinent mouse information with an alcohol resistant marker and mark directionality of the muscle by labeling the terminal tendon location.
    - b. Fill base mold with O.C.T. to the point where it almost overflows from the inset. Do not allow bubbles to form. If they do, use a 100 µL micropipettor and tip to remove the bubbles.
    - c. Place fixed muscle tissue in the base mold and push it evenly to the bottom of the mold. The orientation should be as it was labeled previously.
    - d. Chill 2-methylbutane with dry ice or liquid nitrogen. Immerse muscles in base molds in chilled 2-methylbutane for >30 s for TA muscles and >1 min for gastrocnemius muscles.
    - e. Place chilled blocks on dry ice for 1 h to allow residual 2-methylbutane to evaporate.

f. Wrap blocks in base molds with parafilm and store at  $-80^{\circ}\text{C}$  for at least overnight (16 h).

▮▮ **Pause point:** Cryoblocks can be stored at  $-80^{\circ}\text{C}$  indefinitely.

7. Sectioning of skeletal muscles.

- The evening before cryosectioning, remove cryoblocks from  $-80^{\circ}\text{C}$  and place at  $-20^{\circ}\text{C}$ .
- Label superfrost plus slides with pertinent mouse information.
- Remove block from base mold and section in a cryostat, cutting  $10\ \mu\text{m}$  slices. We try to keep the tissue orientation the same for all blocks. Both longitudinal, as well as cross sectional slices will work with this assay.

**Note:** For optimal adherence of sections, we firmly press cut sections onto room temperature ( $25^{\circ}\text{C}$ ) superfrost plus slides.

d. Store slides at  $-20^{\circ}\text{C}$  until all samples have been collected and sectioned.

▮▮ **Pause point:** Slides can be stored at  $-20^{\circ}\text{C}$  indefinitely.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Alexa Fluor 488 Donkey anti-goat IgG; 1/300 dilution	Thermo Fisher Scientific	Cat# A11055; RRID:AB_2534102
Goat anti-mouse Vcam1; stock made at 1 mg/mL; 1/100 dilution	Fisher Scientific	Cat# PIPA547029; RRID:AB_2609027
Rat anti-CD11b-biotin, clone M1/70; 1/200 dilution	BD Biosciences	Cat# 553309; RRID:AB_394773
Rat anti-CD31-biotin; 1/200 dilution	Fisher Scientific	Cat# 13-0311-85; RRID:AB_466421
Rat anti-mouse CD34-BV421, Clone RAM34; 1/20 dilution	BD Biosciences	Cat# 562608; RRID:AB_11154576
Rat anti-CD45-biotin; 1/500 dilution	BD Biosciences	Cat# 553078; RRID:AB_394608
Rat anti-Ly-6A/E-biotin (Sca1), clone E13-161.7; 1/200 dilution	BD Biosciences	Cat# 553334; RRID:AB_394790
anti-Alpha 7 Integrin Alexa Fluor 647, clone R2F2; 1/25 dilution	Ablab.ca	Cat# 67-0010-05; RRID: AB_2890939
Biotinylated mouse anti-human CD31; 1/50 dilution	Thermo Fisher Scientific	Cat# 13-0319-82; RRID:AB_466423
<b>Chemicals, peptides, and recombinant proteins</b>		
4% paraformaldehyde	Thermo Scientific	Cat# J19943-K2
Antigen unmasking solution (Commercial citrate buffer)	Vector Labs	Cat# H-3300
Bovine Serum Albumin (Fraction V)	Gemini Bio-Products	Cat# 700-100P
Cenpb-Cy3	PNA Bio	Cat# F3002
Dextran sulfate sodium salt	Sigma-Aldrich	Cat# D8906
EDTA	Invitrogen	Cat#15575-020
Ethanol, 200 proof	Decon Labs	Cat# 2701
Ethylene Carbonate	Sigma-Aldrich	Cat# E26258
Formamide	Sigma-Aldrich	Cat# 47670
Immersion oil, type A	Nikon	Cat# MXA20233
OCT	Thermo Scientific	Cat# 6502G
Potassium chloride	Sigma Aldrich	Cat# P9541
Potassium phosphate, monobasic	Sigma Aldrich	Cat# P9791
Prolong gold plus DAPI	Thermo Scientific	Cat# P36935
RNAse A	Fisher Scientific	Cat# AM9780
Sodium Chloride	Sigma Aldrich	Cat# S6191
Sodium Phosphate, dibasic, dihydrate	Sigma Aldrich	Cat# S3264
SSC buffer, 20x	Corning	Cat# 46-020-CM
Streptavidin PE-Cy7	BioLegend	Cat# 405206
Streptavidin Cy3	Thermo Scientific	Cat# SA1010

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sucrose	Sigma-Aldrich	Cat#S0389
TelC-Alexa Fluor 647	PNA Bio	Cat# F1013
Tris-Cl	Quality Biological	Cat#351-006-131
Triton X-100	Fisher Scientific	Cat# BP151
Tween 20	Fisher Scientific	Cat# BP337

**Experimental models: organisms/strains**

Mouse: C57Bl/6J, 2-5-month-old males	Jackson Laboratory	Stock# 000664; RRID:IMSR_JAX:000664
Mouse: IKK2CA: B6.Cg-Gt(ROSA)26Sor <sup>tm4(lkbb)/Rsky</sup> /J, 2-5-month-old males	Jackson Laboratory	Stock# 008242; RRID:IMSR_JAX:008242
Mouse: Pax7 <sup>ERT2Cre</sup> : B6.Cg-Pax7 <sup>tm1(cre/ERT2)Gaka</sup> /J, 2-5-month-old males	Jackson Laboratory	Stock# 017763; RRID:IMSR_Jax:017763
Mouse: mdx/mTR: B6.Cg-Terc <sup>tm1Rdp</sup> Dmd <sup>mdx-4Cv</sup> /BlauJ, 7-month-old males	Jackson Laboratory	Stock# 023535; RRID:IMSR_Jax:023535
Rat: Sprague Dawley, male, 14-month-old male	Charles River	Stock# 001; RRID: MGI:5651135

**Biological samples**

Human skeletal muscle samples, 10-15-year-old male	Obtained in house	N/A
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**Software and algorithms**

Graphpad Prism v. 9.1.2	Licensed Software	<a href="https://www.graphpad.com">https://www.graphpad.com</a>
Image J	Open Source	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>
Java SE 11	Open Source	<a href="https://www.oracle.com/java/technologies/javase-downloads.html">https://www.oracle.com/java/technologies/javase-downloads.html</a>
Microsoft Excel	Licensed Software	<a href="https://www.office.com">https://www.office.com</a>
NIS-Elements	Licensed Software	<a href="https://www.nikon.com/products/microscope-solutions/lineup/img_soft/nis-elements/">https://www.nikon.com/products/microscope-solutions/lineup/img_soft/nis-elements/</a>
Telometer ImageJ Plugin	Open Source	<a href="https://demarzolab.pathology.jhmi.edu/telometer/">https://demarzolab.pathology.jhmi.edu/telometer/</a>

**Other**

Coplin Staining Jar	Thermo Fisher Scientific	Cat# S17495A
Slide Tray for Immuno-Staining with Black Cover	RPI corp.	Cat# 195801
6 well plates	VWR	Cat#10062-892
1.5 mL microcentrifuge tubes	VWR	Cat# 10025-724
15 mL centrifuge tubes	VWR	Cat# 10025-692
50 mL centrifuge tubes	VWR	Cat# 10025-702
Superfrost plus microscope slides	Thermo Fisher Scientific	Cat# 1255015
Super HT PAP Pen, mini	RPI corp.	Cat# 195506
Tissue Path disposable base molds, 15 × 15 × 5 mm	Thermo Fisher Scientific	Cat# 22-363-553
VWR VistaVision Cover Glass, No. 1	VWR	Cat# 16004-096
Compact Digital Rocker	Thermo Scientific	Cat# 88880020
Isotemp Water Bath	Fisher Scientific	Model# 2320
Steamer	Hamilton Beach	Model# VS02
Thermobrite Slide Warmer	Leica	Model# S500-12
Thermomixer	Fisher Scientific	Cat# 05-412-500
Widefield Microscope	Nikon	Model# NiU

**MATERIALS AND EQUIPMENT**

**Buffers**

**TET buffer**

Reagent	Final concentration	Amount
Tris, pH 7.5 (1M)	10mM	0.5 mL
EDTA, pH 8.0 (500mM)	1mM	0.1 mL
Tween-20	0.05%	0.025 mL
ddH <sub>2</sub> O	n/a	49.375 mL
<b>Total</b>	<b>n/a</b>	<b>50 mL</b>

pH 7.6. Buffer can be stored at room temperature (25°C) indefinitely if making a larger batch. Do not reuse buffer.

### Hybridization buffer

Reagent	Final concentration	Amount
Ethylene carbonate	15%	0.15g
Dextran sulfate	20%	0.20g
Sodium chloride, 5M	600mM	120 uL
100x commercial citrate antigen retrieval buffer	1x	10uL
ddH <sub>2</sub> O	n/a	Up to 1 mL
<b>Total</b>	<b>n/a</b>	<b>1 mL</b>

pH 6.0. Use immediately after making buffer.

### Wash Buffer I

Reagent	Final concentration	Amount
SSC (20x)	2x	100 mL
Tween-20	0.1%	1 mL
ddH <sub>2</sub> O	n/a	899 mL
<b>Total</b>	<b>n/a</b>	<b>1000 mL</b>

pH 7.2. Wash buffer I can be prepared in large stocks and stored at room temperature (25°C) for several months.

### Wash Buffer II

Reagent	Final concentration	Amount
SSC (20x)	1x	50 mL
Tween-20	0.1%	1 mL
ddH <sub>2</sub> O	n/a	949 mL
<b>Total</b>	<b>n/a</b>	<b>1000 mL</b>

pH 7.2. Wash buffer II can be prepared in large stocks and stored at room temperature (25°C) for several months.

### Wash Buffer III

Reagent	Final concentration	Amount
SSC (20x)	0.5x	25 mL
Tween-20	0.1%	1 mL
ddH <sub>2</sub> O	n/a	974 mL
<b>Total</b>	<b>n/a</b>	<b>1000 mL</b>

pH 7.2. Wash buffer III can be prepared in large stocks and stored at room temperature (25°C) for several months.

### Wash Buffer IV

Reagent	Final concentration	Amount
SSC (20x)	0.25x	12.5 mL
Tween-20	0.1%	1 mL
ddH <sub>2</sub> O	n/a	986.5 mL
<b>Total</b>	<b>n/a</b>	<b>1000 mL</b>

pH 7.2. Wash buffer IV can be prepared in large stocks and stored at room temperature (25°C) for several months.

### Antibody Blocking Buffer

Reagent	Final concentration	Amount
Bovine serum albumin	3%	1.5g
Triton X-100	0.1%	50 uL
Phosphate Buffered Saline	1x	Up to 50 mL
<b>Total</b>	<b>n/a</b>	<b>50 mL</b>

pH 7.4. Blocking buffer should be stored at 4°C when not in use and is stable for several weeks.

### Phosphate Buffered Saline (PBS), 1×

Reagent	Final concentration	Amount
Sodium Chloride	137mM	8g
Potassium Chloride	2.7mM	0.2g
Sodium phosphate, dibasic, dihydrate	4.3mM	9g
Potassium phosphate, monobasic	1.47mM	0.24g
<b>Total</b>	<b>n/a</b>	<b>1000 mL</b>

pH 7.4. PBS is stored at room temperature (25°C) and is stable for several months.

### Equipment

The following equipment was used for the described protocol.

- –20°C freezer
- –80°C freezer
- 4°C refrigerator
- Compact Digital Rocker (Thermo Scientific, Cat#88880020)
- Isotemp Water Bath (Fisher Scientific, Model #2320)
- Steamer (Hamilton Beach, Model# VS02)
- Thermobrite slide warmer (Leica, Model #S500-12)
- Thermomixer (Eppendorf, Model# F1.5)
- Widefield microscope (Nikon, Model# NiU)

**Alternatives:** A hybridization oven may also be utilized for wash buffer incubations and telomere probe preheating and hybridization, as well as additional heating steps. Common equipment such as freezers, refrigerators, rockers, water baths, steamers, and thermomixers are interchangeable with any other brand and model. Any widefield microscope equipped with the proper filter sets and a 100× objective can complete the imaging described in this protocol.

### Software

The following software are instrumental in analysis of datasets generated using this protocol.

- Java SE 11 or later
- Telometer v3.0.5
- Microsoft Excel 365 or equivalent
- Graphpad prism 9.1.2 or later

## STEP-BY-STEP METHOD DETAILS

### Telomere, centromere, muscle stem cell, and nuclear staining

⌚ Timing: 2 days

1. Remove one slide from each desired sample set from –20°C and warm to room temperature (25°C).
2. Permeabilize sections by immersing slides in 0.5% Triton X-100 in PBS for 10 min in a coplin jar at room temperature (25°C).

**Note:** The coplin jar used in this protocol holds approximately 50 mL of solution and 8 slides. If more slides must be stained, we recommend using a tissue-tek staining dish and 24-place slide holder. This alternative setup requires 200–250 mL of solution volume for adequate coverage of the slides.

3. Wash slides twice with PBS for 3 min each at room temperature (25°C).
4. Perform heat-mediated antigen retrieval with TET buffer.
  - a. Heat buffer to boiling in a microwave first and then transfer buffer to a coplin jar placed in a preheated steamer (95°–100°C).
  - b. Add slides to the coplin jar containing hot TET buffer (95°–100°C) and incubate for 10 min. Remove coplin jar containing the slides from the steamer and let it cool at room temperature (25°C) for 15 min.

**Note:** Take care when handling hot solutions.

△ **CRITICAL:** Do not let the slides dry at any time following antigen retrieval, as doing so greatly increases autofluorescence of the tissue and will make distinguishing MuSCs difficult.

- c. Wash slides with PBS twice for 3 min each in a coplin jar at room temperature (25°C).
5. RNase A treatment
  - a. Remove slides from coplin jar and mark tissue boundaries with a hydrophobic/PAP pen.
  - b. Add sufficient RNase A (100 µg/mL) to cover tissue on the slide (100–200 µL) and cover the slide with a glass coverslip to prevent evaporation.

△ **CRITICAL:** RNase should be DNase-free. We routinely prepare RNase stock concentrations of 10 mg/mL, heat at 100°C for 10 min to destroy DNases and then dilute to 100ug/mL in water and store at –20°C.

- c. Incubate slides at 37°C in a slide warmer or alternative heating source for 20 min.
  - d. Remove coverslip and transfer slides into coplin jars containing PBS. Wash slides twice for 3 min each at room temperature (25°C).
6. Telomere Staining
  - a. Prepare hybridization buffer [adapted from (Matthiesen and Hansen, 2012)] as described in the [materials and equipment](#) section.

**Note:** The hybridization buffer requires extensive vortexing and pipetting to get all components completely into solution. Heating or alteration of pH is unnecessary. When all components are properly dissolved, the solution will be viscous.

- b. Prepare TelC-Alexa Fluor 647 telomere probe and optional Cy3-CENPB centromere probe.
    - i. Dilute 5 nmol probe pellet provided by the supplier with 100uL formamide.
    - ii. Aliquot in 4 µL aliquots in 1.5 mL microcentrifuge tubes and store at –80°C.

**Note:** Probe is light sensitive. All steps proceeding the addition of probe should be done in the dark or low-light conditions.

**Note:** Probes stored at –80°C are stable for at least several months.

**Note:** We recommend using the Alexa Fluor 647-conjugated telomere probe, as it provides the least background in skeletal muscle cryosections.

- c. Remove telomere probe from –80°C storage, thaw, and add probe at a 1/600 dilution to the hybridization buffer (1.67 µL/mL of buffer).



**Table 1. Telomere/centromere probe hybridization conditions**

Steps	Temperature	Time
Initial Denaturation	67°C	10 min
Hybridization	42°C	90 min

**Optional:** Centromere probe staining and analysis is not required, unless problems arise with abnormal nuclear architecture or DAPI staining intensities between samples. If centromere probe staining is desired, use Cy3-CENPB probe at 1/600 dilution and add it at the same time as the telomere probe above. The CENPB probe is prepared identically to the telomere probe.

- d. Heat probe(s) in hybridization buffer for 10 min at 67°C in a thermomixer or alternative heating source in the dark.
- e. Add hybridization buffer containing probe to slides. We typically use 100 µL probe solution per slide. Cover slides with a glass coverslip to prevent evaporation during incubation.

**△ CRITICAL:** It is crucial that bubbles are kept to a minimum, as areas that are not evenly covered will not stain properly.

- f. Incubate slides in a ThermoBrite slide warmer or equivalent heating source, according to [Table 1](#).
- g. Preheat wash buffers I, II, III, IV to 55°C in a water bath or equivalent heating source.
- h. Gently remove coverslips from slides after hybridization, place slides in coplin jars, and incubate with 50 mL prewarmed wash buffer I for 5 min. Keep coplin jar at 55°C during the wash process. Repeat wash once.
- i. Continue washing stepwise with 2 washes each (50 mL per wash) of wash buffer II, wash buffer III, and wash buffer IV, in that order, for 5 min each wash and at 55°C.
- j. Wash slides once with PBS for 5 min at room temperature (25°C).

## 7. Muscle Stem Cell Staining

- a. Block sections in 50 mL antibody blocking buffer for 1 h at room temperature (25°C).
- b. Incubate slides in cold (4°C) blocking buffer with VCAM antibody (1/100 dilution); stock antibody concentration 1 mg/mL, in a dark, humidified staining chamber overnight (16 h) at 4°C. We use 100 µL of antibody solution per slide. Cover slides with a coverslip to ensure even coverage of antibody solution.
- c. Wash slides the following day in coplin jars with 50 mL PBS, twice, 5 min each at room temperature (25°C).
- d. Incubate sections for 1 h with secondary antibody (Alexa Fluor 488 Donkey anti-goat IgG; 1/300 dilution) in antibody blocking buffer at room temperature (25°C). We use 100 µL of antibody solution per slide. Cover slides with a coverslip to ensure even coverage of antibody solution.
- e. Wash slides three times for 5 min each with PBS at room temperature (25°C). PBS washes are carried out in coplin jars and using 50 mL of solution.
- f. Mount coverslips with Prolong gold with DAPI.
- g. Allow mounting media to solidify overnight (16 h) at room temperature (25°C) in the dark.

## Image acquisition

⌚ **Timing:** less than 3 days

**△ CRITICAL:** The imaging must take place as soon as possible after staining, and imaging must be completed for all samples in as short a period of time as possible. Given the

sensitivity of Q-FISH assays, minor changes in fluorescence intensities can result in skewed datasets. Even with antifade agents in the mounting medium, it is imperative to image cells of interest as quickly as possible, so we recommend completing imaging of all samples in 3 days or less.

8. Identify VCAM+ cells present in tissue sections using a Nikon Eclipse NiU widefield epifluorescence microscope.

**Note:** Any widefield epifluorescence microscope with appropriate objectives, filters, and camera should be capable of completing this assay.

**Note:** While VCAM1 is a good marker of MuSCs, it may also label other cells, such as endothelial cells. To prevent selecting the wrong cell types during image acquisition and analysis, we only image MuSCs in the satellite cell position (between the basal lamina and the sarcolemma). Additionally, staining with other non-muscle markers such as CD31 and CD45 (as described in [Tichy et al., 2017](#)) could be performed to exclude such populations from the analysis.

- a. Identify VCAM+ cells (Alexa Fluor 488+) with a low power air objective (i.e., Nikon plan fluor 10x/0.30 objective).
  - b. Switch to a higher-powered 100x objective (i.e., Nikon plan apo100x/1.45 oil) for actual imaging and photographing cells of interest.
9. Identify the optimal exposure settings for DAPI, telomere Alexa Fluor 647 signal, and optional centromere Cy3 imaging. [Troubleshooting 1](#), [Troubleshooting 2](#)

**Note:** Several slides should be examined before determining the optimal exposure settings. It is undesirable to have oversaturated or undersaturated pixels in any of the images. In either situation, fewer regions of interest are identified.

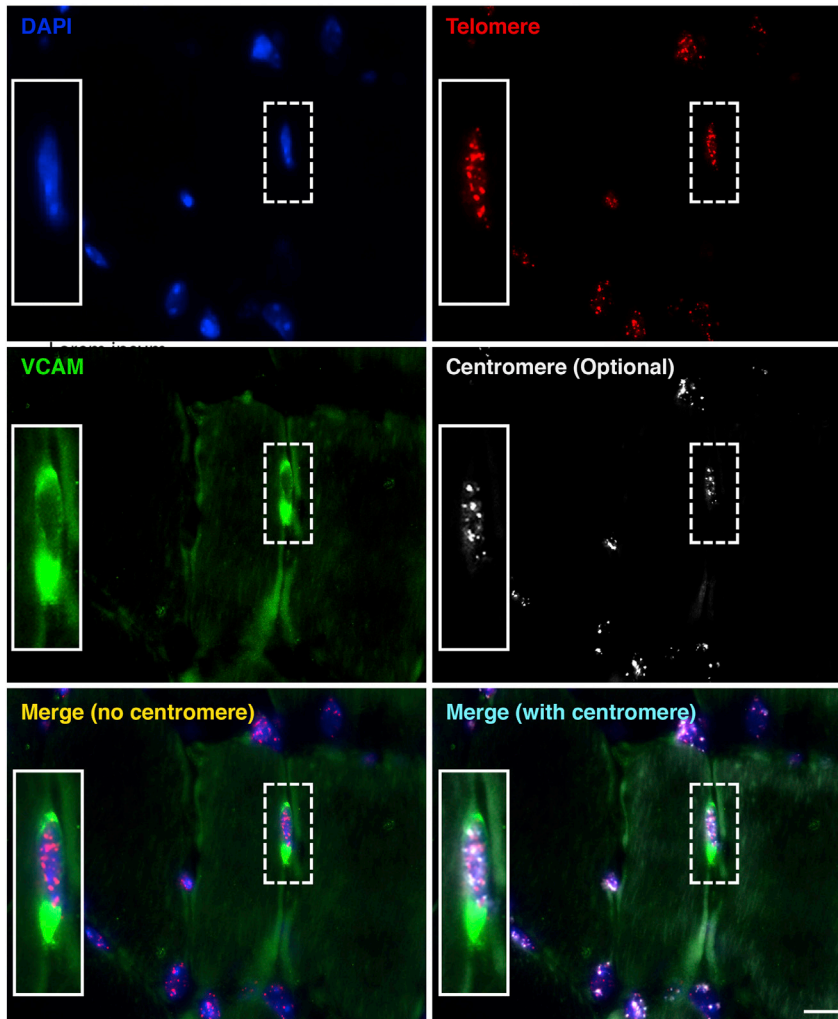
**△ CRITICAL:** Once intensity settings are determined, the exposure settings should not be changed for the duration of the particular experiment.

10. Proceed with multichannel imaging of VCAM, DAPI, telomere, and optional centromere signals ([Figure 1](#)).
  - a. Identify focal planes containing the most telomere signals for every cell of interest.
  - b. A minimum of 35 VCAM+ cells should be imaged per biological sample.
  - c. Save multichannel images in raw file formats to maintain metadata. For Nikon Elements software, this is a .nd2 file.
  - d. Image as many cells as possible during scheduled equipment usage hours from the biological samples. [Troubleshooting 3](#)

**Optional:** Obtaining z-stacks and flattening with an extended depth of focus algorithm will provide more telomere and optional centromere foci per cell, but a single focal plane with many foci in focus is sufficient to yield useable data for analysis.

**Note:** Imaging more VCAM+ cells will produce a better representation of the population of MuSCs throughout the muscle. Ensure that more than one muscle section is examined per slide.

**Note:** If imaging of all desired cells from all samples cannot be completed in one day, stagger the genotypes/treatments during imaging to ensure fading that occurs between imaging sessions will be similar between the groups.



**Figure 1. Representative image of a skeletal muscle cryosection processed for telomere staining**

Blue: DAPI staining. Red: telomere probe. Green: VCAM muscle stem cell marker. White: optional centromere probe staining for alternative normalization. Yellow text: DAPI, VCAM, and telomere merged image (typical processing). Cyan text: DAPI, VCAM, telomere and centromere merged image. Use of centromere probe requires telomere length normalization to DAPI. Then centromere normalization to DAPI. Finally, a ratio of the two values is generated. Scale bar: 10  $\mu$ m.

**Note:** It is best to avoid imaging/assessing cells of interest that are positioned on top of or below other cells/nuclei, as telomere length quantitated from overlapping cells may confound the datasets.

11. Export raw image files in two groups.
  - a. Each channel should be first exported as grayscale/monochrome 16-bit .tiff images for analysis.
  - b. A merged 8-bit color .tiff image is also exported to identify cells of interest (VCAM+) for later analysis.

**Pause point:** After all needed images are collected, downstream analysis can be conducted at the end user's leisure.

### Cryosection MuSC-fluorescent *in situ* hybridization (CRYO MuQ-FISH) telomere length data collection and analysis

⌚ Timing: varies based on sample size. Several hours to several days.

Telomere length is measured using a semi-automated ImageJ-based platform known as Telometer. Telometer provides step-by step instructions to analyze 16-bit monochrome images for telomere length. The premise involves creating a project that is saved on a local machine, identifying cells of interest, and measuring the intensity of telomeres, relative to the total DAPI intensity of that cell. The readout from Telometer produces both total sum intensity of telomeres, which can be normalized to DAPI intensity to account for differences in cell cycle position, as well as the mean intensity of telomeres, which can also be normalized to DAPI. Should nuclear architecture or staining be compromised, telomere measurements can instead be normalized to centromere intensities. In this fashion, Telometer steps are conducted twice. Once, where the telomere signal is assessed relative to DAPI, and then those same cells of interest are analyzed a second time, where the centromere intensities are measured instead of the telomere intensities. The ratio of these measurements would then be used for dataset readouts.

12. Open Telometer software and create a new project in the “Admin” tab (Figure 2A).
13. Click the “Score” tab and follow the on-screen instructions for each step.
14. Select the Project name for your analysis grouping and the Preset objective (in this case 100× telomere) and subtract Cy3 background. The scoring tag adds metadata to the analysis. We do not usually alter this.
15. Select the Telomere 16-bit monochrome exported tiff image when prompted for opening the first image.

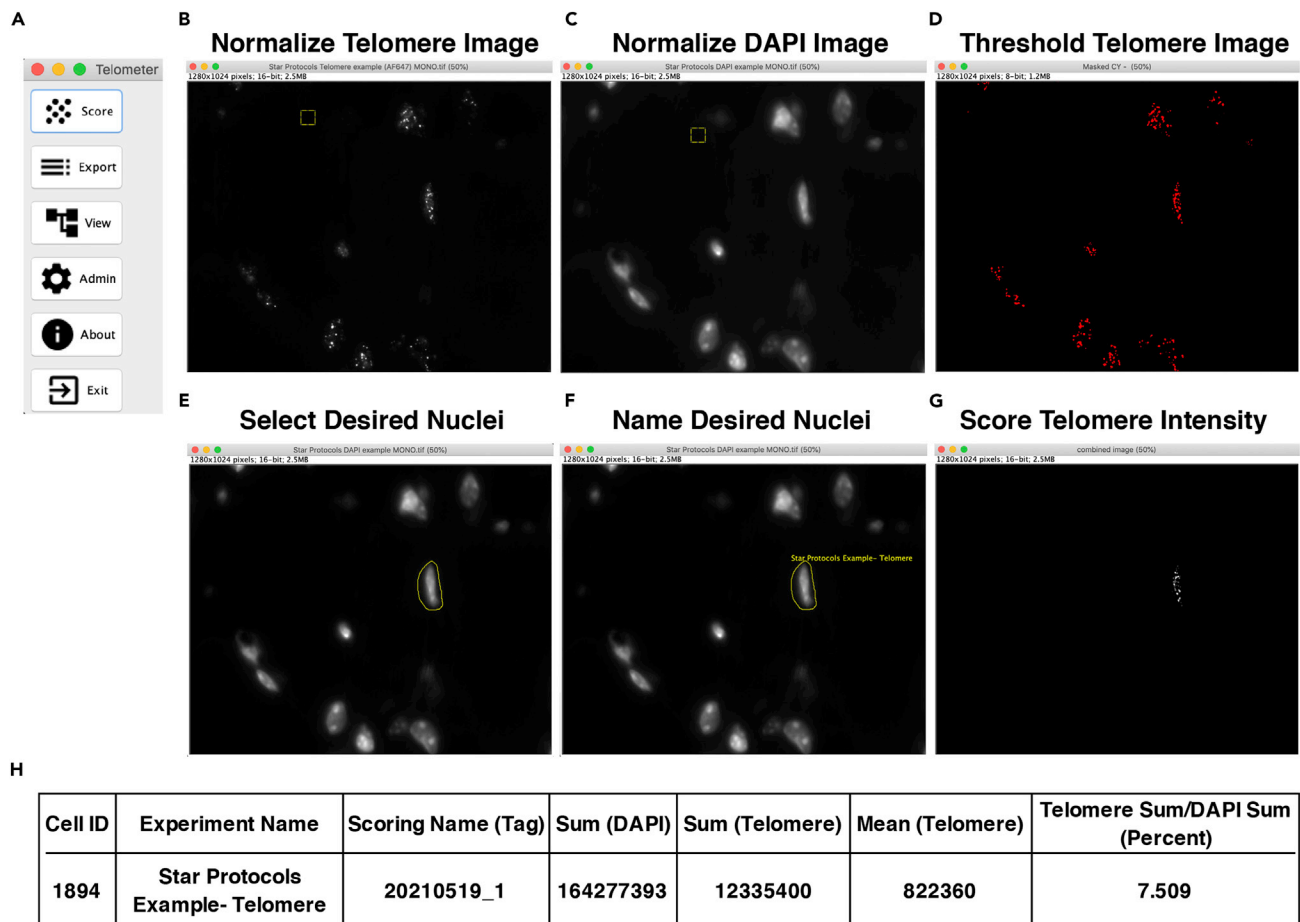
**Note:** Telometer defaults as the telomere signal being a Cy3 image. In this protocol, the telomere image is the Alexa Fluor 647 channel image, and it is this image that is opened first when prompted.

16. Next, open the DAPI 16-bit monochrome exported tiff image when prompted.
17. Normalize the Telomere image by clicking and forming a box of any size in an area absent of features and click “normalize” (Figure 2B).
18. Repeat normalization for the DAPI image when prompted (Figure 2C).
19. Continue following software prompts for creating masked images to narrow down regions in the image that contain telomere foci.
20. Threshold the masked image, if necessary, when prompted to obtain the greatest telomere foci in the image with the least background (Figure 2D).

**Note:** The Telometer software typically does a decent job with auto-thresholding, so the investigator does not often need to further threshold.

21. Manually draw a circle encompassing a nucleus of interest when prompted. Refer to the color image to determine which nuclei are clearly VCAM+ and designate the nucleus of interest. Selection of VCAM nuclei should only occur for cells in the satellite cell position (between the basal lamina and sarcolemma). Name the nucleus as desired (Figure 2E).
22. Select the “+ Add Nucleus” button. Repeat for additional VCAM+ cells in the image, if any. Click the “next” button (Figure 2F).

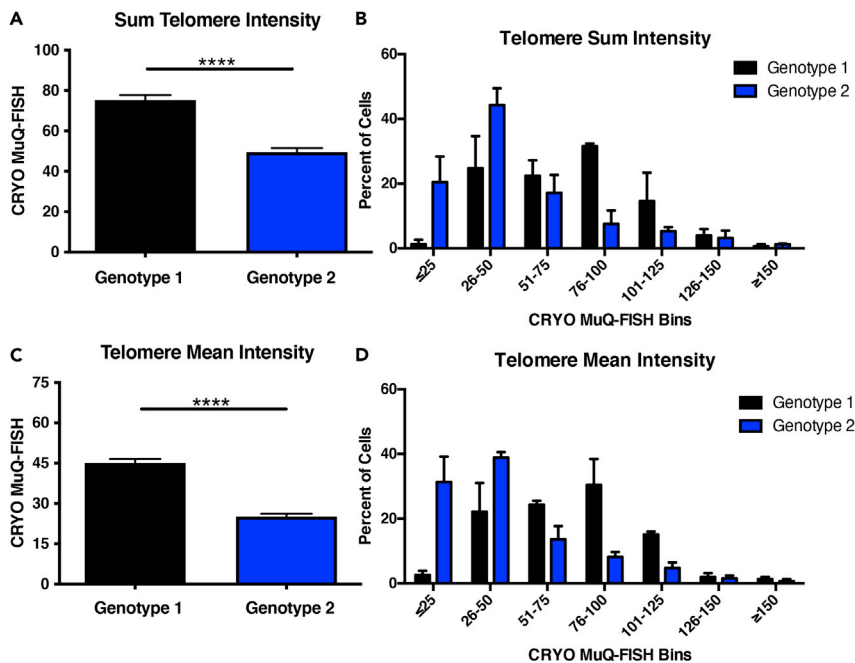
**Optional:** Telometer next gives the option to separate conjoined telomere foci (if counting telomere number) and manually remove halo artifacts from out of focus light. These functions are completely optional for the analysis.



**Figure 2. Processing cells of interest for telomere analysis by Telometer**

(A) Telomere radio button depicting different options for data acquisition and analysis.  
 (B) Representative monochrome image of telomere image from Figure 1. Yellow box is the region chosen for background normalization.  
 (C). Representative DAPI monochrome image from Figure 1. Yellow box is the region chosen for background normalization.  
 (D) Thresholding of telomere channel to determine optimal balance between telomere foci identification with the least background.  
 (E) Selection of VCAM+ nucleus from Figure 1 by manually outlining when prompted by the software.  
 (F) Naming of a selected VCAM+ nucleus.  
 (G) Masked image used for data analysis by Telometer.  
 (H) Selected data outputs provided by Telometer analysis. Cell ID: unique identifier for a particular cell that was analyzed. Experiment name: selected at beginning of analysis (project name). Scoring Name (Tag): prefix added to dataset. Usually the date of analysis. Sum (DAPI): measured DAPI total intensity. Sum (Telomere): Total measured telomere intensity. Mean (Telomere): Mean intensity of Telomere signal per nucleus. Telomere Sum/DAPI Sum (Percent): Value used to graph datasets.

23. Select “complete.” The data will be stored on your local machine. To view images at a later time that have been processed and analyzed, select the “View” option in the Telometer tab (Figure 2G).
24. Repeat step 15–23 for remaining images to analyze. Ensure that they are being saved in the proper project data file and appropriately named.
25. Export data to copy into excel (Figure 2H).
  - a. In the “Export” tab of Telometer, select the project name for data export.
  - b. Click the “Scoring” button that has an arrow.
  - c. Select all data under “Scorings” and click the “Cells” button that has an arrow.
  - d. Select all data under “Cells” and copy and paste into Excel or other spreadsheet software. Ensure that you also note the headings for each column. [Troubleshooting 4](#)



**Figure 3. Example graphical plots from Telometer analysis**

(A) CRYO MuQ-FISH Telomere Sum Intensity/DAPI Sum Intensity (Percent) data graphed from 2 different mouse genotypes subjected to chronic injury. Note the significant reduction of telomere lengths in genotype 2. Genotypes depicted in this figure are those used in our companion article (Tichy et al., 2021) and are control and IKK2CA<sup>MuSC</sup> mice subjected to chronic injuries.

(B) CRYO MuQ-FISH data binned in Microsoft Excel from (A) and plotted as percent of cells per mouse and per genotype. Bins are arbitrarily assigned. Note that the shift of the population of genotype 2 to the left is indicative of telomere shortening.

(C) CRYO MuQ-FISH Telomere Mean Intensity/DAPI Sum Intensity (calculated percent) from analysis in (A).

(D) Histogram representation of data from (C). For all graphs, N=3 mice per genotype, with a minimum of 35 cells imaged per mouse. Depicted are mean  $\pm$  SEM. \*\*\*\* p<0.0001.

26. To analyze datasets using the Telomere sum option, refer to the last column of the dataset. It represents the telomere sum intensity divided by DAPI sum intensity formula\*100.

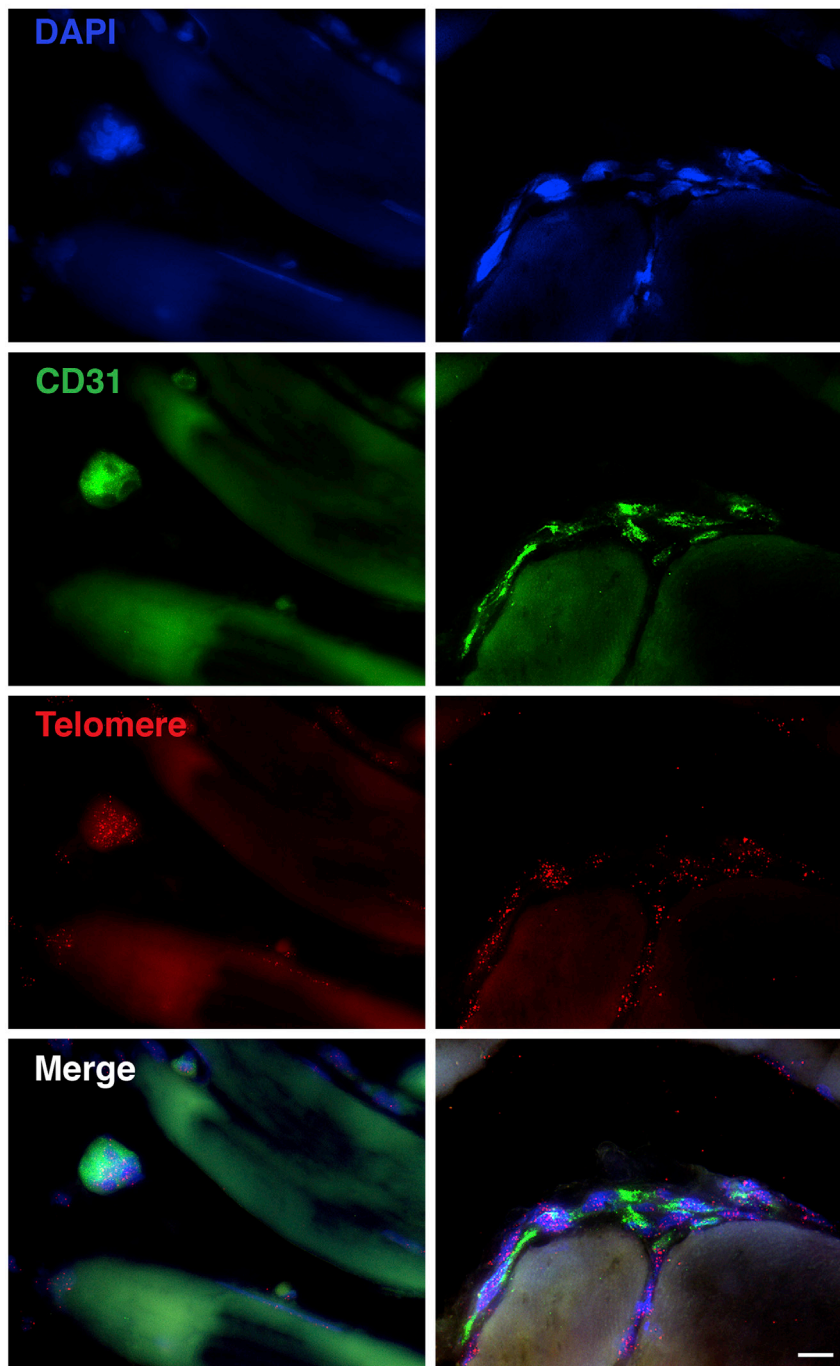
**Optional:** If telomere mean intensities are desired per cell instead of total (sum) intensities, calculate this value by dividing the datasets in the Telomere Mean column by its respective DAPI Sum.

**Optional:** If one desires to normalize to centromere intensity instead of DAPI intensity, the procedure for telomere analysis is repeated, but instead of using the Telomere images on prompt to open, the centromere images are opened instead. The nuclei should be named and selected similarly in the same order as the telomere analysis. Then the last column of data from the telomere analysis is divided by the last column of centromere data analysis. [Troubleshooting 5](#)

27. Graph datasets as desired in Graphpad prism. Data can be graphed as total intensity values, or data can be graphed as histograms to demonstrate minor shifts of telomere length within the populations ([Figure 3](#)). [Troubleshooting 6](#)

**Note:** The telomere and centromere probes are probes that recognize mammalian DNA sequences and are functional in other species, including samples from rat and human. We have found that this protocol is compatible with muscle tissues of human and rat skeletal muscle cryosections ([Figures 4](#) and [5](#)). We also examined the adaptability of this protocol to

## Human Cryosections

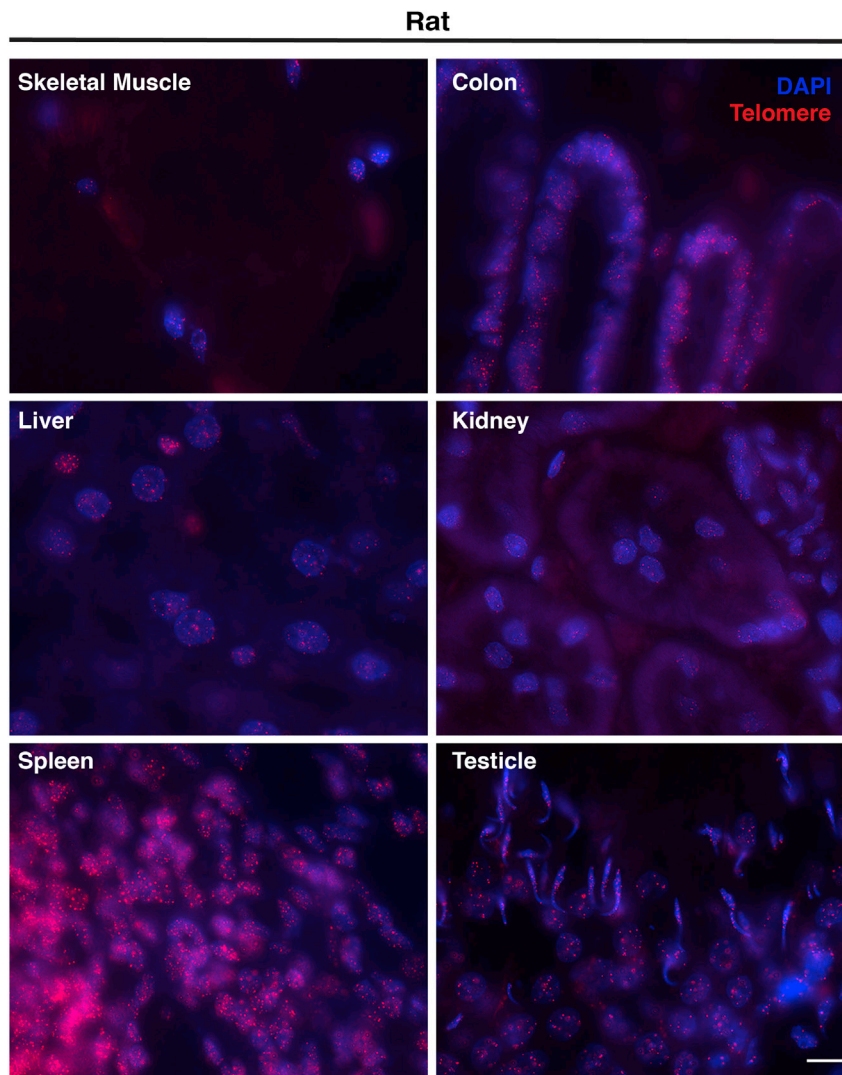


**Figure 4. Application of CRYO MUQ-FISH to human skeletal muscle samples reveals compatibility of the method with human tissues**

Cryosections were stained with the endothelial marker CD31 and processed for CRYO MuQ-FISH. Scale bar: 10  $\mu$ m.

several other tissue types (spleen, liver, kidney, testicle, and colon) and found this protocol does appropriately label telomeres in all tissues examined (Figure 5), increasing the impact and usability of this method. However, in such tissues, it may be necessary to utilize thinner





**Figure 5. Assessment of CRYO MuQ-FISH protocol translatability to additional tissue and organism types**

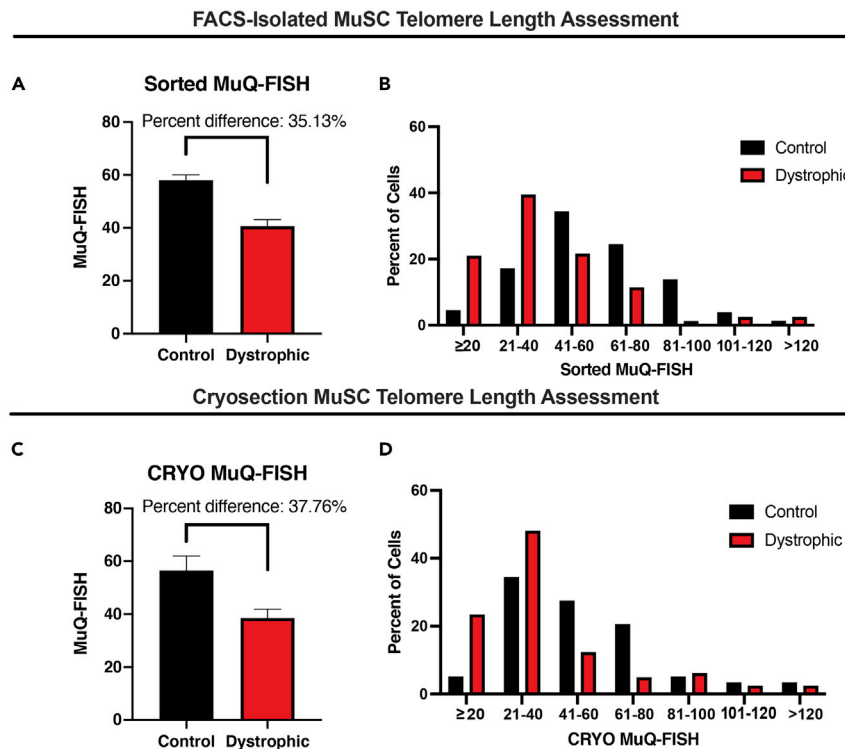
Scale bar: 10  $\mu$ m. Note- background was left intentionally higher to demonstrate surrounding tissue architecture. Adult 17-month-old male Sprague-Dawley rat tissues were collected and processed identically as described in the CRYO MuQ-FISH protocol and imaged for telomere signal and DAPI. We found this protocol capable of staining telomeres in tissues of the tibialis anterior muscle, colon, liver, kidney, spleen, and testicle. Scale bar: 10  $\mu$ m.

sections or more advanced microscopes (confocal microscopy), given the increased number of focal planes exhibiting telomere foci observed.

### EXPECTED OUTCOMES

This protocol demonstrates the methodology to process cryosections of skeletal muscle for telomere analysis. We utilized VCAM as a muscle stem cell marker (Liu et al., 2015; Maesner et al., 2016); however, this protocol can likely be applied to additional resident skeletal muscle populations, once appropriate markers are identified and tested for compatibility. Here, we utilized data from two different genotypes of mice that underwent chronic, repetitive injuries, to demonstrate different ways to represent the data (Figure 3). Additionally, to confirm the validity of the CRYO MuQ-FISH method, we compared CRYO MuQ-FISH with our traditional MuQ-FISH method using isolated MuSCs. We found similar patterns of telomere shortening in a mouse model of Duchenne muscular dystrophy coupled with a telomere defect (Figure 6).





**Figure 6. Comparisons of CRYO MuQ-FISH with previously established telomere length assessments in isolated MuSCs demonstrates no differences in expected data outcomes**

(A) MuSCs were FACS-sorted (CD11b-/CD31-/CD45-/Sca1-/CD34+/ $\alpha$ 7Integrin+) from wildtype mice or dystrophic mice lacking the telomere component Terc (*mdx/mTR<sup>G2</sup>*). MuSCs were subjected to our previously established and validated MuQ-FISH method to assess telomere length. Depicted are mean  $\pm$  SEM. Percent difference between the two groups is displayed.

(B) Data from (A) plotted as a histogram to demonstrate the cellular distribution of telomere lengths.

(C) CRYO MuQ-FISH of VCAM+ cells in skeletal muscle cryosections from mice used in (A). Gastrocnemius muscles were processed for telomere length by the CRYO MuQ-FISH protocol. Note the similar percent difference in telomere length between the groups compared with what is shown in (A).

(D) Histogram representation of data depicted in (C). Note the similarity in structure of the histograms between (C) and (D). For all graphs, N=3 male mice per genotype (5–7 months of age), with a minimum of 35 cells imaged per mouse. Depicted are mean  $\pm$  SEM.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Comparisons of data between genotypes are conducted using unpaired student T-tests with Welch's correction. Cells from at 3 mice or samples per experimental condition should be examined, with at least 35 cells imaged per mouse.

## LIMITATIONS

This protocol utilizes the quantitative fluorescence in situ hybridization method for measuring telomere length. Because measurements of telomere length are based on fluorescent intensities, it is critical to image the slides as soon as possible, since fading will occur over time. Samples should not be imaged between different experiments, but instead they should only be imaged in the same batch in as short a time window as possible, since fluorescent intensities will be variable between experiments. Normalization must be done to respective control samples to combine datasets. Since MuSCs comprise a rare population of cells and are very small in size, a 100 $\times$  or equivalent high-powered objective is necessary to visualize the telomere foci to get meaningful datasets.

### TROUBLESHOOTING

#### Problem 1

No Q-FISH telomere probe signal present following imaging (step 9).

#### Potential solution

Antigen retrieval time was insufficient or not completed with boiling buffer. Ensure that temperatures and times are completed as outlined in this protocol.

Probe was bleached or stored improperly after being prepared. Always use a new aliquot of telomere/centromere probe from  $-80^{\circ}\text{C}$  and ensure that it is protected from light throughout this protocol.

Inappropriate dilution of probe was used, hybridization buffer was not correctly made, hybridization conditions were not followed. If probes are too dilute, increase the amount of the Q-FISH probes used in the protocol. Confirm that hybridization buffer is completely dissolved and that no air bubbles were present when hybridization occurred. Confirm the heating source during denaturation and hybridization is at the proper temperatures.

#### Problem 2

High background staining (step 9).

#### Potential solution

Do not let the tissue dry out at any step following permeabilization. Avoid Cy3/Alexa fluor red/orange dyes except for centromeric region staining.

#### Problem 3

Large numbers of samples that cannot be imaged in the required timeframe (step 10d).

#### Potential solution

While imaging samples in the same time window is optimal, if too many samples are present, a 3-day window may not be sufficient time to complete the acquisition, resulting in fading of the sample and alteration of the data. To overcome this caveat, it may be possible to normalize the intensity of a particular reference sample imaged on different days using fluorescent beads, such as those found in the InSpeck Microscopy Image Intensity Calibration Kit. These kits contain beads of varying but defined intensities from which a standard curve can be generated on each day within the same experiment.

#### Problem 4

Telometer is not displaying recorded datasets (step 25d).

#### Potential solution

Ensure that you are trying to view data in the appropriate project file. It may be necessary to use the "refresh" button to repopulate the project file, particularly if one is trying to view data that was accumulated since Telometer was first opened.

#### Problem 5

DAPI staining intensity is not consistent between experimental groups or nuclear architecture is dissimilar (step 26).

#### Potential solution

Conduct telomere analysis by normalizing to a centromeric probe. First do telomere analysis normalized to DAPI. Then repeat the Telometer analysis on the same cells using the centromere image instead of the telomere image. Ensure to label cells of interest in the same order between the

telomere identification and the centromere identification. Finally, divide the Telomere sum/DAPI sum percent by the Centromere sum/DAPI sum. Utilize those values for graphing.

### Problem 6

Inconsistent data between experiments (step 27).

### Potential solution

Given that Q-FISH is an intensity-based measurement, experimental samples are ideally measured at the same time with the same exposure settings. If too many samples are present, fading can occur between sample sets that would confound downstream analysis. Thus, it is optimal to image all experimental groups as soon as possible, and comparisons between different experiments are not possible. Additionally, telomere probe in hybridization buffer should completely cover the sample without any air bubbles. While the values themselves may be different, due to inherent differences between experimental preparations, calculations of percent difference between groups can be useful in confirming that datasets are similar.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Foteini Mourkioti ([fmour@penmedicine.upenn.edu](mailto:fmour@penmedicine.upenn.edu)).

### Materials availability

This study did not generate new materials or unique reagents.

### Data and code availability

This study did not generate any unique data sets or code.

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## AUTHOR CONTRIBUTIONS

E.D.T. designed and performed experiments, developed, and optimized the protocol, and wrote the manuscript. F.M. designed experiments, interpreted data, and wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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