# Chapter 11

# Isolation, Culture, Functional Assays, and Immunofluorescence of Myofiber-Associated Satellite Cells

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## Abstract

Adult skeletal muscle stem cells, termed satellite cells, regenerate and repair the functional contractile cells in adult skeletal muscle called myofibers. Satellite cells reside in a niche between the basal lamina and sarcolemma of myofibers. Isolating single myofibers and their associated satellite cells provides a culture system that partially mimics the in vivo environment. We describe methods for isolating and culturing intact individual myofibers and their associated satellite cells from the mouse extensor digitorum longus muscle. Following dissection and isolation of individual myofibers we provide protocols for myofiber transplantation, satellite cell transfection, immune detection of satellite cell antigens, and assays to examine satellite cell self-renewal and proliferation.

**Key words** Skeletal muscle, Satellite cells, Muscle stem cells, Myofiber isolation, Extensor digitorum longus, Immunostaining, Proliferation, Self-renewal, Transfection, AraC, EdU, CFDA-SE, Transplant, Tibialis anterior, Mouse

## 1 Introduction

The cellular building blocks of skeletal muscle tissue are post-mitotic, multinucleated, syncytial cells called myofibers. The myonuclei in myofibers are incapable of repair and when damaged, myofibers rely on resident muscle stem cells, called satellite cells, for repair and regeneration [1]. In addition to rebuilding damaged myofibers, satellite cells continuously fuse and contribute myonuclei to myofibers in mature skeletal muscle [2, 3]. Satellite cells reside between the basal lamina and the sarcolemma of myofibers [4] and upon receiving signals to expand, quiescent satellite cells activate, induce myogenic transcription factors, enter into the cell cycle and proliferate, becoming myoblasts or muscle progenitor cells. Quiescent and activated satellite cells express the paired-box

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transcription factor Pax7 and upregulate the myogenic transcription factors MyoD and Myf5 prior to cell cycle entry and proliferation [1]. The majority of myoblasts will then terminally differentiate to fuse and repair muscle, while a subset self-renews to replenish the satellite cell pool for future rounds of muscle regeneration [1]. Satellite cell self-renewal is a quintessential characteristic of stem cells and requires both cell autonomous and cell nonautonomous signals [5, 6].

Elucidating the signals that regulate the finely tuned balance of satellite cell self-renewal and proliferation is critical for understanding muscle regeneration and homeostasis. In vivo assays aimed at understanding satellite cell behavior are cumbersome, time-consuming, and expensive. Isolating single, mononuclear satellite cells from whole muscle is one alternative approach. Post-isolation, satellite cells are cultured on tissue culture plastic or Matrigel; however, these conditions do not accurately recapitulate the niche in which satellite cells thrive. Another alternative involves isolating and culturing myofibers with their associated satellite cells, thereby maintaining the satellite cell niche. The myofiber niche provides a specific substrate stiffness that influences satellite cell behavior [7] as well as provides growth factors such as FGF [8]. Due to the satellite cell's intimate association with a myofiber, satellite cells remain attached to their corresponding myofibers during myofiber isolation. The extensor digitorum longus (EDL) muscle of *Mus musculus* provides hundreds of individual myofibers with associated satellite cells when isolated by enzymatic and mechanical dissociation.

Isolating and culturing isolating individual myofibers from EDL muscles, pioneered in the 1980s [9], has been used extensively by several groups [10–13]. Here we outline (1) how to isolate and culture myofiber-associated satellite cells from a mouse EDL, (2) assays used to study and manipulate myofiber-associated satellite cell behavior, and (3) how to fix and stain myofiber-associated satellite cells. Our approach can be modified to isolate, assay, manipulate, and identify epitopes by antibody staining on myofiber-associated satellite cells from different muscle groups including the gastrocnemius and soleus. We provide a comprehensive reagent list and detailed methods, optimized by over a decade of experience, to probe the behavior of myofiber-associated satellite cells.

## 2 Materials

Prepare all cell culture reagents in a laminar flow hood with sterile technique. Follow all waste disposal regulations when disposing of chemical and other waste materials.

### 2.1 Myofiber Growth Media

The components of myofiber growth media are F12-C media, horse serum, and antibiotics. Some of the following protocols require F12-C media alone, F12-C media with antibiotics or F12-C media with 15% horse serum and no antibiotics. Here we

describe how to make myofiber growth media; however, this protocol can be modified to make F12-C media with antibiotics and no serum, to make F12-C media alone or to make F12-C media with 15 % horse serum and no antibiotics.

1. For 1 L of F12-C media add 700 mL of tissue culture grade water to an Erlenmeyer flask and mix with stir bar.
2. Slowly add one packet of Ham's F-12 Powdered Nutrient Mix (Gibco/Thermo Fisher Scientific, Grand Island, NY, USA) and rinse the F-12 container with a small amount of water and add to mixture.
3. Stir until powder is dissolved.
4. Add 1.176 g $NaHCO_3$ and stir until dissolved. With 10 mL water rinse any residual powdered F-12 and $NaHCO_3$ from the sides and neck of the flask.
5. Adjust the pH to 7.15–7.20.
6. Add 10 mL 100× penicillin–streptomycin stock solution (*see* Subheading 2.1.1) and 4 mL 0.2 M $CaCl_2$ (*see* Subheading 2.1.2). Bring final volume to 1 L.
7. Sterile filter the solution through a 0.22 μm filter and aliquot 425 mL into tissue culture bottles. Store at 4 °C (*see* **Note 1**).
8. Add 75 mL of lot tested horse serum to 425 mL F12-C media to make myofiber growth media (*see* **Note 2**).

#### *2.1.1 Penicillin–Streptomycin 100× Stock Solution*

1. Dissolve 10 M units of tissue culture grade sodium salt penicillin G (Sigma-Aldrich, St. Louis, MO, USA) and 500 mg of tissue culture grade streptomycin sulfate (Sigma-Aldrich) in 900 mL tissue culture grade water.
2. Stir with stir bar until completely dissolved.
3. In tissue culture hood, filter the solution through a 0.22 μm bottle top filter, aliquot into 50 mL plastic conical vials and store at −20 °C until needed.

#### *2.1.2 0.2 M $CaCl_2$ Stock Solution*

1. Dissolve 11.1 g $CaCl_2$ in 450 mL tissue culture grade water and bring volume to 500 mL with tissue culture grade water.
2. In a tissue culture hood, filter the solution through a 0.22 μm bottle top filter into a sterile glass bottle. Store at room temperature.

### *2.2 Myofiber Isolation Components*

1. F12-C media with antibiotics and no serum.
2. 2 mL microcentrifuge tubes.
3. 70 % ethanol in a spray bottle.
4. Two pairs of Dumont #5 forceps (Fine Science Tools, Foster City, CA, USA).
5. Extra fine Bonn scissors with 13 mm cutting edge (Fine Science Tools).

6. ToughCut scissors with 22 mm cutting edge (Fine Science Tools).
7. 2 mL Pipet pump (Bel-Art Products, Wayne, NJ, USA).
8. Low oxygen humidified tissue culture incubator 37 °C, 5% $CO_2$, 6% $N_2$.
9. Six-well tissue culture plates.
10. Stereoscope or dissection microscope with light base.

*2.2.1 Recombinant Human Basic Fibroblast Growth Factor (FGF2) Stock Solution*

1. Resuspend FGF2 (Promega Corporation, Madison, WI, USA) to a concentration of 25 μg/mL (10,000×) in 1% BSA in PBS.
2. Sterile filter through a 0.22 μm filter. Aliquot FGF2 into 20 μL aliquots and store at −20 °C. Once thawed store at 4 °C (*see* **Note 3**).

*2.2.2 Fire-Polished Pasteur Pipettes*

Light a Bunsen burner and gently rotate the tips of glass Pasteur pipettes in the flame (*see* **Note 4**). Polish the end until it becomes smooth and rounded so as not to damage myofibers during transfer.

*2.2.3 20× Saline G Solution*

1. Mix 600 mL $dH_2O$ and 160 g NaCl, 8.0 g KCl, and 3.0 g $KH_2PO_4$ in the order listed.
2. Add 3.0 g $Na_2HPO_4$, 22.0 g dextrose and 100 mg Phenol Red.
3. Stir until dissolved then add 400 mL $dH_2O$ to bring final volume to 1 L.
4. Sterile filter the solution through 0.22 μm filter and store at 4 °C until needed.

*2.2.4 Collagenase Type I 10× Stock Solution*

1. Calculate the amount of collagenase type I (Worthington Biochemicals, Lakewood, NJ, USA) required based upon activity provided (*see* **Note 5**). The final 10× concentration should be 40,000 U/mL.
2. Freshly dilute 20× Saline G to 1× with sterile water and add collagenase.
3. Stir on ice until collagenase is dissolved.
4. Sterile filter the solution through a 0.44 μm filter then through a 0.22 μm filter (*see* **Note 6**).
5. Aliquot the collagenase into 100 μL aliquots and store at −20 °C. Thaw aliquots at room temperature when needed (*see* **Note 7**).

### *2.3 Fixing and Staining Components for Antigen Detection by Immunofluorescence*

1. Phosphate buffered saline (PBS, 10× stock; dilute to 1× before using): 1.37 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 18 mM $KH_2PO_4$.
2. PBS containing 0.5% Triton X-100.
3. Paraformaldehyde: 4% paraformaldehyde diluted in PBS from 10% EM grade paraformaldehyde stock solution (Electron

Microscopy Sciences, Hatfield, PA, USA). For every well of myofibers in a six well plate to be fixed, prepare 2.5 mL of 4% paraformaldehyde. Mix and use immediately.

4. Hydrogen peroxide quench solution: 3% $H_2O_2$ diluted with $dH_2O$ from 30% $H_2O_2$ stock solution (Fisher Scientific/Thermo Fisher). Mix and use immediately.
5. Blocking solutions: 0.25% Triton X-100, 3% bovine serum albumin fraction V (BSA; Sigma-Aldrich) in PBS for initial permeabilization and blocking. 0.125% Triton X-100, 3% BSA in PBS for primary and secondary antibodies. For EdU Click-It chemistry 0.5% Triton X-100, 3% BSA in PBS (*see* **Note 8**).
6. Superfrost Plus Pre-cleaned Microscope Slides (Fisher Scientific/Thermo Fisher).

*2.3.1 Myofiber Staining Baskets*

1. Make basket top by carefully removing the lid and bottom half of a 1.5 mL tube making the edge as flat as possible (*see* **Note 9**).
2. Cut the Nylon Mesh (80 μm pore size, 46-μm thread diameter, Small Parts/Amazon.com Inc, Seattle, WA, USA) into a square slightly larger than the edges of the cut 1.5 mL tube (*see* **Note 10**).
3. Warm a hot plate loosely covered with aluminum foil to a medium heat setting (*see* **Note 11**).
4. Place the nylon mesh on the foil covered hot plate and place the basket top over the mesh. Press down gently and evenly until basket top melts into the nylon mesh.
5. Remove basket from hot plate and allow to cool.
6. Trim the edges of the extra nylon from the 1.5 mL tube using a razor blade or scalpel until the basket fits into a well of a 48 well plate easily (*see* **Note 12**).

*2.3.2 Mowiol Mounting Medium*

1. Place 6 g of glycerol into a 50 mL glass beaker with a stir bar.
2. While stirring, add 2.4 g Mowiol 4-88 (Calbiochem/EMD Millipore, Darmstadt, Germany).
3. Add 6 mL $dH_2O$ and stir for 2 h at room temperature.
4. Add 12 mL 0.2 M Tris-HCl (pH 8.5) and incubate at 50–60 °C for 10 min using a bead or water bath followed by mixing on a stir plate for 10–15 min.
5. Repeat the heating and stirring for 1–2 h until all the Mowiol goes into solution.
6. Transfer the solution into 50 mL conical tubes and centrifuge at 5000 × *g* for 15 min to remove any un-dissolved Mowiol from the solution.
7. Transfer the supernatant into a new 50 mL glass container with a stir bar and mix again. Add 0.569 g (2.5% weight/volume

concentration) anti-fade agent 1,4-Diazabicyclo[2.2.2]octane (DACO) (Sigma-Aldrich).
8. Mix for 5–10 min until DACO is dissolved.
9. Store 1 mL aliquots at −20 °C. Warm aliquots to room temperature before use (*see* **Note 13**).

### 2.4 Myofiber Transplantation Components

1. 27 ½G 0.40×13 mm ½cc Tuberculin syringes (Fisher Scientific/Thermo Fisher Scientific).
2. Sterile filtered 0.9% Saline Solution (*see* **Note 14**).
3. Sterile filtered 1.2% Barium Chloride in 0.9% Saline Solution (*see* **Note 15**).
4. Six-well tissue culture plates.
5. Myofiber growth media and 2.5 ng/mL FGF2.

### 2.5 Myofiber Associated Satellite Cell Transfection Components

1. OptiMEM Reduced Serum Media (Gibco/Thermo Fisher Scientific).
2. Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific).
3. Plasmid DNA to transfect.
4. 25 μg/mL FGF2 stock solution (*see* Subheading 2.1.1).
5. Myofiber growth media.
6. F12-C media with 15% horse serum and no antibiotics.

### 2.6 CFDA-SE Self-Renewal Assay Components

1. Myofiber growth media.
2. 25 μg/mL FGF2 stock solution (*see* Subheading 2.1.1).
3. 10 mM CFDA-SE (Life Technologies/Thermo Fisher Scientific) stock solution in DMSO (*see* **Note 16**).
4. 10 μM CFDA-SE diluted from 10 mM stock in pre-warmed sterile PBS (*see* **Note 17**).

### 2.7 EdU Proliferation Assay Components

1. Click-iT® EdU Alexa Fluor® Imaging Kit (Invitrogen/Thermo Fisher Scientific).

#### 2.7.1 10 mM 5-Ethynyl-2′-Deoxyuridine (EdU; Carbosynth, Berkshire, UK) In Vitro Labeling Stock Solution

1. Dissolve EdU in DMSO to 10 mM final concentration to generate a stock solution.
2. Aliquot in 50 μL volumes and store at −20 °C.

#### 2.7.2 0.5 mg/mL EdU Water for In Vivo Labeling

1. Dissolve 0.5 mg/mL EdU in sterile filtered $H_2O$ with 1% dextrose (*see* **Note 18**).

### 2.8 AraC Assay Components

1. Myofiber growth media.

#### 2.8.1 100 mM Cyotsine β-d-Arabinofuranoside (AraC; Sigma-Aldrich) Stock Solution

1. Generate a 100 mM AraC stock solution in $dH_2O$ and store at −20 °C (*see* **Note 19**).
2. Dilute 100 mM stock solution to previously determined optimal working AraC concentration (*see* **Note 20**).

## 3 Methods

All procedures are carried out at room temperature unless otherwise specified. Follow all waste disposal regulations when disposing of all chemical and other waste materials.

### 3.1 Isolation of Single Myofibers from Mouse EDL Muscle

In mice, the EDL is located between the knee and ankle joints in the hindlimb muscle in a ventral-lateral orientation. The origin of the EDL is the lateral condyle of the tibia and the insertion is split between the four extensor tendons of the 2nd–5th digits. Anatomically, the belly of the EDL is located lateral and inferior to the tibialis anterior (TA) and superficial to the tibia [14]. The function of the EDL is to extend the 2nd–5th digits [15].

#### 3.1.1 Initial Preparation Prior to EDL Harvest (per Mouse)

1. Add 900 μL F12-C media with antibiotics to a 2 mL microcentrifuge tube.
2. On a single six-well plate, add 4 mL of myofiber growth media and 2.5 ng/mL FGF2 to a single well and 3 mL to two additional wells and place into a 37 °C tissue culture incubator.
3. Thaw a single 100 μL frozen 10× collagenase aliquot (*see* Subheading 2.2.4).

#### 3.1.2 Dissection and Harvest of EDL Muscle

The EDL should only be handled by grasping the tendon. If the EDL is grasped by the muscle belly, the integrity of the myofibers will be compromised and the yield of intact myofibers will be low.

1. Euthanize mouse according to IACUC and institutional protocols.
2. Apply 70% ethanol to mouse hindlimb so that the fur is saturated (Fig. 1a).
3. Place mouse on its back under dissection lamp.
4. Pinch the skin above the knee joint and use ToughCut scissors to cut a superficial notch in the skin approximately 0.75 cm in length that is parallel to the tibia (Fig. 1b). Be careful not to cut too deeply into the skin in order to avoid damaging the underlying musculature.
5. Pinch the skin with fingers both proximal and distal to the superficial cut and quickly pull distal portion towards the paw (Fig. 1c). This forceful action will quickly and cleanly remove the skin from the mid-foot to the knee allowing access to the

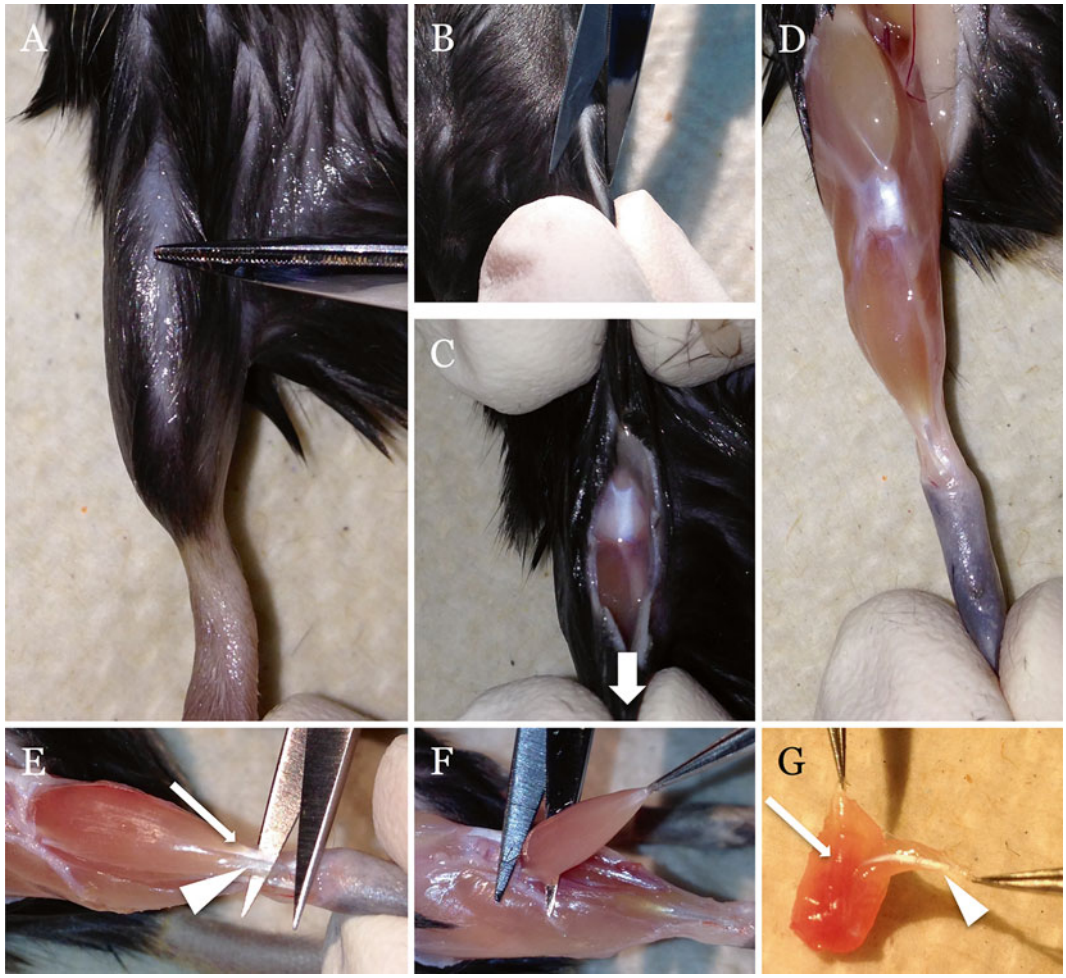


**Fig. 1** Isolation of *Mus musculus* extensor digitorum longus (EDL) muscle. (**a**) The hind limb of *Mus musculus* is soaked with 70 % ethanol. The scissors are pointing to the knee joint. (**b**) The skin is pinched below knee joint and cut just above knee joint. (**c**) The distal portion of cut skin is pulled towards the paw (in the direction of the *white arrow*) to remove the skin. (**d**) The exposed musculature of the *Mus musculus* hindlimb after skin removal. (**e**) The tendons of the tibialis anterior (TA) (*white arrow*) and the EDL (*white arrowhead*) are cut at the ankle joint. (**f**) The TA and EDL are reflected and cut together at the origin point just below the knee joint (**g**) The EDL (*white arrowhead*) and TA (*white arrow*) are gently pulled apart with forceps to isolate the EDL. The isolated EDL is placed in collagenase solution for isolation of single myofibers and the associated satellite cells

EDL (Fig. 1d). Other methods to remove the skin require more tedious reflection of the skin using scalpel blades and forceps [15].

6. Using #5 forceps, gently remove the fascia that overlies the hind limb muscles (focusing on the fascia that is over the TA muscle). The fascia appears as a thin film surrounding the musculature. When the fascia is removed the identification and isolation of the EDL tendons is facilitated.

7. When the skin and fascia are removed there are two prominent tendons on the ventral side of the ankle joint. The medial tendon connects with the TA muscle and the lateral tendon is the confluence of the four insertion point tendons of the EDL. Using #5 forceps, gently isolate both tendons (Fig. 1e).
8. Gently isolate both the EDL and TA muscles by sliding #5 forceps underneath both isolated tendons. Gently slide the forceps up towards the EDL/TA origin. Be careful not to stretch the muscles.
9. Leaving the #5 forceps underneath the muscles, use the extra fine Bonn scissors to cut the two isolated tendons at the ankle joint (Fig. 1e). Be careful not to cut into any TA or EDL muscle.
10. With #5 forceps grasp the proximal portion of the cut tendons and reflect both the EDL and TA muscles proximally.
11. Carefully cut the reflected muscles at the insertion points on the tibia just below the knee joint (Fig. 1f). Be sure to not to stretch the muscle and cut as high as possible to avoid damaging or cutting the EDL musculature. Continue to grasp the distal part of the muscle by the tendons with #5 forceps. At this point the TA and EDL are separated from the mouse as a single unit.
12. Place the isolated muscles on the dissection pad and separate the two tendons on the distal part of the muscle.
13. Using two #5 forceps grab the TA and EDL tendons and gently separate the two by pulling apart. There should be very little resistance (Fig. 1g).

#### *3.1.3 Collagenase Digestion of Isolated EDL Muscles*

1. Immediately after isolation place both EDL muscles from a single mouse into prepared 2 mL microcentrifuge tube containing 900 μL of F12-C media with antibiotics. Be sure to only handle the EDL muscles by holding the tendons, even when placing into the media.
2. Add 100 μL of 10× stock collagenase (*see* Subheading 2.2.4) and incubate at 37 °C while inverting the tube ten times every 10 min for 70–90 min.
3. Retrieve the six well plate with pre-warmed myofiber growth media from the incubator. Stop the enzymatic digestion by decanting the entire contents of the 2 mL microcentrifuge tube (2 EDLs, F12-C media with antibiotics, collagenase) into a well with 4 mL of the pre-warmed myofiber growth media and 2.5 ng/mL FGF2. This well will be referred to as the "bulk well."

#### *3.1.4 Isolation of Single Myofibers from Bulk EDL Muscle*

Throughout the isolation process do not allow the bulk muscle tissue or single myofibers outside of an incubator or off a 37 °C warming plate for more than 15 min at a time. If performing

isolations from two mice, work on one plate for 15 min and then switch to the other plate while the first re-warms in a 37 °C low $O_2$ incubator.

1. Under a stereo dissection scope, examine the collagenase digested EDL. The bulk muscle should resemble a loose bundle of translucent fishing line. If the myofibers appear white/opaque then they were likely damaged in the dissection and are not viable. Live myofibers are shiny and translucent under the light of the dissecting scope.
2. Transfer any completely detached individual myofibers into the well containing 3 mL of myofiber growth media and 2.5 ng/mL FGF2 (referred to as the "pick well") using a fire polished Pasteur pipet (*see* Subheading 2.2.2) and pipet pump. Minimize the amount of media, debris and dead myofibers moved to the pick well. This will minimize the amount of contaminating substances and non-myogenic cells as well as maximize myofiber survival (*see* **Note 21**).
3. Release any remaining, loosely attached myofibers in the bulk well by pipetting the whole muscle into a fire polished glass pipet and expelling it into the media with a pipet pump. This can be done a number of times with some force while still maintaining live myofibers. Be sure not to pinch or trap the bulk muscle of myofibers against the plastic of the six-well dish as this will kill or damage the myofibers.
4. After mechanical separation of the myofibers, transfer all detached individual myofibers to the pick well while minimizing debris transfer.
5. Transfer the individual myofibers from the first pick well to the second pick well. We have found two transfers are sufficient to isolate between 100–300 myofibers per EDL (*see* **Note 22**).

### *3.1.5 Culturing Isolated EDL Myofibers*

Myofibers and their associated satellite cells can be cultured for extended periods of time (greater than 5 days). During this time a number of assays permit monitoring of myofiber-associated satellite cell behavior in culture; a number of these techniques will be described in the following sections (*see* Subheadings 3.2–3.6). Alternatively, myofibers and their associated satellite cells can be fixed immediately post-isolation to examine the in vivo activity of satellite cells and myofibers.

1. Myofibers are grown in myofiber growth media and 2.5 ng/mL FGF2 at 37 °C in a low (5%) $O_2$ tissue culture incubator. Rock six-well plate back-and-forth gently every 24 h to redistribute recombinant FGF2. Add additional FGF2 stock solution (*see* Subheading 2.2.1) to maintain the 2.5 ng/mL concentration every 48 h.

2. Every 24 h examine the culture dish and remove hypercontracted/dead myofibers from the culture along with any remaining debris. If done carefully, this procedure will not considerably decrease the volume of myofiber growth media. If planning a short culture (0–96 h) the myofiber growth media will not need to be changed.
3. If planning a longer culture experiments (greater than 5 days) refer to other published protocols that describe methods to change media and maintain isolated myofibers [15]. For the following assays, longer myofiber cultures are unnecessary.

### 3.2 Transplantation of Myofiber-Associated Satellite Cells

Transplantation of myofiber-associated satellite cells into skeletal muscle results in lifelong hypertrophy, increased satellite cell numbers and resistance to age-related muscle degeneration [16]. The technique has been used to assess age-related muscle degeneration and the effects of the aged environment on young muscle stem cells [11, 16]. The procedure is technically challenging.

#### 3.2.1 Initial Steps to Prepare for Transplantation

1. Pre-warm a six-well plate with 2.5 mL myofiber growth media and 2.5 ng/mL FGF2 in a 37 °C tissue culture incubator. One well of myofiber growth media and 2.5 ng/mL FGF2 will be needed per donor mouse.
2. Gather 27½ G tuberculin syringes (one for each injection) and recipient mice.
3. Add either sterile filtered 1.2% $BaCl_2$ in 0.9% saline or sterile filtered 0.9% (*see* Subheading 2.4) to a 10 cm plate (8–12 mL) and let equilibrate to room temperature.

#### 3.2.2 Transplantation into Recipient Mice

1. For myofiber-associated satellite cell transplantations, pick the cleanest, longest, straightest, translucent myofibers from a fresh (0 h) myofiber isolation (*see* Subheading 3.1) and transfer them into a clean six-well plate containing 2.5 mL of pre-warmed myofiber growth media and 2.5 ng/mL FGF2. Make sure there is no debris.
2. Let the myofibers culture in myofiber growth media and 2.5 ng/mL FGF2 for 3–5 h in a 37 °C, low $O_2$ tissue culture incubator.
3. Seal the FGF2 treated myofiber six-well plate with Parafilm before moving to the location of transplantation (mouse facility).
4. Anesthetize recipient mice according to IACUC and institutional guidelines.
5. Apply 70% ethanol to the mouse hind limb and push aside hair so you can clearly see bare skin over the tibialis anterior (TA) muscle. The TA is the most superficial muscle in the ventral hind limb between the knee and ankle joint [14].

6. Once the mouse is anesthetized use a 27½ G tuberculin syringe to draw up ten myofibers while minimizing the media in the syringe if possible. Eject the myofibers into the 10 cm plate with either 0.9% saline or 1.2% $BaCl_2$ in 0.9% saline. Quickly, void the syringe of any excess media and withdraw the myofibers that were just moved into the 10 cm plate. Make sure to count the number of myofibers you have withdrawn into the syringe. Avoid withdrawing excessive media/saline/$BaCl_2$ into the syringe (*see* **Note 23**).
7. Once withdrawn, let the myofibers settle to the bottom of the syringe and clear air bubbles to a final volume of 50 μL. Keep needle tip up until ready to inject mouse. When ready, move syringe so myofibers are floating in middle of solution closer to syringe needle tip. Place needle bevel point up into the belly of the TA muscle by going parallel to the tibia along the muscle while being careful to not go through the muscle (too deep) or just under the skin (too superficial). Advance the needle until there is minor resistance from the knee joint, at which point retreat the needle slightly (less than 0.5 cm).
8. Once the needle is placed correctly into the TA, quickly eject the solution containing the isolated myofiber-associated satellite cells. Be sure not to retreat the needle all the way out before all the contents are ejected.
9. After the injection into the mouse is complete, withdraw 100–200 μL 1.2% $BaCl_2$ or 0.9% saline into the syringe from the 10 cm plate used earlier then quickly eject back into a clean plate to see if myofibers are still in needle. At this point the myofibers will be hyper-contracted but will come out if you eject quickly/hard several times. Count the number of myofibers remaining following injection. This will tell you how many myofibers you transplanted into the mouse.
10. Put the recipient mouse back into its cage and note recovery. Mice will limp on their injured/transplanted hind limb for a few hours post injection.

### 3.3 Transfection of Myofiber-Associated Satellite Cells

Transfection of myofiber-associated satellite cells results in transient expression of target constructs. Care should be taken as transfection can result in expression by both the myofiber and the associated satellite cells. Careful experimental design is necessary to assess effects on both satellite cells and myofibers. It is important to note that antibiotic free media should be used in all steps with the exception to **step 8**.

1. Isolate myofibers from EDL muscle as described in Subheading 3.1. This protocol works directly following myofiber isolation (0 h myofibers).

2. Immediately following digestion isolate at least 50 myofibers (with minimal debris) per condition into wells of a 24 well dish. Include a transfection only control (*see* **Note 24**).
3. Rinse myofibers with F12-C + 15% horse serum + 2.5 ng/mL FGF2. Place them in a final volume of 500 μL F12-C + 15% horse serum + 2.5 ng/mL FGF2.
4. Mix 1 μg DNA and OptiMEM media to 62.5 μL final volume.
5. Separately mix 2.5 μL Lipofectamine 2000 with 62.5 μL OptiMEM media and let sit at room temperature for 5 min (no more than 25 min).
6. Combine OptiMEM/DNA mixture with OptiMEM/Lipofectamine 2000 mixture to obtain 125 μL final volume. Incubate mixture at room temperature for 25 min, no more than 6 h.
7. Add Lipofectamine 2000/DNA mixture to each well containing myofibers and 500 μL F12-C + 15% horse serum. Incubate myofibers with Lipofectamine 2000/DNA mixture in a 37 °C in low oxygen incubator overnight (*see* **Note 25**).
8. Following transfection, rinse myofibers and change media to myofiber growth media + 2.5 ng/mL FGF2. Culture myofibers until needed, supplementing with 2.5 ng/mL FGF2 every 48 h.
9. Fix myofibers and store at 4 °C as described in Subheading 3.7.

### 3.4 CFDA-SE Self-Renewal Assay

CFDA-SE is a cell-permeant dye, which transforms within the cell, dramatically slowing its outflow rate. CFDA-SE is diluted upon cell division, and thus, can serve as a reliable marker for self-renewal or terminal differentiation [12]. CFDA-SE, which is fluorescein based, is available in several colors with distinct properties from Invitrogen/Thermo Fisher.

1. Isolate myofibers from EDL muscle as described in Subheading 3.1.
2. Culture myofibers with myofiber growth media and 2.5 ng/mL FGF2 for 48 h prior to addition of CFDA-SE.
3. Add previously determined working concentration (*see* Subheading 2.6 and **Note 17**) of pre-warmed CFDA-SE mixture to myofibers and pulse for 15 min at 37 °C.
4. Wash CFDA from myofibers with pre-warmed PBS and then again with pre-warmed myofiber growth media. Keep cells and fresh CFDA-SE away from light, as CFDA-SE is light sensitive (*see* **Note 26**).
5. Continue culturing myofibers in myofiber growth media and 2.5 ng/mL FGF2 until needed then fix and store at 4 °C as described in Subheading 3.7.

### 3.5 EdU Proliferation and Fusion Assay for EDL Myofibers

To assay for actively dividing cells, [$^3$H]Thymidine and BrdU have been historically used but these techniques have drawbacks including generation of radioactive waste and difficulties in anti-BrdU antibody accessibility to incorporated BrdU, respectively. EdU labeling and detection eliminates both these drawbacks and is much more reliable than BrdU staining. Both in vitro/ex vivo satellite cell proliferation percentages as well as in vivo satellite cell proliferation and myonuclear contribution can be determined with these techniques [3].

#### 3.5.1 Ex Vivo S-Phase Labeling of Myofiber-Associated Satellite Cells

1. Isolate myofibers from EDL muscle as described in Subheading 3.1.
2. Culture myofibers in myofiber growth media and 2.5 ng/mL FGF2 until desired time point for collection.
3. Prior to collection, add 10 μM EdU from 10 mM EdU stock solution (*see* Subheading 2.7.1) to the cultured myofibers and incubate in a low $O_2$ tissue culture incubator at 37 °C. EdU incubation time should be empirically determined (*see* **Note 27**).
4. After completion of the EdU incubation, wash myofibers with pre-warmed PBS and fix the myofibers as described in Subheading 3.7.1.
5. Transfer the fixed myofibers to a myofiber staining basket (*see* Subheadings 2.3.1, 3.7.2 and Fig. 2).
6. Block and permeabilize the myofibers with 300 μL 3% BSA, 0.5% Triton X in PBS added to each well containing a myofiber staining basket for 30 min.

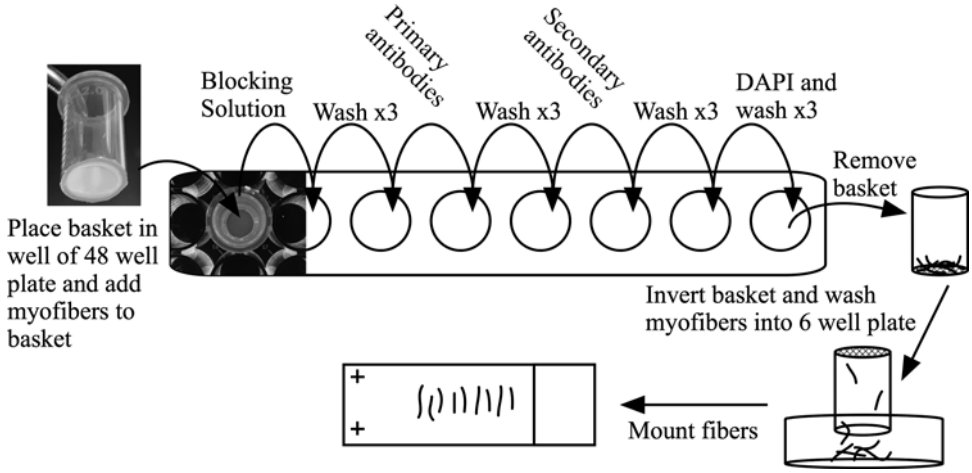


**Fig. 2** Schematic of immunostaining procedure with myofiber staining baskets. Place a prepared myofiber staining basket (*see* Subheading 2.3.1) into a well of a 48 well plate and transfer myofibers into the basket. Move the basket into a new well and add blocking solution. Continue moving the basket into a new well for each step of the staining procedure. When complete, remove basket, invert basket and wash myofibers into six well dish with PBS then mount myofibers onto a charged slide (*see* Subheading 3.7.3)

7. Add 300 μL EdU Click-iT® cocktail (as described by the Click-iT® EdU Alexa Fluor® Imaging Kit listed in Subheading 3.7.1) to each well containing a myofiber staining basket and incubate for 30 min in the dark.
8. Continue immunolabeling for additional antigens (*see* Subheading 3.7.2) or mount myofibers (*see* Subheading 3.7.3).

#### *3.5.2 In Vivo EdU Labeling of Satellite Cells and Myonuclei*

1. Prepare appropriate volume of 0.5 mg/mL EdU drinking water as described in Subheading 2.7.2.
2. Place the sterile EdU drinking water in a mouse water bottle and keep the bottle full until the desired time point.
3. Sacrifice the mice and isolate myofibers as described in Subheading 3.1.
4. Fix the myofibers in 4% paraformaldehyde immediately post-isolation as described in Subheading 3.7.1.
5. Continue with the EdU Click-iT® chemistry as described in Subheading 3.5.1, **steps 5–8**.

### *3.6 AraC Treatment*

AraC incorporates into DNA, inhibiting DNA replication and killing dividing cells. Myofibers, which are terminally differentiated are immune to AraC, but dividing satellite cells are affected [12]. While CFDA-SE (*see* Subheading 3.4) will label self-renewed cells brightest, AraC specifically selects for cells that have self-renewed or undergone terminal differentiation as neither of these cells are mitotically active. Immunostaining the satellite cells for Myogenin will distinguish between terminally differentiated cells and quiescent satellite cells.

1. Isolate myofibers from EDL muscle as described in Subheading 3.1.
2. Culture myofibers in myofiber growth media and 2.5 ng/mL FGF2 for 48–72 h.
3. After 48–72 h in culture, add previously determined concentration of 10 mM AraC stock solution (*see* Subheading 2.8.1 and **Note 20**) to media.
4. Change media on myofibers daily, gently washing with growth media to remove residual AraC before re-treating myofibers with AraC (*see* **Note 28**).
5. Culture myofibers in growth media containing AraC changing media and re-treating with AraC daily until fixation (*see* Subheading 3.7).

### *3.7 Fixation and Immunofluorescence of EDL Myofibers*

EDL myofiber-associated satellite cells can be fixed and stained at different time points post-isolation depending on the experimental parameters to visualize proteins on or within both myofibers and satellite cells. Myofiber staining baskets, which we developed,

provides simpler method for myofiber staining that minimizes myofiber loss and myofiber damage compared to currently used techniques.

*3.7.1 Paraformaldehyde Fixation of Isolated EDL Myofibers*

1. To fix isolated myofibers: make 2.5 mL of 4% paraformaldehyde in PBS for every well of myofibers to be fixed (*see* Subheading 2.3).
2. Transfer only single, straight and translucent myofibers to an empty well of a six well dish using a horse serum coated, fire polished, glass Pasteur pipet (*see* Subheading 2.3 and **Note 29**).
3. Remove as much growth media as possible (without losing myofibers) and add 2.5 mL of PBS to wash the myofibers.
4. Transfer the myofibers to an empty well of a six well dish using a horse serum coated, fire polished, glass pasture pipet.
5. Remove as much PBS as possible (without losing myofibers) and add 2.5 mL of 4% paraformaldehyde in a drop-wise manner to avoid myofiber clumping.
6. Incubate the myofibers in 4% paraformaldehyde for 10 min at room temperature. The myofibers should transform from shiny and translucent to white and opaque.
7. Transfer the fixed myofibers to an empty well of a six well dish and remove as much 4% paraformaldehyde as possible without losing myofibers. Then add 4.0 mL of PBS to the well to wash the myofibers.
8. Transfer the myofibers to an empty well and remove as much volume as possible without losing any myofibers and again add 4.0 mL of PBS. At this point the myofibers can be stored in PBS at 4 °C for up to 3 weeks or until you are ready to immunostain.

*3.7.2 Immunostaining of Isolated EDL Myofibers*

1. Transfer 4% paraformaldehyde fixed myofibers, using a horse serum coated, fire polished, glass pasture pipet, into a myofiber staining basket (*see* Subheading 2.3.1) sitting in a single well of 48 well dish (Fig. 2 and *see* **Note 30**).
2. Once 20–40 myofibers have been transferred, move the myofiber staining basket to an empty well and wash with 300 µL PBS as shown in Fig. 2 (*see* **Note 31**).
3. Add 300 µL 3% $H_2O_2$ (*see* Subheading 2.3) for 3–5 min and then move the myofiber staining basket to another well of the 48-well dish and rinse with PBS (*see* **Note 32**).
4. Move the myofiber staining basket to another well and add 300 µL 0.25% Triton X-100, 3% BSA in PBS and incubate for 60 min at room temperature.
5. Transfer the myofiber staining basket to an empty well and wash by adding 300 µL of PBS.

6. Transfer the myofiber staining basket to an empty well and add 300 μL primary antibody(s) diluted in 3% BSA; 0.125% Triton X in PBS. Incubate at 4 °C overnight or 1.5 h at room temperature.
7. After the primary antibody incubation, wash the myofibers three times in PBS by transferring the myofiber staining basket between wells (Fig. 2).
8. Incubate with the appropriate secondary antibodies diluted in 2% BSA; 0.125% Triton X in PBS for 1 h in the dark.
9. After the secondary antibody incubation, wash the myofibers three times in PBS by transferring the myofiber staining basket between wells.
10. Incubate the myofibers in 1 μg/μL DAPI for 10 min at room temperature.
11. Wash once with PBS by transferring the myofiber staining basket to an empty well.

### 3.7.3 Dry Mounting Myofiber-Associated Satellite Cells

1. To mount the myofibers, transfer the myofibers from the basket to a six-well dish or watch glass by turning the basket upside-down and then flushing the bottom of the basket with PBS to remove myofibers to the plate (*see* **Note 33**).
2. Under a dissection scope, transfer the myofibers individually to a charged glass slide by picking them up from one end with #5 forceps and placing them on the glass slide. Do not stretch the myofibers, as they will break. The charge from the slide will help the myofibers attach (Fig. 2).
3. Once all myofibers are transferred, mount with Mowiol (*see* Subheading 2.3.2 and **Note 34**), let stand at room temperature for at least 2–4 h to allow Mowiol to harden. Slides should be stored at room temperature in the dark until ready for imaging.

## 4 Notes

1. To make larger volumes of F12-C media adjust volumes of water, $NaHCO_3$, 100× penicillin–streptomycin, and 0.2 M $CaCl_2$ accordingly.
2. Multiple lots of horse serum should be tested to determine the lot that best supports myofiber and associated satellite cell growth. Typically, we test 8–15 lots of horse serum. Briefly, isolated satellite cells are cultured for 5 days then passaged and cultured for an additional 5 days. Each lot of horse serum is tested in quadruplicate 100 mm tissue culture plates. Cell numbers are counted each day to determine the doubling efficiency.

At the conclusion of the second round of 5 day culture, three plates from each lot are fixed and immunostained for skeletal muscle myosin heavy chain protein (MF20 antibody Hybridoma Studies Bank). For each lot, one plate is transferred to 1% serum supplemented with 1:100 ITS (Insulin, Transferrin, Selenium; Corning 25-800-CR) and cultured for 48 h to induce differentiation. The differentiated plates are stained for myosin heavy chain and the extent of differentiation determined for all lots. The lot that exhibits the best growth with the least differentiation in growth medium and the best differentiation when switched to low serum is purchased and stored at −80 °C. Prior to use each 0.5 L horse serum bottle is aliquoted into 75 mL volumes and stored at −20 °C. Aliquots are thawed at 37 °C when needed. Typically, one or two lots of horse serum of ten lots (10–20%) permit robust satellite cell growth and differentiation. Although laborious, this is a critical and key requirement for producing growth media.

3. FGF2 is very basic and will adhere to any glass surface. Always store FGF2 in plastic containers and use plastic pipette tips to add FGF2 to solutions and cell culture plates. If needed, aliquot media into a plastic conical tubes before adding FGF2.
4. Care should be taken to avoid overheating and sealing the pipette end. Once cool, rinse the glass-fired pipettes with media containing horse serum prior to their first use to prevent myofibers from sticking to the inside of the pipette during transfer.
5. Multiple lots of collagenase type I should be tested to determine the best lot for myofiber digestion and long term myofiber survival. We find there is significant lot to lot variability where some lots will dramatically reduce satellite cell viability.
6. Either syringe filters or bottle top filters will work depending upon volume filtered. Pre-filtering through the 0.44 μm filter allows lower applied pressure filtering through the 0.22 μm filter, especially if you are using syringe filters.
7. Do not freeze/thaw the collagenase 10× stock solution. Multiple freeze/thaws will decrease the enzyme efficiency.
8. PBS with Triton X-100 can be made in advance and stored at room temperature. Blocking solutions can be made in advance and frozen in 5–10 mL aliquots and stored at −20 °C. Thaw aliquots completely at room temperature prior to use.
9. Ideally, tubes are trimmed using a hand-held rotary tool with a cut-off wheel. Alternatively, a heated scalpel can be used to cut the tube. The ideal cut point on the tube is between the straight and tapered point of the tube.
10. Various mesh sizes were tested and 80 μm was found to be optimal. <80 μm mesh did not allow sufficient liquid flow-thru while >95 μm allowed myofibers to fall through.

11. The hot plate should be hot enough to melt basket tops but cool enough not to melt the nylon mesh. Foil covering serves two purposes—(1) allows easy cleanup of hot plate and (2) allows stuck tubes to be removed quickly without the risk of injury.
12. The tubes are examined for partial attachment or melted plastic within the mesh. Tubes with melted plastic in the mesh are discarded. The mesh, which is partially attached, can be remelted. Ideally tubes should sit slightly above the bottom of a 48 well plate to allow fluid flow.
13. The protocol can be scaled to make larger quantities. Aliquots can be freeze/thawed multiple times without consequence. Once added to slides, store in the dark to allow solution to harden. The solution will completely harden overnight and does not need any other sealing agents.
14. For 300 mL of 0.9 % saline solution, add 2.7 g NaCl to 250 mL $dH_2O$ and stir until dissolved. Bring to final volume of 300 mL with $dH_2O$ then sterile filter through 0.22 μm Steritop bottle filter into a sterile container and store at room temperature.
15. For 300 mL of 1.2 % $BaCl_2$ in 0.9 % saline solution, add 2.7 g NaCl and 3.6 g dehydrate $BaCl_2$ to 250 mL $dH_2O$, stir until dissolved. Bring to a final volume of 300 mL with $dH_2O$ then sterile filter through 0.22 μm Steritop bottle filter into a sterile container and aliquot into 10 mL volumes for use with transplantation. Store at room temperature.
16. The CFDA-SE 10 mM stock solution should be made fresh for every experiment and stored in the dark, as CFDA-SE is light sensitive.
17. The optimal CFDA-SE concentration needs to be determined empirically. Satellite cells and myofibers, which autofluoresce green, uptake the dye at different rates. We recommend performing a titration curve of CFDA-SE concentrations ranging from 1 to 10 μM.
18. To dissolve EdU into sterile $H_2O$ with 1 % dextrose place solution in 37 °C water bath for 1–2 h, occasionally mixing, until EdU goes into solution. Dextrose is added to the water to encourage the mice to drink the water.
19. Keep all AraC solutions away from light. A 100 mM stock solution is good for 1 year at −20 °C.
20. At high concentrations, AraC affects cellular processes other than DNA replication. An optimal working AraC concentration that kills as many cycling satellite cells as possible without killing the myofiber should be empirically determined. We recommend performing a titration curve of AraC concentrations ranging from 0.1 to 100 μM.

21. Other published protocols [15] suggest a number of rinses to remove excess collagenase from the bulk muscle. We have found that keeping the bulk muscle in diluted collagenase allows for a more complete digestion and less mechanical manipulation, ultimately leading to an increased single, live myofiber yield. Additionally, we have also found picking myofibers between multiple wells dilutes the collagenase sufficiently to avoid damage to the myofibers.
22. During the second transfer, be sure to only pick single, translucent, straight myofibers that have no associated debris or other live myofibers attached to them. Myofibers with bends, curls, kinks or hyper-contractions will often die within the first hours of culture.
23. To withdraw myofibers with minimal liquid, place the bevel of the syringe directly above a myofiber like a vacuum cleaner over a carpet. Withdraw the syringe minimally but swiftly.
24. Fifty myofibers is the minimum recommended for transfection. >100 myofibers should be done in a 12 well plate and amounts listed doubled. >200 myofibers should be done in a six well plate with all volumes quadrupled.
25. Myofibers and associated satellite cells should be monitored for death post transfection. Transfection time can be shortened to 4–6 h if necessary.
26. Wait at least 30 min before examining or fixing myofibers to determine if CFDA-SE pulse was effective.
27. The longer the myofibers spend cultured in the presence of EdU the higher the percentage of EdU labeled myofiber-associated satellite cells. The precise time-point used is under the discretion of the experimenter. When cultured in the presence of 2.5 ng/mL FGF2 for 48–72 h a 2-h EdU incubation will label approximately 70% of the myofiber-associated satellite cells.
28. AraC is only stable in culture for 24 h. For experiments lasting longer than 24 h where cycling cells need to be killed, myofibers will need to be washed with growth media and AraC replaced every 24 h.
29. It is critical to only transfer live myofibers during this step. Once myofibers are fixed it is difficult to distinguish between live and dead myofibers. This step is also a good opportunity to remove remaining debris or clumped myofibers.
30. If the volume inside the myofiber staining basket reaches capacity before all the myofibers are transferred move the myofiber staining basket to an empty well and finish transferring myofibers.
31. Baskets should be moved into empty wells and solution applied post move. Adding baskets to wells with solution present can

push myofibers to the top of the basket and can affect further staining steps. Depending upon basket length, volumes can be decreased if myofibers are covered with solution.

32. Do not leave quenching solution on myofibers longer than 3–5 min or it will hinder future staining. This step will reduce the autofluorescence of the myofibers during immunofluorescent microscopy.
33. Myofiber staining baskets can be reused for staining by autoclaving baskets in $dH_2O$ and allowing them to air-dry. Alternatively, baskets can be rinsed thoroughly with $dH_2O$ and allowed them to air-dry before their next use. However, be sure to check under a dissection microscope that no myofibers remain in the baskets before reuse.
34. For a 22 × 60 mm coverslip 40–60 μL of mowiol is sufficient. Do not add >100 μL mowiol as this will cause the coverslip to move and the myofibers will detach from the slide and become tangled. If too much mowiol is added, you can use a vacuum along the edges of the coverslip to remove the extra.

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