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Isolation and Culture of Skeletal Muscle Myofibers as a Means to Analyze Satellite Cells

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Summary

Myofibers are the functional contractile units of skeletal muscle. Mononuclear satellite cells located between the basal lamina and the plasmalemma of the myofiber are the primary source of myogenic precursor cells in postnatal muscle. This chapter describes protocols used in our laboratory for isolation, culturing and immunostaining of single myofibers from mouse skeletal muscle. The isolated myofibers are intact and retain their associated satellite cells underneath the basal lamina. The first protocol discusses myofiber isolation from the flexor digitorum brevis (FDB) muscle. Myofibers are cultured in dishes coated with Vitrogen collagen and satellite cells remain associated with the myofibers undergoing proliferation and differentiation on the myofiber surface. The second protocol discusses the isolation of longer myofibers from the extensor digitorum longus (EDL). Different from the FDB myofibers, the longer EDL myofibers tend to tangle and break if cultured together; therefore, EDL myofibers are cultured individually. These myofibers are cultured in dishes coated with Matrigel. The satellite cells initially remain associated with the myofiber and later migrate away to its vicinity, resulting in extensive cell proliferation and differentiation. These culture protocols allow studies on the interplay between the myofiber and its associated satellite cells.

Keywords

Satellite cells; skeletal muscle; myofiber isolation; single myofiber culture; flexor digitorum brevis; extensor digitorum longus; mouse; Vitrogen collagen; Matrigel

1. Introduction

Myofibers are the functional contractile units of skeletal muscle. While they are established during embryogenesis by fusion of myoblasts into myotubes, processes involved in their growth and repair continue throughout life. The development of myofibers and their regenerative potential depends on the availability of myogenic precursor cells. Mononuclear satellite cells located between the basal lamina and the plasmalemma of the myofiber are classically considered to be the myogenic precursors in postnatal muscle (1,2). While in a growing muscle at least some of the satellite cells are proliferating and adding myonuclei to the enlarging muscle fibers, in a normal adult muscle most satellite cells are quiescent. However, in response to a variety of conditions, ranging from increased muscle utilization to muscle injury, quiescent satellite cells can enter the cell cycle, replicate and fuse into existing myofibers or form new myofibers (reviewed in (2)). The cascade of cellular and

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molecular events controlling satellite cell myogenesis is therefore of interest for understanding the mechanisms of muscle maintenance during life span, as well as for developing strategies to enhance muscle repair after severe trauma or during myopathic diseases.

Two main in vitro approaches have been employed in the study of satellite cells: i) myogenic cultures prepared from mononucleated cells dissociated from the whole muscle (i.e., primary myogenic cultures), and ii) cultures of isolated myofibers where the satellite cells remain in their in situ position underneath the myofiber basal lamina. Protocols for obtaining primary myogenic cultures aim at releasing as many satellite cells as possible from the entire muscle. Steps of mincing, enzymatic digestion and repetitive trituration of the muscle are required for breaking down the connective tissue network and myofibers in order to release the satellite cells from the muscle bulk. These steps are followed by procedures aimed at removing tissue debris and reducing the contribution of nonmyogenic cells typically present in primary isolates of myogenic cells (3-5). In contrast, approaches for isolating myofibers aim at releasing intact myofibers that retain the satellite cells in their native position underneath the basal lamina (4). The first method, based on breakage of the myofibers and release of satellite cells, provides a means for studying parameters affecting the progeny of satellite cells as they proliferate, differentiate and fuse into myotubes. The second method, based on isolating intact myofibers, allows studying satellite cells in their in-situ position as well as studying their progeny after migrating from the myofibers.

This chapter describes the two approaches used in our laboratory for isolation and culture of single myofibers from mouse skeletal muscle. One approach, first introduced by Bekoff and Betz (6) and further developed by Bischoff (7,8), has been adopted by us for studies of satellite cells in isolated myofibers from both rat (4,9) and mouse (10). In this case, myofibers are isolated from the flexor digitorum brevis (FDB) muscles of the hind feet, and multiple myofibers are typically cultured together. The FDB has been used as donor muscle because it consists of short myofibers that do not tangle (and consequently break) when cultured together. A second approach, introduced by Rosenblatt and colleagues (11,12), is suitable for the isolation of longer myofibers from a variety of limb muscles (e.g., extensor digitorum longus (EDL), tibialis anterior (TA) (11,13,14). Different from the FDB myofibers, the longer myofibers tend to tangle and break if cultured together. Hence, typically when working with muscles such as EDL or TA, the isolated myofibers are cultured individually. In both approaches, the culture dishes are coated with commercially available matrixes that facilitate rapid and firm adherence of the myofibers to the dish surface.

Table 1 compares in brief the two approaches for myofiber isolation and the specific use of each procedure. Both protocols for myofiber isolation can produce high yields of intact myofibers retaining their satellite cells underneath the basal lamina. However, delicate manipulation of the donor muscle at the tendons when harvesting and processing, the type and specific source of the digesting enzyme, the length of the enzymatic digestion period and the degree of trituration of the digested muscle are all important factors that should be well controlled during the isolation procedure. Myofibers that are damaged in the course of the isolation procedure will not survive and can be easily distinguished from the intact myofibers since they typically hypercontract.

Protocols for immunocytochemical analysis of satellite cells in cultures of FDB and EDL myofibers are also included in the chapter.

Representative micrographs of FDB and EDL myofiber cultures are shown in Figure 1 (FDB, panels A-D) and Figure 2 (EDL, panels A-D).

2. Materials

2.1. General comments

1. As a general rule, only sterile materials and supplies are to be used. All solutions, unless otherwise noted, are sterilized by filtering through 0.22 μm filters, all glassware and dissection tools are sterilized by autoclaving, and all cell culturing steps are performed using sterile techniques.
2. The cultures are maintained at 37.5°C and 5% CO₂ in a humidified tissue culture incubator.
3. All culture media are stored at 4°C and used within 3 weeks from preparation.
4. Before starting isolation, the tissue culture medium is pre-warmed to 37°C and then held at room temperature throughout the procedures (do not leave medium at 37°C for an extended period of time). Before transferring solutions/media into the tissue culture hood, spray the glass/plastic containers with 70% ethanol.
5. The quantities of glassware, media and reagents as well as the time intervals for enzymatic digestion described in this chapter are appropriate for the isolation of myofibers from one adult mouse of the age and strain detailed below. Adjustments are needed when isolating myofibers from younger/older mice, other mouse strains, mutant mice, or other laboratory rodents such as rats.
6. Muscles used for preparing isolated myofibers are harvested from the hind limbs.

2.2. General equipment

The following facilities are required for the cultures described in this chapter:

1. Standard humidified tissue culture incubator (37.5°C, 5% CO₂ in air).
2. Tissue-culture hood.
3. Phase contrast microscope.
4. Stereo dissecting microscope with transmitted light base (microscope is placed inside a tissue culture hood).
5. Low speed agitator placed in the tissue culture incubator (Labline instruments, Inc. model no. 1304). The agitator is used for gently agitating the muscle during enzymatic digestion; a shaking water bath set at 37°C can be used instead of the low speed agitator.
6. Bunsen or alcohol burner inside the tissue culture hood.
7. Water bath (37°C).
8. Hair trimmer (optional, for shaving hair from the hind limbs prior to muscle dissection).

2.3. Surgical tools

1. Straight Operating Scissors: V. Mueller, fine-tipped, Sharp/Sharp stainless steel, 165 (6¹/₂) (VWR Scientific Inc. cat. no. 25601-142), for delicate cutting and fine incisions.
2. Dissecting Scissors: stainless steel, 140-mm (5.5") length. Both blades blunt (VWR Scientific Inc. cat. no. 25877-103), protects the surrounding tissue from any unwanted nicks.

3. Dressing Forceps: V. Mueller, serrated, stainless steel, rounded points, 140-mm (5¹/₂" length (VWR Scientific Inc. cat. no. 25601-072).
4. Two, very fine point forceps: extra-fine tips, smooth spring action, stainless steel. Straight, 110-mm (4¹/₄"; VWR Scientific Inc. cat. no. 25607-856).
5. Micro scissors Vannas Scissors: 8 cm long, straight 5-mm blades, 0.1-mm tips (World Precision Instruments, cat. no. 14003).
6. Scalpel handle: size 3 for blades 10-15 (Bard-Parker, cat. no. 371030) and sterile blade (#10; Bard-Parker, cat. no. 371110).
7. Placement instrument (VWR Scientific Inc. cat. no. 1790-034) 8. Two straight, 5" Hemostatic Forceps (VWR Scientific Inc. cat. no. 25607-302). 9. Dissecting board.

2.4. Animals

C57BL/6 mice, 2-5 month-old, maintained according to institutional animal care regulations. Various other mouse strains have been used in our studies following the same myofiber isolation procedures.

2.5. Muscles

The information in this section is provided to assist in the identification and isolation of the FDB and EDL muscles.

2.5.1. Flexor digitorum brevis (FDB)—The FDB is a superficial, multipennate, broad and thin muscle of the foot and paw (8,17); it arises from the tendon of the plantaris as three slender muscles converging into long tendons. At the base of the first phalanx it divides into two, passes around the tendon of the flexor hallucis longus obliquely across the dorsum of the foot, and ends as the tendons insert into the second phalanx of the 2nd through the 5th digits. As the FDB contracts, digits 2-5 are flexed. For additional details about the anatomy of the FDB muscle (*see Note 1*).

2.5.2. Extensor digitorum longus (EDL)—The EDL muscle is situated at the lateral part of the hind limb running from the knee to the ankle, extending to the 2nd to 5th digits (17). The EDL actually consists of four combined muscle bellies and their tendons; the bellies arise from the lateral condyle of the tibia and the front edge of the fibula (2 tendons at the origin of the muscle). The tendons lie close to each other and appear as one glistening white tendon that continues down to the surface of the ankle. At the ankle joint it separates to 4 tendons, each attached to one of the 2nd-5th digits. As the EDL contracts, the 4 digits are extended. For additional details about the anatomy of the EDL muscle (*see Note 1*).

2.6. Plastic and glassware for myofiber isolation and culture

2.6.1. FDB myofiber isolation and culture

1. Standard 9" Pasteur pipettes (VWR Scientific Products, cat. no. 0035904).
2. Standard 5" Sterile glass Pasteur pipettes (VWR Scientific Products, cat. no. 0035901).
3. Wide-mouth pipettes prepared from the standard 5" Pasteur pipettes. Cut the tip of a pipette about 3" from its narrow end using a file or a diamond knife. Shake the pipette to remove any glass fragments.
4. Use flame in hood to fire polish the distal ends of all Pasteur pipettes listed above to smoothen sharp edges that can damage myofibers. Syringe filters, 0.2 and 0.45

μm (Millex-GS, Millipore, cat. no. SLGS0250S and SLHA0250S, respectively) and 10 cc syringes.

5. Sterile conical tubes, 15 and 50 mL (BD Biosciences/Falcon, cat. no. 352098 and 302097, respectively).
6. Three glass Corex tubes, 15 mL (Sorvall centrifuge tubes; or alternatively 15 mL bicarbonate Sorvall tubes).
7. Wide-bore 100 μL micropipette tips. Trim 100 μL tips 3-mm from the end to minimize myofiber shearing when transferring or dispensing FDB myofibers.
8. Tissue culture dishes, 35-mm (Corning Incorporated, cat. no. 430165).
9. Two L-shape bent pipette spreaders prepared from standard 9" Pasteur pipettes. Use flame to first seal the distal end, then flame about 3/4" from the sealed end until the pipette starts to bend. The bent pipettes are used to spread the coating solution on the tissue culture dishes. Spreaders should be prepared in advance and allowed to cool before used.

2.6.2 EDL myofiber isolation and culture

1. Standard 9" and 5" sterile Pasteur pipettes, syringe filters and conical tubes listed and treated as described in items 1-6 under **subheading 2.6.1**.
2. Three gradually narrower-bore pipettes prepared from standard 5" Pasteur pipettes. Use a file or a diamond knife to prepare a set of pipettes with bore diameter of approximately 2.5, 2, and 1 mm. Shake the pipette to remove any glass fragments and fire polish sharp ends. These pipettes are used to triturate the digested muscle in order to release single myofibers.
3. Six plastic Petri dishes, 60X15-mm (BD Biosciences, Falcon, cat. no. 351007).
4. Twenty four-well Falcon multiwell tissue culture dish (BD Biosciences, cat. no. 353047) (*see Note 4*).

2.7. Media and Cell Culture Reagents

2.7.1. FDB myofiber isolation and culture

1. DMEM (Dulbecco's Modified Eagle Medium; high glucose, with L-glutamine, with 110mg/L sodium pyruvate, with piridoxine hydrochloride. GIBCO-Invitrogen Life Technologies, cat. no. 11995065) supplemented with 50U/mL penicillin and 50 μg /mL streptomycin (GIBCO-Invitrogen, cat. no. 15140-122).
2. Horse serum (HS); standard, not heat inactivated; (HyClone, cat. no. SH30074.03); Stored at -20°C (*see Note 5*).
3. HS, 20 mL, freshly filtered (on the day of use) through a 0.45 μm filter.
4. DMEM, 100 mL, containing 10% filtered HS. All Pasteur pipettes and micropipette tips are pre-flushed with DMEM containing 10% HS to prevent sticking of myofibers during manipulation.
5. Controlled Process Serum Replacement (CPSR; Sigma-Aldrich, stored at -20°C). Alternative serum replacement products (e.g., Sigma-Aldrich, cat. no. S9388; (19) can also be used depending on experimental requirements (*see Note 6*).
6. FDB myofiber culture medium is made up of DMEM (supplemented with antibiotics), 20% Controlled Process Serum Replacement and 1% HS.

7. Vitrogen Collagen In Solution (Cohesion Technologies, cat. no. FXP-019) for coating 35- mm tissue culture dishes. Vitrogen Collagen In Solution is the recommended product and the use of collagen from other companies would require pre-screening to ensure compatibility (*see Note 2*).
8. 7X DMEM made from powder DMEM (1 liter package, Sigma-Aldrich, cat. no. D3656); used to prepare isotonic Vitrogen collagen (*see Note 2*).
9. Collagenase (type I, Sigma-Aldrich, cat. no. C-0130) used for muscle digestion as described under the Methods section.

2.7.2. EDL myofiber isolation and culture

1. DMEM and horse serum as listed and prepared in items 1-4 under **subheading 2.7.1**.
2. Fetal bovine serum (FBS; standard, not heat inactivated; Sigma-Aldrich, cat. no. F-2442; stored at -20°C) (*see Note 7*).
3. Chicken embryo extract (CEE, GIBCO-Invitrogen, cat. no. 16460024), stored at -20°C ; or, as in our studies, prepared by the investigator (*see Notes 8, 9*).
4. EDL myofiber culture medium is made up of DMEM (supplemented with antibiotics), 20% fetal bovine serum, 10% HS and 1% CEE.
5. Matrigel (*see Note 3*) for coating 24-well multiwell dishes. Matrigel can be purchased in its standard format (BD Biosciences, cat. no. 354234) or in its growth factor reduced format (BD Biosciences, cat. no. 354230). In our studies we use the growth factor reduced format.
6. Collagenase, as listed in item 9 under **subheading 2.7.1**.

2.8. Reagents and solutions for fixing and immunostaining myofiber cultures

2.8.1. FDB myofiber cultures

1. Pre-fixation rinse solution: DMEM as in item 1 under subheading **2.7.1**.
2. Fixative: ice-cold 100% methanol (*see Note 10*).
3. Rinse solution: Tris buffered saline (TBS); 0.05 M Tris, 0.15 M NaCl, pH 7.4 (*see Note 11*).
4. Detergent: Tween 20 (Sigma, cat. no. P1379).
5. Detergent solution: TBS containing 0.05% Tween 20 (TBS-TW20).
6. Blocking reagent: Normal goat serum (Sigma-Aldrich, cat. no. G9023).
7. Blocking Solution: TBS containing 1% normal goat serum (TBS-NGS).
8. Mounting medium: Vectashield (Vector Laboratories, Inc. Burlingham; cat. no. H-1000).
9. Cover glass, 22 mm² (Corning Labware and Equipment, cat. no. 48371-045).

2.8.2. EDL myofiber cultures

1. Fixative: 4% paraformaldehyde containing 0.03M sucrose (*see Notes 12, 13*).
2. Rinse solution: TBS as item 3 under **subheading 2.8.1**.

3. Detergents: Triton X-100 (Sigma, cat. no. T6878); Tween 20 as item 4 under **subheading 2.8.1**.
4. Detergent solution: TBS containing 0.5% Triton X-100 (TBS-TRX100); TBS-TW20 as in item 5 under **subheading 2.8.1**.
5. Blocking reagent and solution: same as items 6 and 7 under **subheading 2.8.1**.
6. Mounting medium: same as item 8 under **subheading 2.8.1**.
7. Microcover glass (VWR Scientific Inc., cat. no. 12CIR-1).

3. Methods

3.1. Isolation of single myofibers from the Flexor Digitorum Brevis (FDB) muscle

3.1.1. Initial steps prior to harvesting the muscle and preparation of digestive enzyme

1. Add 3 mL of DMEM to six 35-mm tissue culture dishes and place the dishes in the tissue culture incubator until muscle dissection begins.
2. Add 3 mL of DMEM containing 10% HS to three 35-mm tissue culture dishes and place them in the tissue culture incubator until needed for the isolated single myofibers.
3. Add 6 mg of collagenase type I to 3 mL of DMEM in order to prepare 0.2% (wt/vol) collagenase type I solution. Use a 0.22 μ m filter attached to a 10 cc syringe to filter the collagenase solution into a 35-mm tissue culture dish (*see Note 14*).

3.1.2. Dissection of FDB muscle

1. Euthanize one mouse according to institute regulations.
2. Shave the hind limbs and spray them lightly with 70% ethanol.
3. Secure the mouse, lying on its back, to the dissecting board by pinning down the forelimb diagonally across from the limb being dissected.
4. Use a scalpel to cut through the skin all around and just above the ankle (after this initial circular cut the skin below resembles a sock).
5. Cut the skin in a straight line along the center of the ventral part of the foot almost all the way to the digits (the cut as viewed from the front of the foot should resemble a "T").
6. Clamp a hemostatic forceps to one of the upper corners of the cut tissue (at the junction of the circular and longitudinal cuts) shifting the skin away from the foot.
7. Hold the scalpel with its blade parallel to the longitudinal axis of the partially exposed muscle and carefully cut away the connective tissue. Be especially careful not to cut into the muscle tissue at the back of the leg, as the FDB is the most superficial muscle of the back of the foot.
8. Clamp the second hemostat to the other corner of the cut tissue and repeat step 7.
9. When the skin is completely cut away from the foot, the FDB should be exposed all the way to the tendons reaching the digits.
10. Turn the mouse over so that it lies on its stomach, and identify the FDB. During the next steps of the surgery, to avoid blood cell-contamination of the myofiber preparation, be careful not to injure the small medial plantar artery that supplies

blood to the FDB. This artery passes along the medial part of the sole of the foot and branches into the digits.

11. Carefully run the tip of the scalpel along each side of the FDB to disrupt the connective tissue holding the muscle in place.
12. When the FDB is separated from the surrounding muscles, insert the tip of the placement instrument underneath the FDB and gently lift the muscle so that the flat side of the scalpel may be inserted horizontally underneath it.
13. With the blade of the scalpel underneath the muscle, running horizontal and parallel to the muscle, cut away the underlying connective tissue. It is best to cut towards the heel and only lift that portion of the muscle directly over the scalpel.
14. Cut underneath the tendon to separate the muscle and a large portion of its tendon from the heel bone.
15. Clamp the freed tendon as far as possible from the muscle tissue with a hemostat.
16. Use the hemostatic forceps to gently lift the FDB away from the leg. Use the scalpel, running parallel to the muscle, to cut through the connective tissue while holding the FDB down.
17. Continue cutting through the connective tissue until the tendons that connect the FDB muscle to the digits have been exposed. When about half the length of the three tendons has been exposed, cut the tendons and release the entire muscle from the leg. The fourth small lateral tendon (attached to the 5th digit) and its attached myofibers can be trimmed off.
18. Retrieve from the incubator three 35-mm tissue culture dishes containing DMEM and place them close to the dissection area.
19. Place the harvested FDB in one of the 35-mm tissue culture dishes.
20. For harvesting the FDB from the other hind foot repeat steps 11-17, and place the muscle in a second 35-mm tissue culture dish.
21. Place the 35-mm tissue culture dishes, one at a time, under the stereo dissecting microscope.
22. Use fine point forceps to pull the connective tissue perpendicular to the line of the muscle and use scissors to cut it off.
23. Once the muscle is clean, shorten the tendons but do not cut all of them off.
24. Use a wide-bore Pasteur pipette to transfer the cleaned muscle to another 35-mm tissue culture dish containing DMEM.
25. Repeat steps 21-24 to clean the second FDB muscle.

3.1.2.1. Enzymatic digestion

1. Transfer the two cleaned FDB muscles to a 35-mm tissue culture dish with the 0.2% collagenase I solution.
2. Place this 35-mm tissue culture dish on the low speed agitator inside the tissue culture incubator to allow gentle and continuous collagenase digestion for 2.5 h (see **Note 14**).
3. At the end of the digestion period, transfer each muscle to a 35-mm tissue culture dish containing 10% HS.

3.1.3. Separation of the 3 tendons and release of myofibers—All Pasteur pipettes used are pre-flushed with 10% HS as described in item 4 under **subheading 2.7.1.**

1. Place one muscle at the time under the stereo dissecting microscope.
2. Identify the two grooves running between the three tendons separating the middle from the two lateral tendons.
3. Being careful not to touch the muscle, insert the tip of a forceps into one of the grooves and by securing the connective tissue between the tendons to the dish, hold the muscle in place.
4. Use another pair of forceps to gently pull the connective tissue that holds the tendons and their attached muscle tissue together.
5. Continue removing the connective tissue until the lateral tendons are separated from the middle tendon and its attached myofibers.
6. Holding the muscle only at its tendons, transfer the muscle preparation to a 35- mm dish containing 3 mL of 10% HS.
7. While grasping one end of the middle tendon with a pair of forceps, use a second pair of forceps to grip its surrounding connective tissue sheath and pull gently. If the sheath does not come off easily, use fine point forceps to pull the connective tissue perpendicular to the line of the muscle and cut it off.
8. Repeat steps 1-7 with the second FDB muscle until all six tendons and their attached myofibers are in the 35-mm tissue culture dish containing 10% HS.
9. For one tendon at a time: hold one end of the tendon with a pair of forceps and with the tip of a second pair gently separate the myofibers from the tendon. The liberation of the myofibers from the two lateral tendons should be easy, while the middle tendon requires patience since the myofibers are attached to it more firmly.
10. Use a wide-bore Pasteur pipette to gently triturate the clumps of myofibers until they disengage into single myofibers.
11. Remaining clumps should be transferred to another 35-mm tissue culture dish containing 10% HS and further triturated until disengaged into single myofibers.
12. Set the stereo dissecting microscope magnification so that the small pieces of connective tissue floating around in the suspension are visible and use a pair of forceps to pick them out. Continue until the myofiber suspension is clean of any connective tissue debris.
13. Triturate the myofiber suspension 10 more times using a 9" Pasteur pipette with a fire-polished tip to further separate small clumps of myofibers.

3.1.4. Further purification of FDB myofibers

1. Add 10 mL of 10% HS to each of the 3 glass Corex tubes.
2. Using the trimmed 100 μ l pipette tip, transfer the myofiber suspension to the top of the 10% HS column in the first Corex tube. Allow the myofibers to settle (at 1g) through the HS column for 15 min at room temperature (see **Note 15**). This step is important for purifying the myofibers from free mononucleated cells, debris and occasional broken myofibers.
3. As soon as the myofibers are settled, aspirate about 11 mL of the supernatant (leaving about 1-1.5 mL). Triturate the myofiber suspension gently with a 5" fire-

polished Pasteur pipette and transfer the suspension to the next Corex tube as described in step 2.

4. Allow myofibers to settle and transfer the myofiber suspension to the third Corex tube as in steps 2 and 3.
5. Allow myofibers to settle and harvest the final myofiber suspension. Following the third purification, the residual volume of medium to be left with the myofiber suspension depends on the number of culture dishes and the desired myofiber number per dish. Typically in our studies the volume of the final myofiber suspension is 300 μ l, which is sufficient for culturing 4-6 dishes.

3.1.5. Preparation of isotonic Vitrogen Collagen—Isotonic Vitrogen collagen can be prepared during the settling of myofibers. The isotonic mixture should be kept on ice. Stock Vitrogen is an acidic solution and when made isotonic it gels rapidly if not maintained at 4°C (*see Note 2*).

1. Place Vitrogen collagen stock bottle, 7X DMEM and one 15 mL conical tube on ice.
2. On ice: Add 1 volume of 7X DMEM and 6 volumes of Vitrogen to the 15 mL conical tube and mix gently. Calculate the volume of stock Vitrogen needed for the experiment based on using 120 μ l isotonic Vitrogen collagen to coat each 35-mm tissue culture dish. Use pH paper strips to ensure a neutral pH of the Vitrogen collagen in DMEM solution. The pH of this solution rises slightly after coating the culture dish. If the pH remains acidic after coating a test dish, add 1-2 drops of 1 M NaOH to the Vitrogen collagen in DMEM solution.

3.1.6. Coating culture dishes with isotonic Vitrogen collagen and myofiber culturing

1. On ice: Transfer 120 μ l of isotonic Vitrogen collagen to the center of a 35-mm culture dish and immediately use the L-shape spreader to coat the dish evenly.
2. Gently swirl the myofiber suspension (in the 15 mL tube) for even distribution of myofibers throughout the residual medium.
3. Remove one culture dish at a time from ice to allow rapid warming to room temperature.
4. Use a wide-bore 100 μ l micropipette tip to dispense about 50 μ l of the myofiber suspension per each culture dish.
5. Gently swirl the culture dish to allow even distribution of the myofibers.
6. Repeat steps 2-5, one dish at a time, for additional culture dishes.
7. Transfer the cultured dishes to the tissue culture incubator for a minimum of 20- 30 min to allow the formation of Vitrogen collagen matrix and the adherence of the myofibers to the matrix.
8. Remove dishes from the incubator. Gently add 1 mL of myofiber culture medium to each dish without agitating the myofibers and return dishes to the incubator. When the effect of growth factors on satellite cell proliferation/ differentiation is investigated, parallel cultures are maintained in myofiber culture medium with/ without additives and the medium is replaced every 24 h to ensure that growth factors do not become rate limiting. Except for harvesting myofiber cultures for early time points, cultures should be left undisturbed for the initial 18 h to allow good adherence of myofibers to matrix.

3.2. Isolation of single myofibers from the EDL muscle

3.2.1. Procedures prior to muscle harvesting

3.2.1.1. Preparation of Matrigel working mixture and coating tissue culture dishes with Matrigel

1. Thaw the required amount of stock Matrigel by placing frozen aliquots on ice for approximately 20 min. When diluted into the final working solution, a 200 μ l stock aliquot should be sufficient for coating at least four 24-well multiwell dishes.
2. Pre-chill a 15 mL conical tube on ice and transfer the thawed Matrigel into the tube. Add ice-cold DMEM to dilute the Matrigel to a final concentration of 1 mg/mL.
3. Place 3-4 drops of diluted Matrigel solution in the center of each of the 24-wells, using a glass 1 mL pipette.
4. Swirl the 24-well multiwell dish to allow even coating of the wells.
5. Allow the Matrigel-coated dish to sit at room temperature for 30 min in the tissue culture hood.
6. Transfer excess Matrigel solution from the wells back to the original tube with diluted Matrigel that is kept on ice. Use this Matrigel solution to coat additional dishes within the next 2 hours. Do not keep diluted Matrigel for re-use on subsequent days.
7. Incubate the Matrigel-coated multiwell dishes in the tissue culture incubator until the end of the enzymatic digestion period, but for at least 30 min.

3.2.1.2. Coating glassware and plasticware dishes with HS for the initial steps of myofiber isolation

1. Coat six plastic Petri dishes with undiluted filtered HS, prepared as described in item 3 under **Subheading 2.7**. Transfer 1 mL of HS to each Petri dish and swirl the dish to coat evenly.
2. Allow the dishes to sit with HS solution for 5 min at room temperature, then aspirate the HS and add 9 mL of DMEM to each Petri dish.
3. Incubate Petri dishes in the tissue culture incubator until needed following muscle digestion.
4. Coat the fire-polished Pasteur pipettes, prepared as described in items 1 and 2 under **Subheading 2.6.2**, with HS by passing 10% HS solution through the pipettes several times.

3.2.1.3. Preparation of the digesting enzyme solution: Prepare 0.2% (wt/vol) collagenase type I solution in 3 mL of DMEM and filter the solution into a 35-mm tissue culture dish using a 0.22 μ m syringe filter (see **Note 14**).

3.2.2 Dissection of EDL muscle

1. Euthanize one mouse according to institute regulations.
2. Shave the hind limbs and spray them lightly with 70% ethanol.
3. Secure the mouse, lying on its back, to the dissecting board by pinning down the hind limb to be operated on and the diagonal forelimb.

4. Use the straight rounded-tip scissors to cut through the skin, opening a small incision above the knee.
5. Holding the skin with fine forceps, insert the rounded-tip scissors beneath the incision and carefully open the scissors to loosen the skin from the underlying muscles.
6. Extend the incision to a point just in front of the digits.
7. Loosen the skin as you go, being careful not to cut the underlying muscles or blood vessels.
8. Cut and remove the skin from the knee to the paw.
9. Identify the 2 tendons at the origin of the EDL.
10. Use micro scissors to cut these tendons as far as possible from the muscle itself.
11. Identify the 4 tendons at the insertion of the EDL, each extending to one of the digits but not the toe.
12. Use the micro scissors to cut all 4 tendons.
13. Using fine forceps, gently pull the portion of the tendon before its division (to the 4 tendons) until the 4 tendons slide from the paw up to the ankle.
14. Grasp the 4 tendons and carefully pull them in order to remove the EDL muscle.
15. The EDL should slide underneath the TA muscle and should pull out easily. It is very important not to apply any force; if the muscle does not slide out easily, one or both tendons at the origin of the muscle may still be attached to the bone. In that case identify the attached tendon and cut it.
16. The muscle should only be handled by its tendons to prevent damage to the myofibers. Be careful not to injure the anterior tibial artery that supplies blood to the EDL, to avoid blood cell contamination of the myofiber preparation. In the upper third of its course, this artery lies between the tibialis anterior and EDL muscles (very close to the origin of the EDL muscle); in the middle third, it lies between the TA and extensor hallucis longus. The lower third of the artery starts at the ankle, crossing from the lateral to the medial side, lying between the tendon of the extensor hallucis longus, and the first tendon of the insertion of EDL muscle.

3.2.3 Enzymatic digestion

1. Holding the muscle by its 4 tendons, transfer it to the 35-mm tissue culture dish containing 0.2% collagenase I solution.
2. Place the dish on the low speed agitator located inside the incubator. Allow gentle and continuous agitation for collagenase digestion for 60 min (see **Note 14**).

3.2.4 Liberation of single myofibers from muscle bulk—Use a stereo dissecting microscope (placed inside tissue culture hood) throughout the procedure.

1. Inspect the muscle under the stereo dissecting microscope to make sure that the myofibers are loosened from the muscle bulk. If the myofibers are not loosened, continue enzymatic digestion for another 10 min and check again.
2. Retrieve two Petri dishes containing 9 mL of DMEM from the incubator. Use the widest bore Pasteur pipette to transfer the muscle from the collagenase solution to the DMEM to rinse away the collagenase.

3. Transfer the muscle to the second Petri dish for further dilution of any possible collagenase remains.
4. Use another wide-bore pipette (diameter ~2 mm) to triturate the muscle along its length. This orientation of the EDL muscle during triturations is critical to prevent myofiber breakage.
5. When single myofibers are liberated from the muscle, its diameter decreases. Therefore, use a narrower bore pipette for subsequent triturations.
6. When 20-30 viable single myofibers are released, transfer the muscle bulk to another DMEM-containing Petri dish and place the dish with the single myofibers in the tissue culture incubator. The transfer of the muscle bulk to a second dish ensures that the already released myofibers do not break during subsequent muscle triturations.
7. Repeat trituration and muscle bulk transfer to new Petri dishes until the desired number of viable isolated myofibers is acquired.

3.2.5. Culturing single myofibers in 24-well multiwell dishes

1. Transfer a Matrigel coated 24-well multiwell dish from the incubator to the tissue culture hood and open its lid to allow moisture, generated during the incubation period, to evaporate.
2. Bring two Petri dishes containing single myofibers to the tissue culture hood.
3. Use a 9" glass Pasteur pipette to lift one myofiber, with minimal residual medium, from the suspension and gently release the myofiber in the center of a well. Alternate between the two myofiber containing dishes in order to have both early and late isolated single myofibers in each 24-well multiwell dish.
4. After myofibers are dispensed to all 24 wells, look under the stereo dissecting microscope and make sure that indeed there is a myofiber in each well. This step is necessary since occasionally myofibers adhere to the Pasteur pipette and are not released to the well.
5. If needed, add a myofiber to any empty well.
6. Approximately 10 min after distributing myofibers, slowly add 500 μ l of warm culturing medium to each well, avoiding myofiber agitation.
7. Transfer the 24-well multiwell dish to the tissue culture incubator for a minimum of 18 h (overnight).
8. Repeat steps 1-7 until the required number of cultured myofibers is reached.
9. Additional 500 μ l of fresh culturing medium is provided to each well one week after culturing the myofibers. Then, to replenish the medium, every 3 days half of the medium is aspirated and 500 μ l of fresh medium is added.

3.3 Immunolabeling of satellite cells in FDB and EDL myofiber cultures

This section details current protocols used in our laboratory to fix myofiber cultures for immunofluorescence studies of satellite cells. FDB myofiber cultures are typically fixed with ice-cold methanol (the preferred fixative when working with Vitrogen collagen coated dishes). EDL myofiber cultures are typically fixed with paraformaldehyde warmed to 37°C. These protocols allow recovery of intact myofibers at the end of the fixation procedure. It should be noted that the ideal fixatives for FDB or EDL myofiber cultures are not necessarily the optimal fixatives for antigen detection. Thus, when analyzing single

myofibers via immunofluorescence, fixatives should be optimized for both preserving the myofibers and the antigens being analyzed.

Fixation protocols described in this section are also appropriate for detecting proliferating satellite cells in single myofibers by autoradiography following labeling with ^3H -thymidine (7,18) or when analyzing proliferation using bromodeoxyuridine (16,19).

3.3.1. Protocols for fixing and immunofluorescent staining of isolated single myofiber cultures

3.3.1.1. FDB myofiber cultures

1. Warm DMEM in a water bath set at 37°C.
2. Rinse cultures with warm DMEM three times. Following the final rinse add 1 mL ice-cold 100% methanol to each 35-mm tissue culture dish and transfer the dishes to 4°C for 10 min.
3. Return dishes to room temperature, aspirate the methanol and allow the dishes to air-dry for 10-15 min in the tissue culture hood (see **Note 16**).
4. Add 1.5 mL of blocking solution (TBS-NGS) to each culture dish, to block nonspecific antibody binding.
5. Cultures are then kept at 4°C for overnight or longer.
6. Dilute the appropriate primary antibody in the blocking solution.
7. Rinse the cultures three times with TBS-TW20.
8. Aspirate the final TBS-TW20 rinse and add 100 μl of the primary antibody solution for 1 hour at room temperature followed by an overnight incubation at 4°C in a humidified chamber (see **Notes 17, 18**).
9. Dilute the appropriate secondary antibody in the blocking solution.
10. Rinse cultures with TBS-TW20 three times.
11. Aspirate the final TBS-TW20 rinse and add 100 μl of the diluted secondary antibody for 1-2 h at room temperature.
12. Aspirate the secondary antibody and wash three times with TBS-T20.
13. For nuclear visualization, add 100 μl of DAPI solution (4',6-diamidino-2-phenylindole, dihydrochloride; Sigma-Aldrich, cat. no. D8417; stock concentration 10 mg/mL, working concentration 1 $\mu\text{g/mL}$ diluted in TBS-NGS prior to use) for 30 min at room temperature (see **Note 19**).
14. Rinse the cultures twice with TBS-TW20 followed by a final rinse with TBS.
15. Aspirate the TBS and mount in VECTASHIELD mounting medium (1 drop at the center of each culture dish or well) and cover with a cover slip. Mounting medium prevents the stained cultures from drying and retards fading of the immunofluorescent signal.

3.3.1.2. EDL myofiber cultures

1. Warm the needed volume of paraformaldehyde fixative solution in a water bath set at 37°C.
2. While observing each myofiber under the stereo dissecting microscope, use a pipette to gently, without agitating the culture or touching the myofiber, add

500 μ l of the warm paraformaldehyde-fixative solution to the culturing media of each of the 24 wells, for 10 min at room temperature.

3. Use a pipette to remove the parformaldehyde-fixative solution and rinse each well three times with TBS.
4. Add 0.5 mL of TBS-TRX100 for 5 min at room temperature.
5. Add 0.5 mL of blocking solution (TBS-NGS) to each of the 24 wells, to block nonspecific antibody binding.
6. Follow steps 5-15 as described under **subheading 3.3.1.1**.

Notes

1. For additional details about the FDB-muscle anatomy refer to:

<http://www.bartleby.com/107/illus443.html>

<http://www.bartleby.com/107/131.html>

For additional details about the EDL muscle anatomy refer to:

<http://www.bartleby.com/107/illus437.html>

<http://www.bartleby.com/107/illus441.html>

<http://www.bartleby.com/107/129.html>

We recommend these links as good resources for anatomical description and schematic images of the muscles although they refer to human muscles.

2. Vitrogen Collagen In Solution, is a sterile solution of purified, pepsin-solubilized bovine dermal collagen type I dissolved in 0.012N HCl and stored at 4°C until used (Cohesion Technologies, Palo Alto, CA). In our studies, Vitrogen collagen is made isotonic by mixing 6 volumes of stock Vitrogen collagen with 1 volume of 7x DMEM. The isotonic solution is prepared just prior to coating dishes because it gels rapidly at room temperature. To obtain consistent coating, the culture dishes should be pre-cooled and coated on ice. When removed from the ice, these dishes warm up rapidly and are ready for myofiber addition. Vitrogen Collagen In Solution is the recommended product and the use of collagen from other companies would require pre-screening to ensure compatibility.
3. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, entactin, and heparan sulfate proteoglycan (20). To ensure Matrigel stability, we follow the manufacturer's handling instructions and aliquot 200 μ l each into 2 mL cryogenic vials sealed with O-rings (Corning Incorporated, cat. no. 430488). These aliquots are stored at -20°C.
4. Falcon Primaria (BD Biosciences) 24-well multiwell dishes have typically been used for single myofiber isolation; however, we find that the standard, less expensive, Falcon 24-well multiwell dishes are as good.
5. Horse serum (HS) should be pre-selected by comparing sera from various suppliers. We select HS based on its capacity to support proliferation and differentiation of primary chicken myoblasts cultured at standard and clonal densities (21).

6. The Controlled Processed Serum Replacement 2 (CPSR-2; Sigma-Aldrich) that had been routinely used in our earlier studies (4,9,10,15,18) has been discontinued. The source of both the discontinued CPSR-2 and the currently available CPSR-3 is dialyzed bovine plasma; the discontinued product was further processed in a manner that also reduced lipids. The alternative serum replacement product contains bovine serum albumin, insulin and transferrin and its use for mouse myofiber cultures has been previously described (19).
7. Fetal bovine serum (FBS) should be pre-selected by comparing sera from several suppliers. We select FBS based on the capacity of the serum to support proliferation and differentiation of mouse primary myoblasts cultured at various cell concentrations. Only sera able to support growth and differentiation over a wide range of concentrations are employed in our studies. Primary myogenic cultures are prepared as described in references 4 and 22.
8. We prepare chicken embryo extract (CEE) in our laboratory using 10-day old White Leghorn embryos (23). The procedure is similar to a previously described method (24) but uses the entire embryo. We recommend this approach over purchasing CEE if the investigator can obtain embryonated chicken eggs, as the quality is higher and the cost lower than that of purchased CEE.
9. Preparation of chicken embryo extract:

All steps are performed in a sterile manner.

- a. Embryonated chicken eggs (8 dozen, White Leghorn; from Charles River) are maintained in a standard egg incubator (incubation conditions: a dry temperature of 38°C, a wet temperature of 30°C and relative humidity of 56%). The following egg incubator is well suited for basic research use: Marsh Automatic Incubator, Model # PRO-FI cat. no. 910-028, manufactured by Lyon Electric Company Inc. Chula Vista, CA.
- b. After 10 days, batches of 15-30 eggs are removed from the incubator and transferred into the tissue culture hood.
- c. Place the eggs lengthwise in the rack and spray with 70% ethanol to sterilize. Wait for several minutes until the ethanol evaporates.
- d. Crack open one egg at a time into a 150-mm Petri dish.
- e. Remove the embryo from surrounding membranes by piercing it with fine forceps. Rinse the embryo by transferring it through three 150-mm Petri dishes containing Minimal essential medium (MEM; Gibco, Invitrogen Corporation cat. no. 11095-080, MEM is supplemented with antibiotics as described for DMEM in **Subheading 2.7**). Swirl embryo a few times in each dish for a good rinse.
- f. Empty the egg remains from the initial 150-mm dish (described in step d) into a waste beaker and repeat steps d-f until the final rinse dish contains about 30 embryos.
- g. The embryos are transferred with fine forceps into a 60-mL syringe, forced through with the syringe plunger, and the suspension is collected into a 500-mL sterile glass bottle.
- h. The extract is diluted with an equal volume of MEM and gently agitated for 2 h at room temperature. To ensure good agitation, keep maximum volume to one-half bottle capacity.

- i.** The extract is frozen at -80°C for several days or a minimum of 48 h. It is then thawed, dispensed to sterile glass Corex tubes, and centrifuged at $15,000g$ for 10 min to remove particulate material.
 - j.** The supernatant is pooled, divided into 5 mL aliquots and kept frozen at -80°C until needed.
 - k.** Prior to use, the CEE should again be centrifuged at about $700g$ for 10 min to remove aggregates.
- 10.** Methanol is a colorless flammable liquid with an alcohol-like odor. Use nitrile gloves, safety goggles, and a fume hood when handling. It is important to refer to the MSDS instructions and institutional regulations for further information regarding storage, handling and first aid.
- 11.** Preparation of Tris buffered saline (TBS):

To make one liter of 10X TB:

 - a.** Weigh 60.5 gms of Tris-Base into a beaker.
 - b.** Add 700 mL deionized water to the beaker.
 - c.** Place the beaker on top of a magnetic stirrer.
 - d.** When the powder has dissolved, adjust the pH to 7.4.
 - e.** Add deionized water to bring the volume up to 1 liter, mix well and store at 4°C .

To make one liter of TBS:

 - a.** Weigh 8.766gr NaCl in a beaker
 - b.** Add 100 mL of 10X TB to the beaker and mix vigorously.
 - c.** When the powder has dissolved, add deionized water to bring the volume up to 1 liter; mix well and store at 4°C .
 - d.** Filter through a $0.45\ \mu\text{m}$ disposable filter unit (Nalgene, cat. no. 0001530020) into a bottle.
 - e.** Store at 4°C .
- 12.** Paraformaldehyde is a white powder with a formaldehyde-like odor. It is a rapid fixative and a potential carcinogen. When handling paraformaldehyde wear gloves, mask and goggles. It is important to refer to the MSDS instructions and institutional regulations for further information regarding storage, handling and first aid.
- 13.** Preparation of 100 mL of 4% paraformaldehyde with 0.03M sucrose in a fume hood:
 - a.** Mix 4 gms of paraformaldehyde powder and 80 mL of deionized water in a glass beaker; cover with parafilm.
 - b.** Warm the solution to 60°C with continuous stirring to dissolve the powder.
 - c.** Allow the solution to cool to room temperature.
 - d.** Add about 1-4 drops of 1N NaOH, until the opaque color of the solution clears.

- e. Add 10 mL 1M Sodium phosphate.
 - f. Adjust the pH to 7.2-7.4 using color pH strips.
 - g. Add 1.026 gms of sucrose.
 - h. Bring volume to 100 mL.
 - i. Filter through a 0.45 μ m disposable filter unit (Nalgene, cat. no. 0001530020) into a bottle.
 - j. Store at 4°C in an aluminum foil-wrapped bottle for no more than 1 month.
14. Collagenase concentration, as well as the optimal time for enzymatic digestion, should be adjusted for younger or older mice and for different strains of mice.
 15. The time required for the myofiber suspension to settle (at 1g) through 10 mL of 10% HS can vary between 5-15 min and the investigator should adjust this time. A prolonged period results in a preparation with more debris and remaining single cells released from the digested tissue. Depending on mouse age, the number of rounds of myofiber settling in the 15 mL glass Corex tubes, as well as the amount of medium in the tube, may also need to be adjusted.
 16. The tissue culture dishes are dry when the bottom appears opaque white.
 17. For some antibodies the cultures may be blocked for just 2-4 h at room temperature if overnight blocking is not desired.
 18. For even and continuous distribution of the antibodies (both primary and secondary), it is recommended to place the dishes on a 3-dimensional rotator (VWR Scientific Products, Labline Maxi Rotator, cat. no. 57018-500). It is especially important when staining myofibers in 24-well multiwell dishes since the antibody aliquots tend to rapidly accumulate at the well periphery, leading to uneven staining across the culture.
 19. DAPI is potentially harmful. Avoid prolonged or repeated exposure; we typically dissolve the entire powder in its original container and generate a concentrated stock solution. A ready-made DAPI reagent is available from Molecular Probes. It is important to refer to the MSDS instructions and institutional regulations for further information regarding storage, handling and first aid.

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Fig 1.

Phase and immunofluorescent micrographs of an isolated FDB myofiber with associated satellite cells undergoing myogenesis. Myofibers were isolated from a 3-month-old mouse and cultured in 35-mm tissue culture dishes coated with isotonic Vitrogen collagen. Cultures were maintained for 4 days in basal medium containing fibroblast growth factor 2 (FGF2, 2 ng/ml) and fixed with methanol as described under **subheading 3.3.1.1**. The culture shown in this figure was reacted via double immunofluorescence with a monoclonal antibody against myogenin which stains the nuclei of myogenic cells that have entered the differentiated step of myogenesis (panel C) and a polyclonal antibody against ERK1/ERK2 mitogen activated protein kinases (MAPK), which stains the cytoplasm of all fiber-associated cells (panel D). Reactivity with the monoclonal and polyclonal antibodies was traced with a fluorescein- and rhodamine- labeled secondary antibody, respectively. Parallel phase image (panel A) and DAPI staining image (panel D; both myofiber nuclei and satellite cell nuclei are stained) are shown as well. Arrows in parallel panels point to the location of the same cell. Additional immunopositive cells present on the myofiber are not shown, as not all positive nuclei or cells on the fibers are in the same focal plane. All micrographs were taken with a 40x objective. Additional details regarding the source of the antibodies and the rationale of using these antibodies are provided in our previous publications (10,15).

Fig. 2.

Phase micrographs of EDL myofibers depicting the temporal development of myogenic cultures from cells emanating from individual myofibers. Myofibers were isolated from 3 month- old mice and cultured individually in 24-well multiwell tissue culture dishes coated with Matrigel. Cultures were maintained in serum-rich/mitogen-rich growth medium and fixed with paraformaldehyde, as described under **subheading 3.3.1.2**. Satellite cells begin to emigrate from the myofiber within the first day in culture and continue to emigrate during subsequent days. Satellite cells that have emigrated from the myofibers proliferate, differentiate and fuse into myotubes, establishing a dense myogenic culture. Satellite cells remained attached to the muscle fiber during the first hours after culturing (panel A). Nineteen hours after culturing, 2-3 cells detached from the fiber but remained in close proximity to the fiber (panel B). Four days following culturing more cells are seen in the vicinity of the myofibers (only 4 cells shown in panel C). By day 7 progeny of satellite cells that emigrated from the myofiber have established a culture containing mostly proliferating myoblasts and some myotubes (panel D). Micrographs in panels A-C were taken with a 40x objective to show details of the few cells that emigrated from the myofiber, while the micrograph in panel D was taken with a 10x objective to show the establishment of a dense myogenic culture

Table 1

Characteristics of myofiber cultures from FDB and EDL muscles of adult mouse

Donor muscle	Flexor digitorum brevis (FDB)	Extensor digitorum longus (EDL)
Relative myofiber length	Short	Long
Number of fibers per culture dish	~50-100	1
Typical tissue culture dish	35-mm dish	24-well multiwell dish
Dish coating	Thick, gel-like layer of native collagen type I prepared from bovine dermal collagen (Vitrogen, Cohesion Technologies (9,10,15)). (see Note 2).	Thin coating of diluted, growth factor reduced Matrigel. Matrigel is a basement membrane preparation isolated from a mouse tumor (BD Biosciences; (11). (see Note 3).
Medium	DMEM-based, serum-rich / mitogen-poor; specific exogenous growth factors are added to study their effect on satellite cell activation, proliferation, and differentiation (9,10,15).	DMEM-based, serum-rich / mitogen-rich; medium can be modified to a serum-poor / mitogen-poor one to allow analysis of satellite cell activation (11,12,16).
Satellite cell profile after culturing	Satellite cells remain at the surface of the parent myofiber as they proliferate and differentiate. Satellite cells undergo a limited number of proliferative cycles and rapidly differentiate without fusing with the parent myofiber.	Satellite cells emigrate from the parent myofiber and undergo multiple rounds of proliferation, giving rise to an elaborate network of myotubes, resembling regular primary cultures of cells dissociated from whole muscle.
Summary	Cultures may model in vivo behavior of satellite cells in intact fibers during growth and routine muscle utilization. Cultures typically have been maintained short-term and employed for studies on recruitment of satellite cells into the cell cycle. Steps of proliferation and differentiation are highly synchronous (9,10). Cultures can be further used to study cells emigrating from the myofibers as described for the EDL fiber cultures.	Cultures may model events after muscle trauma where new myofibers are formed. Cultures typically have been maintained long-term and employed in studies of myogenic cells, progeny of satellite cells that emigrate from the myofiber to the myofiber surrounding (11). Cultures can also be used for analysis of molecular and cellular events associated with the first round of satellite cell proliferation, as in FDB cultures (16).