Differentiation of skeletal muscle and integration of myotubes with silicon microstructures using serum-free medium and a synthetic silane substrate

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This protocol describes a cell culture model to study the differentiation of fetal rat skeletal muscle cells. The model uses serum-free medium, a nonbiological substrate *N*-1[3(trimethoxysilyl)propyl] diethylenetriamine (DETA) and fabricated microcantilevers to promote the differentiation of dissociated rat myocytes into robust myotubes. In this protocol, we also describe how to characterize the myotubes on the basis of morphology, immunocytochemistry and electrophysiology. Here, four major techniques are employed: fabrication of cantilevers, surface modification of the glass and cantilever substrates with a DETA SAM, a serum-free medium and refined culture techniques. This culture system has potential applications in biocompatibility studies, bioartificial muscle engineering, skeletal muscle differentiation studies and for better understanding of myopathies and neuromuscular disorders. The model can be established in 26–33 d.

INTRODUCTION

Differentiation of skeletal muscle is a highly controlled, multistep process, during which single muscle cells initially freely divide and then align and fuse to form multinucleated myotubes. This process of muscle differentiation *in vivo* is governed by a complex interplay of a wide range of growth and trophic factors. Several such factors have been discovered, which have been observed to promote muscle differentiation *in vivo*^{1–3}. However, very little systematic research efforts have been undertaken to use this extensive *in vivo* knowledge of growth factors to develop a chemically defined medium that promotes muscle differentiation *in vitro*.

Most of the existing *in vitro* culture methods for studying skeletal muscle differentiation use serum-containing medium as well as a biological growth substrate^{4–6}. The presence of many unknown components in serum-containing medium and the technical difficulties in creating reproducible biological substrates has led to extensive variations in the results from experiment to experiment. In order to remove the inherent drawback of utilizing serum-containing medium and biological substrates, a defined culture system consisting of a serum-free medium based on the extensive knowledge of growth factors from *in vivo* studies and a synthetic silane substrate to study skeletal muscle differentiation was developed⁷.

The system has three features that differentiate it from all other previously reported systems. First, a unique chemically defined serum-free medium supplemented with specific growth factors was

developed to study the muscle differentiation process. Second, a synthetic, nonbiological, patternable^{8,9}, cell growth promoting substrate, *N*-1[3(trimethoxysilyl)propyl] diethylenetriamine (DETA), coated on glass coverslips^{10–12}, was used as a template to grow the skeletal muscle cells. Third, it was demonstrated that when the dissociated muscle cells were plated on fabricated microcantilevers, they aligned along the long axis of the cantilever to form aligned myotubes.

In this protocol, step-by-step instructions on how to fabricate and modify the cantilever and glass substrates and then grow the muscle cells on the chemically modified substrates using this serum-free medium, to form robust myotubes, is described. **Figure 1** shows a diagram of the general outline of the whole procedure, while **Figure 2** outlines the microcantilever manufacture process. Note that all fabrication procedures should be performed in a clean room. The protocol consists of two figures: **Figure 3**, which shows a microcantilever array micrograph, and **Figure 4**, which outlines the cell culture process; as well as substrate modification of either a cantilever or a glass substrate (Steps 1–22), preparation of the serum-free medium, as well as both intracellular and extracellular solutions (REAGENT SETUP), dissection of the skeletal muscle tissue (Steps 23–24), purification of the tissue and the culture of the myocytes to form myotubes (Steps 25–38), and characterization of the myotubes morphologically, immunocytochemically and electrophysiologically (Steps 39–40A–C).

MATERIALS

REAGENTS

- Charles River or Sprague Dawley, fetal rats age E18 **! CAUTION** Experiments involving live animals must conform to appropriate National and Institutional regulations.
- H₂SO₄ (EMD) **! CAUTION** Toxic and corrosive. Use gloves, goggles and other chemical use safety measures that are recommended by the appropriate concerned agencies.
- HCl (EMD) **! CAUTION** Toxic and corrosive. Use gloves, goggles and other chemical use safety measures that are recommended by the appropriate concerned agencies.
- Toluene (BDH) **! CAUTION** Toxic.

- Hydrofluoric acid (HF) (Sigma) **! CAUTION** Toxic.
- Diethylenetriamine trichmethoxysilane (United Chemical Technologies)
- AZ 5214E photoresist (Clariant Corporation)
- AZ 9245E photoresist (Clariant Corporation)
- AZ 400K photoresist developer (Clariant Corporation)
- Deep reactive ion etcher (PlasmaTherm)
- Ethanol
- 4',6-Diamidino-2-phenylindole (DAPI)
- Leibovitz medium (L15) (Invitrogen, cat. no. 11415064)
- Medium 199 (Invitrogen, cat. no. 11150059)
- B27 supplement (50×) (Invitrogen, cat. no. 17504044)

Figure 1 | General outline of the entire procedure.

- Basic fibroblast growth factor (b-FGF; Invitrogen, cat. no. 13256029)
 - Brain-derived neurotrophic factor (BDNF; Invitrogen, cat. no. 10908019)
 - Glial-derived neurotrophic factor (GDNF; Invitrogen, cat. no. 10907012)
 - Cardiotrophin-1 (CT-1; Cell sciences, cat. no. CRC700B)
 - Sodium bicarbonate (Fisher, cat. no. 5233500)
 - Embryonic myosin heavy chain (MHC) (F1.652, IgG; Developmental Studies Hybridoma Bank)
 - Primary antibody (mouse anti- α -actin, Sigma, cat. no. A2172)
 - Secondary antibody (Alexa Fluor 488-conjugated donkey antimouse; Molecular Probes, cat. no. A21202)
 - Citifluor-mounting solution (Ted Pella)
 - Clean glass coverslips 22 $\times$ 22 mm² no. 1 (Thomas Scientific, cat. no. 6661F52)
 - O₂ plasma cleaner (Harrick PDC-32G; Harrick)
 - DETA (United Chemical Technologies Inc., cat. no. T2910KG)
 - 0.1% (vol/vol) mixture of organosilane in freshly distilled toluene (Fisher, cat. no. T2904)
 - PBS (calcium- and magnesium-free) (GIBCO, cat. no. 14200075)
 - Trypsin-EDTA (GIBCO, cat. no. 25300054)
 - L15 medium (Invitrogen, cat. no. 41300021)
 - BSA (Sigma, cat. no. A3059)
 - Alexa Fluor 488-conjugated donkey antimouse antibody (Molecular Probes, cat. no. A21202), for immunocytochemistry only
 - Mouse anti- α -actin (Sigma, cat. no. A2172), for immunocytochemistry only
 - HEPES (Fisher, cat. no. BP310), for electrophysiology only
 - HEPES-sodium (Fisher, cat. no. BP410), for electrophysiology only
 - EGTA (Fisher, cat. no. O2783), for electrophysiology only
 - K-gluconate (Sigma, cat. no. G4500), for electrophysiology only
 - MgCl₂ (Fisher, cat. no. S320), for electrophysiology only
 - Na₂ATP (Fisher, cat. no. BP413), for electrophysiology only **▲ CRITICAL** Keep at -20 °C.
 - Antibody against embryonic MHC (F1.652, IgG, Developmental Studies Hybridoma Bank)
 - Serum-free medium (see REAGENT SETUP)
 - Intracellular solution (see REAGENT SETUP)
 - Extracellular solution (see REAGENT SETUP)
 - Absolute MeOH
 - KOH
 - Dry nitrogen
 - Saponin (permeabilization solution)
 - Fetal calf serum
 - Cy3-conjugated antimouse
- EQUIPMENT**
- Pyrex bottles (VWR)
 - Pyrex beakers (VWR)
 - 4-inch silicon-on-insulator (SOI) wafers (MXF)

PROCEDURE

Microcantilever fabrication and surface modification of microcantilevers and glass coverslips **● TIMING** 2–3 weeks

1 | Fabrication of microcantilevers (Figs. 2 and 3). Start procedure with a 4-inch double-sided polished SOI wafers that contain a 5- μ m thick top layer of crystalline silicon bonded onto a 500- μ m thick silicon dioxide layer.

- Hotplate (VWR)
- Ceramic racks (VWR)
- Glove box (MBraun)
- Thermometer (VWR)
- Dissecting instruments
- Microscope—regular and confocal
- Carbon dioxide incubator
- Contact angle goniometer (KSV Instruments, Cam 200 (or similar instrument))
- X-ray Photoelectron Spectrometer (Kratos Axis 165 (or similar instrument))
- Axioscope 2 FS plus upright microscope (Zeiss, Jena, Germany), for electrophysiology only
- PCS-5000 piezoelectric three-dimensional (3D) micromanipulator (Burleigh EXFO, Quebec, Canada), for electrophysiology only
- Gibraltar platform (Burleigh EXFO, Quebec, Canada), for electrophysiology only
- Vibration isolation table (TMC, Peabody, MA), for electrophysiology only
- Multiclamp 700B patch clamp amplifier (Axon/Molecular Devices, Sunnyvale, CA), for electrophysiology only
- Digidata 1320 A/D converter (Axon/Molecular Devices, Sunnyvale, CA), for electrophysiology only
- pClamp 9.0 software (Axon/Molecular Devices Sunnyvale, CA), for electrophysiology only
- P97 pipette puller (Sutter, Novato, CA), for electrophysiology only
- Electrode puller
- Inline solution heater (self-made but available from Warner Instruments, Hamden, CT), for electrophysiology only
- Balance (Mettler-Toledo)
- Osmometer (Fiske), for electrophysiology only
- Suss ACS200 wafer coater
- Suss MA6 aligner PlasmaTherm 770 DRIE etcher
- Metroline M4L plasma resist etcher
- Nitto Model 3195V thermal release tape
- Zeiss LSM 510 confocal microscope
- Countertop oven
- Borosilicate glass pipettes
- Dissecting hood
- Laminar flow hood
- Pasteur pipette
- pH meter

REAGENT SETUP

Serum-free medium Combine the 375 ml Leibovitz medium (L15), 125-ml medium 199, 10 ml B27 Supplement (50 $\times$), 10 ng ml⁻¹ of b-FGF, 1 ng ml⁻¹ of BDNF, 1 ng ml⁻¹ of glial GDNF, 10 ng ml⁻¹ of CT-1 and 0.70 g sodium bicarbonate. Adjust the pH of the medium to 7.3 with 1 N NaOH and the osmolality to 320–325 mOsm with distilled water.

Note: The components can be added all at once, in no particular order.

▲ CRITICAL Store in 50-ml sterile polycarbonate tubes at 4 °C. The medium will last for 2 months.

Intracellular solution Combine 0.15 ml HEPES-sodium salt (from a 1 M solution), 11.4 mg EGTA, 983.6 mg K-gluconate, 0.3 ml MgCl₂ (from a 200 mM stock solution), 30.7 mg Na₂ATP and 30 ml deionized water. Adjust the pH to 7.2 with HEPES, and adjust the osmolality to 276 mOsm, with distilled water. **▲ CRITICAL** The intracellular solution should be prepared in the given order. EGTA dissolves only at basic pH. Store the aliquot intracellular solution in 1 ml freezing vials at -20 °C.

Extracellular solution Take 50 ml of the Leibovitz medium (L15), check the pH and adjust to 7.34 with HEPES, also adjust the osmolality to 320 mOsm with distilled water.

Figure 2 | Outline of microcantilever manufacture procedures.

▲ CRITICAL STEP All fabrication procedures must be performed in the clean room.

2| Perform photoresist spinning and developing in a Suss ACS200 wafer coater/developer (or similar instrument). First, prime the device layer (5 μm silicon) with a 100 $\text{\AA}$ layer of hexamethyldisilazane (HMDS). Then place the wafer, silicon layer up, on a resist spinner vacuum holder. Center the wafer and apply the AZ5214e photoresist to the center of the wafer. Begin spinning the wafer to spread the resist to a final thickness of 2.1 μm (spin at 1,900 r.p.m.).

3| Bake the resist at 110 $^{\circ}\text{C}$ for 50 min.

4| Expose the wafer on a Suss MA6 aligner to the photomask containing the pattern for the cantilever. After exposure, develop the resist in a 1:1 mixture of AZ320 and dH_2O .

5| Perform a deep reactive ion etching (DRIE) in a PlasmaTherm 770 DRIE etcher (or similar instrument) at a rate of 4 $\mu\text{m min}^{-1}$. Etch the 5- μm device layer through to the silicon dioxide layer. Remove the photoresist using a Metroline M4L plasma resist etcher (or a similar instrument).

? TROUBLESHOOTING

6| Flip the wafer over and prime the silicon dioxide layer with a 100 $\text{\AA}$ layer of HMDS. Next, coat with AZ9245 photoresist to a final thickness of 10 μm (1,000 r.p.m.) and softbake at 100 $^{\circ}\text{C}$ for 2 min. Place the coated wafer in a Suss MA6 aligner (or similar instrument) and expose the resist to the mask containing the pattern for the back side pattern.

Note: The Suss MA6 aligner is capable of front/back alignment. For this process it is necessary to incorporate the alignment marks in the photomasks.

7| After exposure, immerse the wafer in AZ 400K developer to develop the patterned resist. This is followed by a hard bake at 120 $^{\circ}\text{C}$ for 30 min. Etch until approximately 50 μm etch depth is left.

8| Mount the wafer on a dummy wafer using Nitto Model 3195V thermal release tape. Complete the DRIE.

Figure 3 | SEM micrographs of microcantilever array. (a) Top down view of cantilevers. (b) 45 $^{\circ}$ angle view of cantilever array.

9| Demount the wafer by heating to 170 $^{\circ}\text{C}$ on a hotplate. Remove the photoresist by plasma etching in a Metroline M4L plasma resist etcher (or similar instrument). Perform the final etch in 49% HF for 10 min to remove the buried oxide layer.

! CAUTION HF is extremely toxic.

Surface modification of the cantilevers with DETA ● TIMING 2–3 d

10| Immerse the cantilever die in a 1:1 solution of absolute MeOH and concentrated HCl for 15–30 min, followed by a 3 $\times$ wash in dH_2O . Next, place the die in a bath of concentrated sulfuric acid for 30–45 min. Note that, at this point you need to ensure that the die are not touching each other as they may stick together during this step.

11| Gently rinse the cantilever die in dH_2O . Finally, boil the cantilever die in deionized water for 30 min. Then place the cantilevers in a 120 $^{\circ}\text{C}$ oven overnight.

Figure 4 | Defined system for growing skeletal muscle. Outline of the cell culture process.

12| Before the surface modifications, prepare all glassware and reagents for the reaction. Clean all glassware in a base bath (saturated KOH in 100% methanol) overnight and then rinse in dH₂O (18 MΩ resistance). Bake the glassware overnight in a 120 °C oven. Distill toluene over metallic sodium in order to ensure minimal water content.

▲ CRITICAL STEP This is necessary to minimize the presence of water in the reaction mixture. Note that excess water causes the polymerization of the silane monomer.

13| Transfer the desired volume of toluene to a clean/dry (see Step 2) Pyrex bottle. Before sealing the bottle, blow dry nitrogen into the unused volume in order to minimize the gaseous oxygen. Seal the bottle and place in the antechamber of the glove box. Evacuate the antechamber for 10 min and then vent with dry nitrogen, repeat three times. Transfer the toluene to the main chamber of the glove box.

14| Add stock DETA solution to the toluene for a final concentration of 0.1% (e.g., 0.2 ml in a final volume of 200 ml). Remove the 0.1% DETA solution from the glove box immediately and transfer into a glass beaker containing the cantilever die.

15| Place the reaction mixture on a hotplate and heat to approximately 80 °C for 30 min. After 30 min, remove the reaction mixture from the hotplate and cool for 30 min or until the reaction mixture can be handled with bare hands.

16| Rinse the die three times in clean/dry toluene. Transfer the cantilever die into a fresh beaker and cover with distilled toluene.

17| Place the beaker back onto the hotplate and heat to approximately 80 °C for 30 min. Finally, remove the cantilevers from the beaker and bake overnight at 120 °C.

Cleaning glass coverslips and subsequent surface modification with DETA

18| Clean glass coverslips (22 × 22 mm no. 1) using an O₂ plasma cleaner for 20 min at 400 mTorr.

19| Form the DETA film by reacting the cleaned surface with a 0.1% (vol/vol) mixture of organosilane in freshly distilled toluene.

20| Heat the DETA coated coverslips to just below the boiling point of the toluene, rinse with toluene, reheat to just below the boiling temperature, and then dry in an oven.

Characterizing the glass coverslips using contact angle measurements and x-ray photoelectron spectroscopy (XPS)

21| Characterize surfaces by contact angle measurements using an optical contact angle goniometer.

22| Use XPS to characterize the monolayer of DETA by monitoring the N 1s peak^{9,13}.

■ PAUSE POINT Samples should be stored in dessicator before use. DETA monolayers should be stable under anhydrous conditions for several weeks to months. Samples more than a couple of months old should be reanalyzed to ensure that the monolayer has not degraded.

Muscle cell isolation and culture: isolation of the myoblasts ● TIMING 4–6 h

23| Dissect the skeletal muscle from the hind limb thighs of a rat fetus at fetal rat age of E18.

24| Collect the tissue samples in a sterile 15-ml centrifuge tube containing 1 ml of PBS (calcium- and magnesium-free).

? TROUBLESHOOTING

25| Enzymatically dissociate the tissue samples using 3 ml of 0.05% of trypsin–EDTA solution for 60 min in a 37 °C water bath, with agitation of 100 r.p.m.

26| After 60 min, remove the trypsin solution and add 6 ml of L15 containing 10% FBS to terminate the trypsin action.

27| Mechanically triturate the tissue using a sterile narrow bore Pasteur pipette.

▲ CRITICAL STEP Please ensure that air bubbles are not formed, as formation of air bubbles damages the dissociating cells.

? TROUBLESHOOTING

28| Allow the dissociated tissue to settle for 3 min.

29| Transfer the 6-ml supernatant to a 15-ml centrifuge tube.

30| Repeat the same process (Steps 26–29) twice by adding 6 ml of L15 + 10% FBS each time.

31| Suspend the 18 ml cell suspension obtained after mechanical trituration on a 6 ml, 4% wt/vol BSA (prepared in L15 medium) cushion and centrifuge at 300g for 10 min at 4 °C.

32| Resuspend the obtained pellet in 10 ml L15 + 10% FBS and plate in 100-mm uncoated dishes for 20–30 min depending on the amount of tissue. During this step, remove the contaminating fibroblasts. The fibroblasts should settle down and attach to the bottom of the 100 mm dish faster than the myocytes. The supernatant should contain pure myocytes.

33| Gently remove the nonattached cells that are present in the supernatant after 20–30 min by using a sterile Pasteur pipette.

34| Transfer the supernatant to the top of a 6 ml 4% BSA cushion, and centrifuge at 300g for 10 min at 4 °C.

35| Resuspend the pellet in 1 ml of serum-free medium.

36| The cells are now ready for plating. At this point, conduct a cell count and plate the cells at a density of 500–800 cells mm⁻² on either coverslips or fabricated cantilevers.

37| Add the medium 1 h after plating. Maintain the cultures in a 5% CO₂ incubator (relative humidity, 85%).

38| Change half of the medium after every 4 d.

? TROUBLESHOOTING

Characterization of the myotubes: morphological quantification and fusion index

39| Quantify the myotube yield using the fusion index, which is defined as the number of nuclei contained in the myotubes divided by the total number of nuclei counted in a given microscope field.

40| The myotubes can now be immunostained for α -actin (option A) or embryonic myosin heavy chain antibody^{5,7} (option B) or characterized using electrophysiology (option C).

(A) Immunostaining myotubules for α -actin ● TIMING 2 d

- (i) Rinse coverslips in PBS, fix in cold ethanol (100%) for 20 min, and rinse again in PBS.
- (ii) Block the cultures by incubating in 5% BSA in PBS for 2 h at 4 °C.
- (iii) Add the primary antibody (mouse anti- α -actin, 1:800 dilution in blocking solution) and incubate for 12 h at 4 °C.
- (iv) Then add the secondary antibody (Alexa Fluor 488-conjugated donkey antimouse, 1:200 dilution in PBS) to the cultures and incubate for 2 h at 37 °C.
- (v) Rinse with PBS.
- (vi) Mount the coverslips with Citifluor-mounting solution onto slides.
- (vii) Visualize the coverslips, using a Zeiss LSM 510 confocal microscope; or a similar instrument could be used.

(B) Immunostaining myotubes with the embryonic myosin heavy chain antibody ● TIMING 2 d

- (i) Rinse coverslips with PBS.
- (ii) Fix the cells in -20 °C methanol for 5–7 min.
- (iii) After 5 min, gently wash the coverslips in PBS in order to remove the excess fixing agent and then further incubate in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution) for 5 min.
- (iv) Block the cells for 30 min with 10% goat serum and 1% BSA.
- (v) Incubate the cells overnight with the primary antibody against embryonic MHC (F1.652, IgG₁) diluted (1:5) in the permeabilization solution.
- (vi) Wash the cells with PBS and incubate with the secondary antibody (Cy3-conjugated antimouse, 1:200 dilution in PBS) for 2 h.
- (vii) After incubating in the secondary antibody for 2 h, the coverslips are finally rinsed with PBS and then the coverslips with Citifluor-mounting solution onto slides are mounted. Visualize the coverslips using a confocal microscope or similar instrument. Cultures used for immunostaining are simultaneously counterstained with DAPI, a classic fluorescent nuclear and chromosome counterstaining marker, and used to identify the nuclei and indicate the chromosome-banding patterns. DAPI selectively binds to double-stranded DNA (dsDNA) and, thus, shows little to no background staining in the cytoplasm. Immunostained coverslips can be stored for more than 1 year. The counting is done at the time of fusion index analysis of the success of the culture system. Generally, we perform this analysis soon after preparing the coverslips. Count nuclei in 20 randomly chosen microscope fields from a minimum of six separate muscle culture experiments.

Figure 5 | Phase pictures of myotubes. (a) Phase picture of 7-day-old myotubes. (b) Phase picture of a 7-day-old myotube with a patch-clamp electrode on its surface for electrophysiological studies.

Figure 6 | Immunostaining pictures of myotubes. (a) α -Actin immunostained picture of 7-day-old myotubes. (b) Embryonic myosin heavy chain stained picture of a 7-day-old myotube.

(C) Electrophysiological characterization of the myotubes using patch-clamp electrophysiology ● TIMING 1 d

- (i) Place the glass coverslips with the cultured skeletal muscle myotubes in a chamber on the microscope stage.
- (ii) Continuously perfuse the chamber (2 ml min^{-1}) with the extracellular solution through the inline heater (35°C) by gravity.
- (iii) Pull a glass pipette (three-step pull, heat = ramp value, pull = 0, velocity = 30, time = 200, pressure = 300) with the electrode puller and fill with the intracellular solution. The electrode resistance should be 6–8 M Ω .
▲ CRITICAL STEP Electrode resistance can be slightly influenced by the heat settings (± 10). If there are lesser or more than three steps, change the velocity value (± 5). Electrode properties are highly dependent on the properties of the glass tube. We prefer to use a 3-mm wide box heating filament (which heats more evenly) in the puller than the traditional u-shape filament.
- (iv) Place the electrode into the headstage; apply positive pressure (approximately 2 cm^3 from a 10 cm^3 syringe) to the pipette before touching the extracellular solution.
- (v) Bring the electrode close to the target cell; compensate for the pipette offset; touch a cell with the tip of the electrodes (seal position) under visual control.
- (vi) Apply a -5 mV seal test; compensate pipette capacitance.
- (vii) Apply a -70 mV holding (voltage clamp mode).
- (viii) Form a gigaseal.
- (ix) Rupture the cell membrane by the application of short suction pulses; measure resting membrane potential in $I = 0$ mode; compensate for whole cell capacitance and resistance.
- (x) Set the amplifier gain to 2 and apply a 2 kHz low-pass filter. Digitize signals at 20 kHz.
- (xi) Correct all membrane potential data by subtracting a 15 mV tip potential. This is calculated using the Axon's pClamp 8 program.
- (xii) Measure membrane resistance and capacitance using 50 ms voltage steps, from -85 to -95 mV , without any whole-cell or series resistance compensation followed by single-exponential fitting ($\tau = R_m C_m$).

Figure 7 | Seven-day-old pictures of the myotubes growing on the cantilevers. Myocytes are aligning along the long axis of the cantilever and forming contracting myotubes.

Figure 8 | Electrophysiological recordings. (a) Representative voltage clamp traces obtained after patching a 7-day-old myotube. (b) Representative current clamp traces obtained after patching a 7-day-old myotube.

- (xiii) Measure the sodium and potassium currents in voltage clamp mode using 10 mV voltage steps from a -85 mV holding potential. Measure the sodium channel current amplitude at -25 mV , where no potassium channel activation is observed. Measure the potassium channel current at $+15 \text{ mV}$ at the end of the voltage step, when sodium channels are inactivated.
- (xiv) Evoke action potentials with 1 s depolarizing current injections from a -85 mV holding potential.

● TIMING

From microcantilever fabrication through immunocytochemical and electrophysiological characterization:

Steps 1–8, microcantilever fabrication (days 1–14/21): 2–3 weeks
 Steps 10–22, clean and coat the microcantilevers and glass coverslips with DETA (days 14–21): 2–3 d
 Steps 23–40, cell culture including dissection, tissue isolation and plating (days 16–23): 4–6 h
 Steps 40A–C (days 26–33) electrophysiological characterization: 1 d; immunocytochemical characterization: 2 d; immunostaining myotubes for α -actin: 2 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible cause	Solution
5	A cloudy film developing on the cantilever die or on the glass coverslip	Polymerization of the <i>N</i> -1[3(trimethoxysilyl) propyl] diethylenetriamine (DETA) monomer; excess ambient humidity	Make sure all excess water is removed from the reaction mixture; ensure glassware is properly cleaned and oven dried (120 °C); ensure atmospheres are 60% relative humidity or less, if humidity is too high, perform the reaction in a controlled glovebox
23–24	Contamination of cells after plating	Contamination introduced before plating, possibly in the dissection	Before dissection, spray instruments with ethanol (70%); perform dissection under a hood; change gloves between dissection and culture of the cells
25–27	Difficult trituration	Tissue was not digested enough	Confirm water bath temperature is 37 °C; with regards to the enzymes—check the expiration date, confirm length of time in water bath before use (less than 1 h)
25–27	Low yield of cells with unnecessary debris in culture	Over-trituration or excessive air during trituration	Use a larger-bore pipette; reduce bubbles created in the process by being more vigilant
25–27	Low yield of cells with tissue pieces remaining intact	Under-trituration	Use a smaller-bore pipette
23–38	Unhealthy cells after plating	Wrong pH of medium, wrong temperature/atmosphere in incubator; over-trituration or digestion	Verify medium pH adjust to 7.4 as necessary; verify incubator temperature and CO ₂ pressure (37 °C and 5%); reduce trituration and digestion time

ANTICIPATED RESULTS

Glass coverslips and cantilevers coated with DETA are analyzed by contact angle and XPS. XPS has been shown to provide a good quantitative indicator of monolayer formation. The contact angle, along with XPS data, indicates that the surfaces are covered by a complete monolayer of DETA.

In the cultures, myoblast fusion begins after 24 h in the defined culture system and after 36–48 h maximal fusion is reached. Spontaneous contractions of the multinucleated myotubes are observed by the end of the second day (Fig. 5).

Immunocytochemistry combined with confocal microscopy and electrophysiological methods are then used to characterize the myotubes. Myotubes are observed to be labeled by the skeletal muscle markers for α -actin and the embryonic myosin heavy chain to facilitate unambiguous identification (Fig. 6).

In half of the cantilevers from each experiment, one will observe that dissociated muscle cells are aligned along the long axis of the cantilever to form contracting myotubes (Fig. 7).

Electrophysiological recordings from the myotubes indicate the presence of voltage-dependent inward and outward currents in voltage clamp mode. In current clamp mode, the myotubes fire single action potentials (Fig. 8).

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