

Supplementary information

Electrically Regulated Differentiation of Skeletal Muscle Cells on Ultrathin Graphene-based Films

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Experimental section

Materials

The used materials followed by their manufactures are listed as follows: penicillin/streptomycin (P/S) and PNIPAm (Sigma-Aldrich Chemical Co., USA); fetal bovine serum (FBS) (Bioserum, Japan); Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, MEM essential amino acid, MEM nonessential amino acid, and insulin (all from Invitrogen, USA); graphite powders (Alfa Aesar, USA); (Tokyo Chemical Industry, Japan).

Fabrication of GO

GO and TR-Graphene were prepared as detailed in our previous works.^[3-5] In brief, graphite powder was oxidized by KMnO_4 and H_2SO_4 at 0°C while stirring. The obtained mixtures were kept at 35°C for 2 h and slowly diluted with the de-ionized (DI) water. H_2O_2 was added to the solution, followed by filtering and washing with diluted HCl and DI water for several times. The obtained samples were dialyzed against DI water for over 1 week to remove residual ions in the solution and the obtained product was the graphite oxide. The graphite oxide was then sonicated in DI water for ~2 h to exfoliate it to single layer GO.

Fabrication of ultrathin GO and TR-Graphene substrates

Aqueous solution containing GO (~0.3 mg/mL, 80 μl) was spun or drop casted on a glass slide with a surface area of ~1 cm \times 1 cm. The solution deposited on the glass slide was kept untouched to obtain a dried and homogenous ultrathin GO substrate.

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The ultrathin GO substrates were then thermally reduced at 150 °C for 1.5 h to generate ultrathin TR-Graphene substrates. To obtain the TR-Graphene on the PNIPAm coated glass slide, the 5 wt% PNIPAm in isopropanol was spin-coated on the glass slide as described elsewhere^[6] prior to the GO deposition. The GO is then thermally reduced to prepare TR-graphene on PNIPAm using the same preparation method of TR-Graphene on glass slide.

Characterization of GO and TR-Graphene substrates

The GO and TR-Graphene substrates were analyzed using the MultiMode 8 atomic force microscope (Bruker Co., USA). A nanomechanical mapping technique using the atomic force microscopy (AFM) and based on the force-distance curve measurements as thoroughly described in our previous works^[7,8] was used to investigate the mechanical properties of the GO and TR-Graphene substrates at ambient conditions. The X-ray photoelectron spectroscopy (XPS) spectra of GO and TR-Graphene substrates were recorded by the PHI 1600 spectrometer. Both the electrical and field effect measurements were conducted by the Agilent 4156C Precision Semiconductor Parameter Analyzer (Agilent, USA) by using gold source-drain electrodes and highly doped silicon as the gate.

Cell culture

C2C12 myoblasts (American Type Culture Collection (ATCC), USA) were cultured in the high glucose-contained DMEM supplemented with 10% FBS and 1% P/S. The

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cells were trypsinized using trypsin/EDTA after reaching 70-80% confluency. The cells were kept at 37°C and 5% CO₂ atmosphere during the culture period.

Cell culture on the substrates

C2C12 myoblasts were seeded on substrates made from glass slide, GO, and TR-Graphene at a density of 5×10^4 cells/cm². Before the cell seeding, the glass slide substrates were rinsed with soap and water, followed by sonication with acetone and then ethanol both for 5 min. The GO and TR-Graphene substrates were also sterilized by UV irradiation for 1 h. Cell loaded substrates were then cultured after adding sufficient culture medium. After 1 day of culture, the growth medium was replaced with the differentiation medium, containing high glucose-contained DMEM supplemented with 2% horse serum, 1 nM insulin, and 1% P/S. During the culture period, the differentiation medium was replenished every 2 days.

Analysis of cell viability

Calcein AM/ethidium homodimer live/dead assay (Invitrogen, USA) was used according to the manufacturer recommendations to quantify the percentage of viable cells on the glass slide, GO, and TR-Graphene substrates. Calcein AM is a cell-permanent dye that is converted to the green fluorescent calcein for live cells as a result of intracellular esterases. Ethidium homodimer is a DNA-binding dye that enters damaged cell membranes and stains them as red color.^[9] The NIH ImageJ software package (<http://rsbweb.nih.gov/ij/>) was used to quantify the percentage of

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live cells, and at least five 10x magnified images of two replicated experiments were used for the cell viability analysis.

Quantification of nuclear shape index (nuclear circularity) and cell shape

After 2 and 4 days of culture, C2C12 myoblasts were fixed with 3-4% paraformaldehyde for 15 min, followed by washing with the phosphate buffered saline (PBS). The cells were then exposed to 0.3% Triton X-100 for 5 min at ambient temperature to make the cell membrane permeable. They were then treated with 5% bovine serum albumin for 15 min to block nonspecific bindings. The immunostaining procedure was done with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, USA) and phalloidin (AlexaFluor® 594, Invitrogen, USA) according to the manufacturer's instructions in order to reveal cell nuclei and filamentous F-actin, respectively. Nuclear shape index (nuclear circularity) and cell shape were quantified from the stained cells as reported in our previous work.^[10] The cell nuclei circularity is defined as $4 \times \pi \times \text{area}/\text{perimeter}^2$ of each individual cell nucleus. A circularity of 1 represents a perfect circular and non-elongated cell nucleus. Both circularity and cell shape parameters were calculated using the ImageJ software package. At least 200 cells for two independent experiments were considered to determine the nuclear circularity and cell shape parameters.

Characterization of C2C12 myotubes

The C2C12 myotubes were fixed and permeabilized as described in our previous works.^[11] The primary mouse monoclonal IgG antibody (ab-7784, Abcam®, Japan)

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was used to detect the fast skeletal myosin-positive cells as to incubate them at 4°C for 24 h with 1:1000 diluted antibody in PBS. The underlying samples were then washed 3 times with PBS and exposed to the 1:1000 diluted secondary goat anti-mouse AlexaFluor® 488 (Invitrogen, USA) in PBS and then kept at 37°C for 1 h. Fluorescence microscopy images of the stained myotubes were taken for the myotube quantification, and at least 50 cells were considered for this work. The myotube length was quantified using the AxioVision Rel. 4.8 software package, and the myotube coverage area was computed with the NIH ImageJ software package.

Electrical stimulation of C2C12 myotubes

On day 8 of culture, C2C12 myotubes on the glass slide, GO, and TR-Graphene substrates were electrically stimulated through the Pt electrodes placed in close proximity (1.5 cm apart) to the samples using a waveform generator (WF 1946B Multifunction Synthesizer; NF Co., Japan). The electrically stimulated samples were exactly placed in the middle of the electrodes. Therefore, the distance between Pt electrodes and substrate was about 0.25 cm. The electrical stimulation (ES) setup has been pictured and shown in our previous work.^[12] For the ES, the differentiation medium was switched to stimulation medium, including high glucose-contained DMEM with 2% horse serum, 1% MEM nonessential amino acid, 2% MEM essential amino acid, 1 nM insulin, and 1% P/S for both electrically stimulated and control samples. Electrical pulses were applied to muscle myofibers using the waveform generator under a specified regime (voltage 8 V, frequency 1 Hz, and duration 10 ms) for 2 days. The ES protocol was approximately the same ones used in our previous

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studies and it was confirmed as an efficient protocol to induce the differentiation of C2C12 myoblasts.^[10-12] The generated electric current was confirmed using an oscilloscope (wave surfer 424; LeCroy Co., Japan). In addition, high conductivity of stimulation medium ($\sim 1830 \text{ mS m}^{-1}$ as measured by the SG 3 conductimeter (Mettler Toledo, Zürich, Switzerland)) and simulation of applied electric field (Figure S3) confirmed the efficiency of ES setup. During the ES of C2C12 myotubes, the stimulation medium was changed every day to refresh the medium and decrease deleterious effects of collected charges in the medium.

RNA extraction and complementary DNA (cDNA) synthesis

The muscle cells were fixed using the liquid nitrogen and thoroughly ground with a mortar and pestle. The RNA was extracted using β -mercaptoethanol and purified in accordance with the manufacturer's protocol (RNeasy[®] microkit; Qiagen, Venlo, Netherlands). Reverse transcription was performed as recommended by the manufacturer (Quantitech Reverse Transcription; Qiagen, Venlo, Netherlands) for up to 3 μg of total RNA. The protocol and temperature profile to synthesize the cDNA were as follows: 12 μl of sample (3 μg of total RNA) was diluted with 14 μl of RNase-free water and 4 μl of gDNA wipeout buffer and incubated for 2 min at 42°C and then cooled down to 4°C. Quantiscript Reverse Transcriptase and Reverse Transcriptase primer mix were subsequently added and the mixture was kept for 15 min at 42°C, followed by the incubation for 3 min at 95°C. The samples were kept at 4°C until use for the quantitative PCR (qPCR).

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Real time PCR

All primer sets (*i.e.*, GAPDH, integrin, talin, vinculin, FAK, collagen type I, MRF4, sarcomeric actin, α -actinin, and MHC-II α /x) that were purchased from Operon Biotechnologies (Tokyo, Japan) were validated for qPCR experiments. The primer sequences are listed in the Supplementary information, **Table S1**. The real-time PCR was performed using Roche Lightcycler 1.5 (Roche, Mannheim, Germany) while 2 μ l of cDNA, 2 μ l of the primer set, and 14 μ l of Lightcycler FastStart DNA Master SYBR Green 1 (Roche, Mannheim, Germany) were used. Following an initial denaturation step at 95°C for 10 min, real-time PCR was performed over 45 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 20 s, followed by a melt curve analysis. The expression of target genes was assessed using the comparative method as to normalize to the mouse GAPDH gene as the internal reference.^[13] The reported gene expression values were the average of at least four independent experiments.

Statistical analysis

The independent Student's *t* test was used to reveal the statistical difference between two groups of data. All repeated experiments were quantified as average \pm standard deviation, and p-values less than 0.05 were considered statistically significant.

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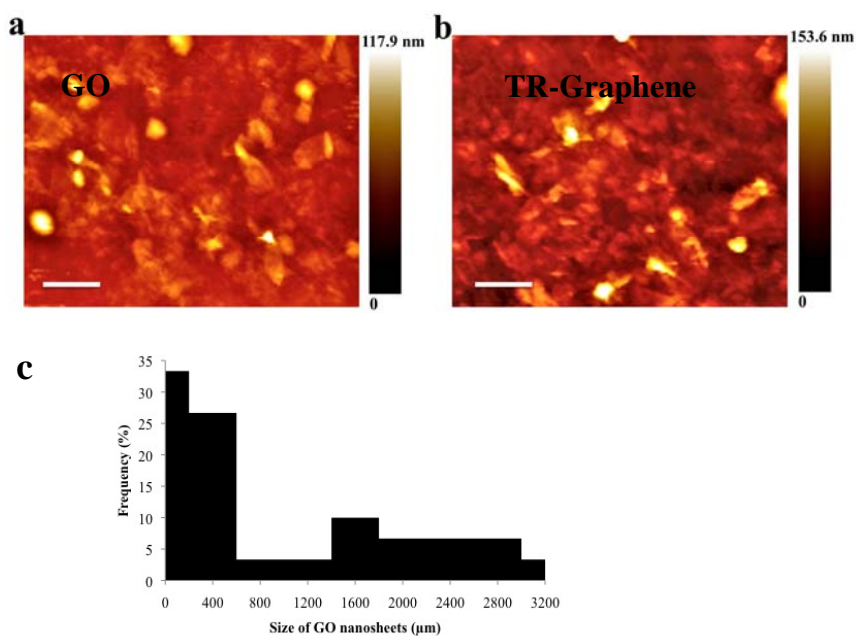


Figure S1. AFM observations of GO and TR-Graphene substrates at room temperature (a,b) and size distribution of GO nanosheets (c). Scale bars show 500 nm.

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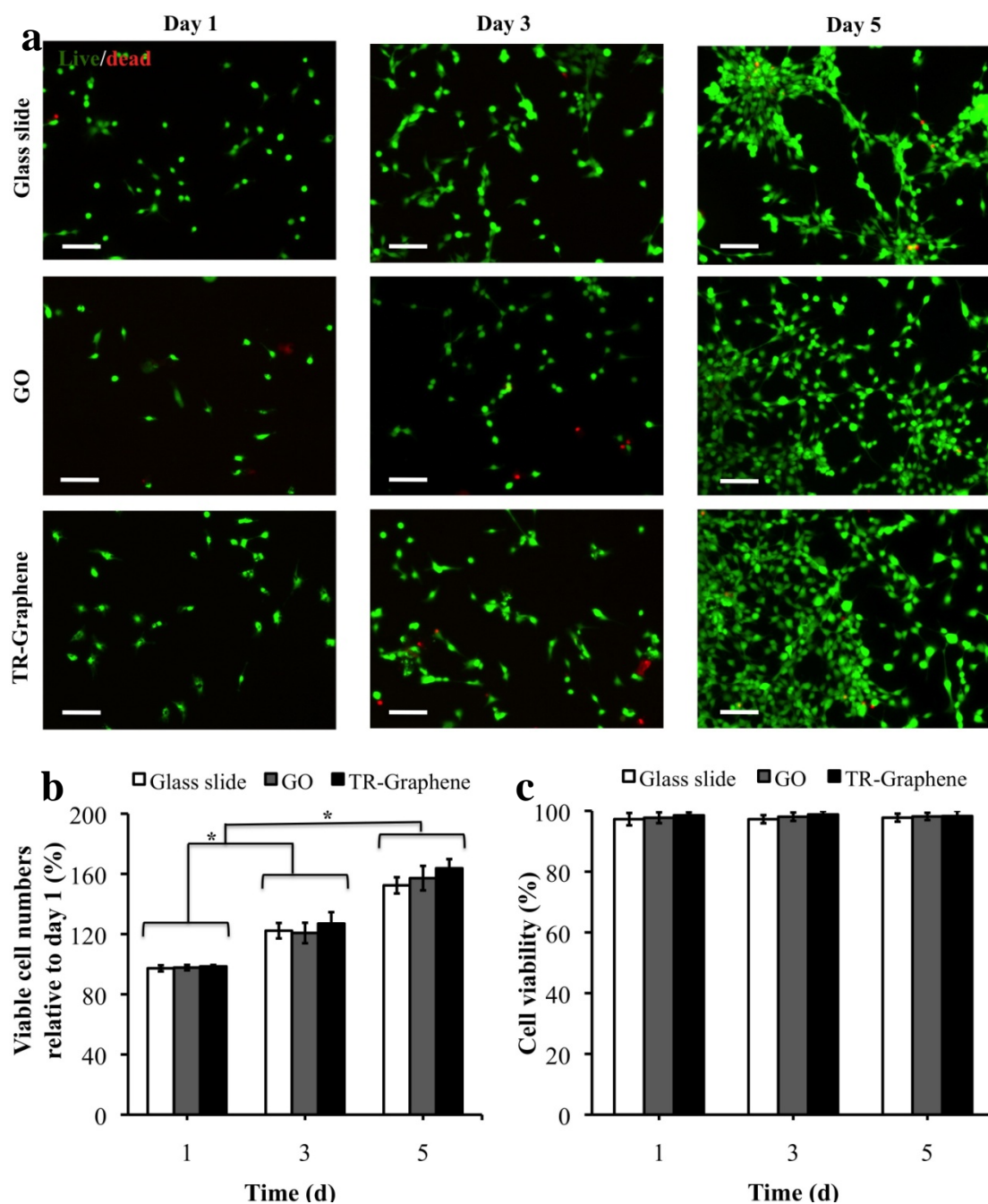


Figure S2. Biocompatibility and proliferation of C2C12 muscle cells on the glass slide, GO, and TR-Graphene substrates. **a**, Live/dead assay was used to quantify the viable cells and proliferation rate at different days of culture. The live and dead cells were stained as green and red, respectively. **b,c**, The cells proliferated on all underlying substrates (**b**) and there was no significant difference between the cell viability for the substrates at different culture periods (**c**). Scale bars in **a** show 50 μm (* $p < 0.05$).

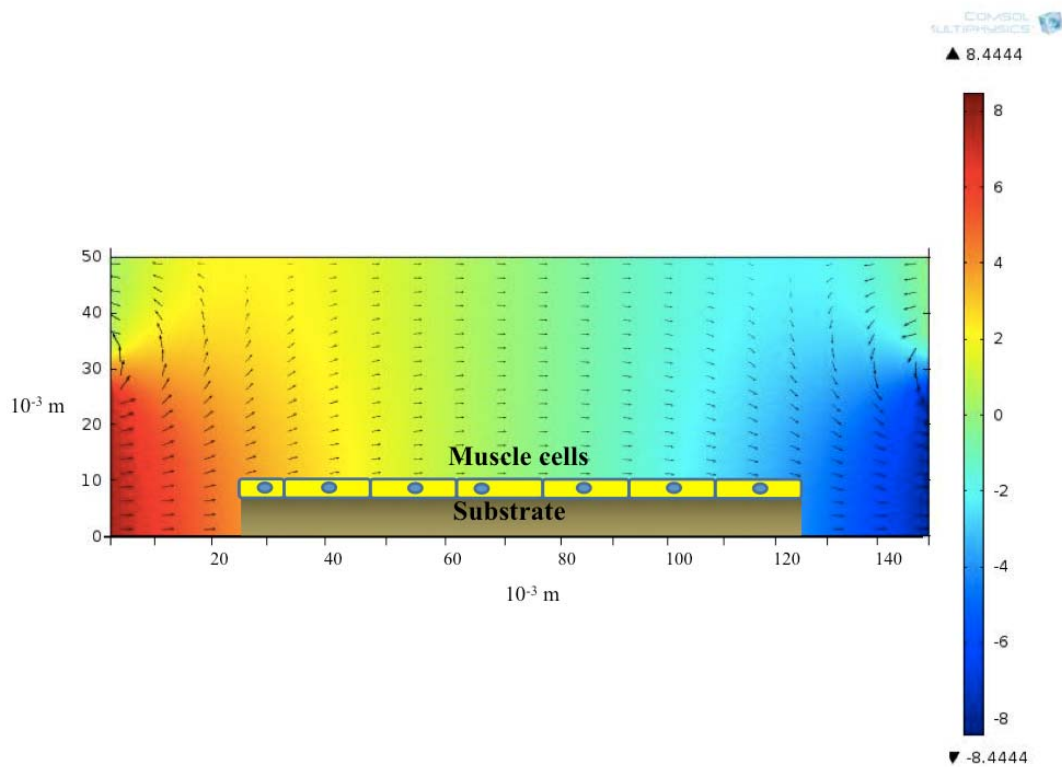


Figure S3. Cross-sectional view of the numerically calculated electric field (V/m) in Petri dish due to applied ES. The simulation data was obtained using COMSOL Multiphysics 4.2 software package. Arrows show current density.

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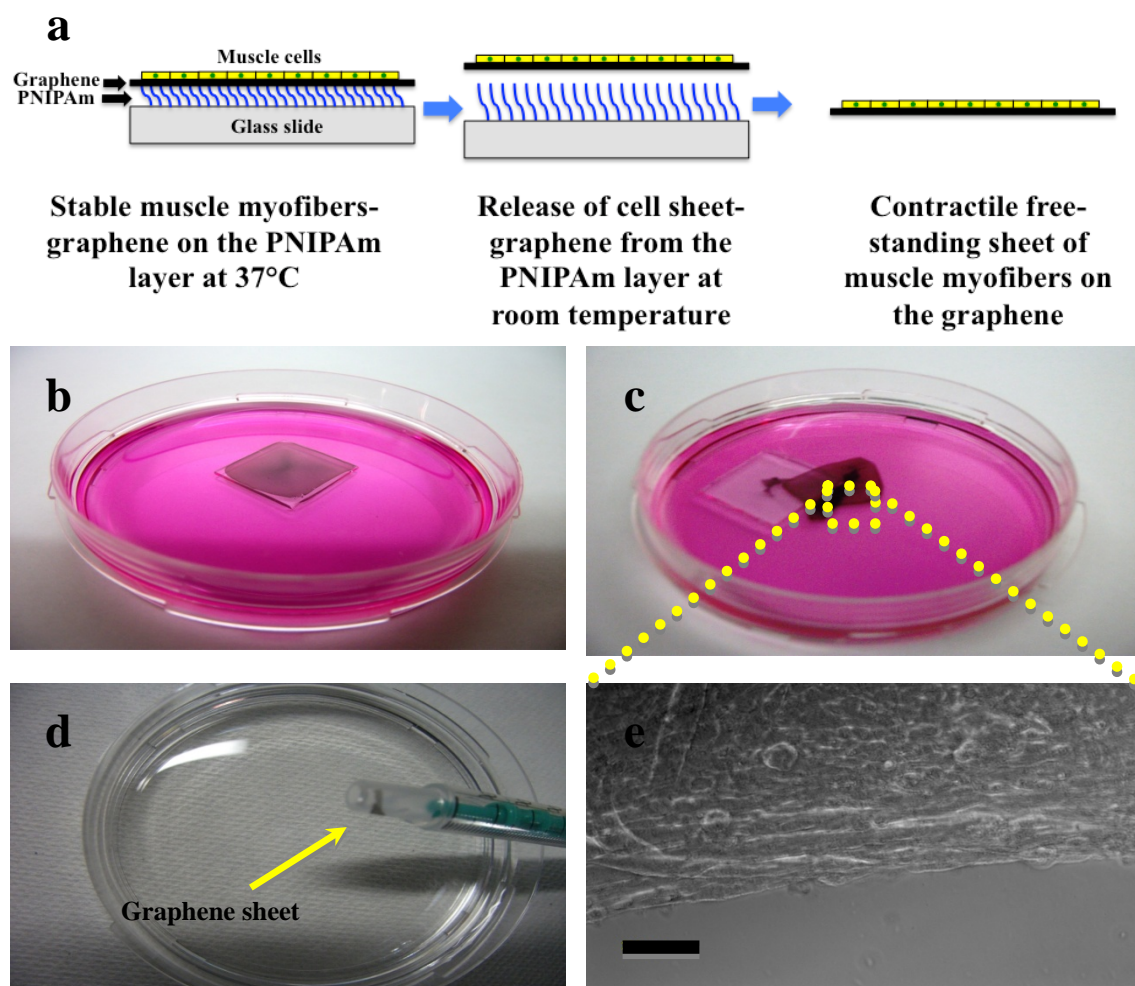


Figure S4. Fabrication and characterization of free-standing and contractile graphene-muscle myofibers. **a**, Schematic picture of fabricating free-standing muscle myofibers on the graphene substrate. Poly(N-isopropylacrylamide) (PNIPAM) was spin coated on the glass slide to provide a thermo-responsive substrate for the graphene-cell sheet. **b,c**, Fabricated C2C12 myotubes on the graphene sheet placed on the 60 mm Petri dish before the detachment (temperature 37°C) (**b**) and after the detachment at the room temperature (**c**). **d**, Highly flexible graphene sheet on a syringe. **e**, C2C12 myotubes as fabricated on the graphene sheet. Scale bar in **e** shows 400 μm .

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Table S1. Primers sequences used in this work.

No.	Name	Left Primer	Right Primer
1	Integrin ^[1]	5'-GACCTGCCTTGGTGCTGTGC-3'	5'- AGCAACCACACCAGCTACAAT-3'
2	Talin ^[1]	5'-GAAATTGAGGCCAAGGTCCG-3'	5'- GCCTTCAGTCGTCTGACTG-3'
3	Vinculin ^[1]	5'- AGCAGGAGTTGACTCACCAG-3'	5'-TCTAAGATCTGCCTGATGGC- 3'
4	FAK ^[1]	5'- GTAGTGAGCCAACCACCTGG-3'	5'- GCCCTTGTCTGTCAGGTAAC- 3'
5	Collagen type I ^[1]	5'-ACTGGTACATCAGCCCGAAC-3'	5'- GGTGGAGGGAGTTTACACGA- 3'
6	MRF4 ^[2]	5'-CGAAAGGAGGAGACTAAAG-3'	5'-CTGTAGACGCTCAATGTAG-3'
7	Sarcomeric actin ^[2]	5'-ATGGTAGGTATGGGTGTCAG-3'	5'-GATCTTCTCCATGTCGTC-3'
8	α -actinin ^[2]	5'-TCATCCTCCGCTTCGCCATTC-3'	5'- CTTCAGCATCCAACATCTTAGG-3'
9	MHC-II α /x ^[2]	5'-GCGACAGACCTCCTTCAAG-3'	5'-TCCAGCCAGCCAGCGATG-3'

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