Supplementary Information

Engineered "coffee-rings" of reduced graphene oxide as ultrathin contact guidance to enable patterning of living cells

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† Electronic supplementary information (ESI) available.

KEYWORDS: reduced graphene oxide, evaporative assembly, cell adhesion, cell guidance

Experimental Section

Preparation of rGO Solution: Expandable graphite (Grade 1721) was purchased from Asbury Carbon Co. Ltd. (USA). Concentrated sulfuric acid (H_2SO_4), potassium permanganate ($KMnO_4$), hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), and 1-methyl-2-pyrrolidinone (NMP) were purchased from Aldrich Co. Ltd (USA). All chemicals were used as received without further purification. Firstly, the GO was synthesized from expanded graphite according to modified Hummers method after thermal expansion of expandable using an Apex microwave oven (Preekem Scientific Instruments Co. Ltd., China) for 10 s at 300 W.[1] In general, 5 g of expanded graphite was gradually charged into the 500 mL of concentrated H_2SO_4 while stirring and keeping the temperature to 0 °C. Then, 30 g of $KMnO_4$ was slowly added while keeping the temperature below 20 °C. The mixture was stirred for 1 h and subsequently diluted with 5 L of deionized (DI) water followed by adding 50 mL of H_2O_2 (30 wt%). Then, the suspension was centrifuged and washed with 10% HCl solution and DI water in sequence to remove the metal ions. To prepare rGO solution, an aqueous GO suspension (10 mg mL^{-1} in DI water) was diluted with NMP to 1 mg mL^{-1} and was sonicated for 20 min to create a homogeneous suspension. Next, the solvothemal reduction of GO was carried out using microwave oven for 10 min at 800 W (Fig. S13). The rGO was washed with copious amount of DI water several times using centrifuge system, and then dispersed in DMF solvent in appropriated concentration.

Sphere-on-flat Confined Geometry: A hemispherical lens (R = 10.94 mm, D = 10.15 mm) made of fused silica, SiO_2/Si wafer, and glass coverslip were used as the upper and lower surfaces, respectively, to construct a confined geometry. The lens, SiO_2/Si, and glass coverslip substrates were cleaned with piranha solution (i.e., a mixture of hydrogen peroxide and sulfuric acid, volume ratio of 0.3/0.7), rinsed with DI water and dried by blowing nitrogen gas. To implement a confined geometry, a micro translation stage and inch-worm motor with a step motion of a few micrometers were used

to place the upper spherical lens into contact with the lower stationary flat substrate (i.e., glass or SiO_2/Si substrate); Before they contacted (i.e., separated by approximately a few hundred micrometers apart), a small amount of the aqueous rGO solution (25 µL) was loaded and trapped within the gap between two surfaces due to the capillary force as schematically illustrated in Figure 1, and then the upper spherical lens was slowly brought into fully contact with the lower stationary flat substrate by the inch-worm motor at a speed of 2 µm S^{-1} such that a capillary-held rGO solution forms with evaporation rate highest at the extremity. This leads to unstable stick-slip motion of the three-phase contact line, which moves toward the center of the spherical lens/Si contact during the course of solvent evaporation. All procedures were conducted in a sealed chamber to maintain constant temperature (~50 °C) during the evaporation process. The evaporation, in general, took 40 min to complete. Afterward, the two surfaces were separated.

Characterization: Optical microscopy (Olympus BX51) was performed in the reflective mode and the surfaces of the patterned rGO with different dimensions were imaged by atomic force microscope (AFM, Park Systems, XE-100) in non-contact mode. Microscopic images were captured via field-emission scanning electron microscopy (SEM, S-4800, Hitachi) operated at an accelerating voltage of 5-10 kV.

Cell Cultures and Conditions: A murine fibroblast cell line (L-929 cells from subcutaneous connective tissue) and a mouse myoblast cell line (C2C12 cells from muscle) were purchased from the American Type Culture Collection (ATCC® CCL-1™ and CRL-1772™, respectively; Rockville, MD). A mouse hippocampal neuronal cell line (HT-22 cells, sub-line derived from their parent HT-4 cells) is immortalized with a SV40 antigen but express neuronal properties.[2] L-929, C2C12 and HT-22 cells were all routinely maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% antibiotic antimycotic

solution (including 10,000 U penicillin, 10 mg streptomycin, and 25 μg amphotericin B per mL, Sigma-Aldrich) at 37°C under a humidified atmosphere of 5% CO_2 in air.

Cell Proliferation Assay: Cell proliferation was measured by using a cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan), which contains a highly water-soluble tetrazolium salt [WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] reduced to a yellow-color formazan dye by mitochondrial dehydrogenases. Each suspension of L-929 and C2C12 cells was seeded at a density of 2×10^5 cells mL^{-1} on either bare or rGO-patterned glass substrates lying in a 48-well plate and then incubated for up to 7 days. After incubation, all cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS, Gibco BRL, Rockville, MD) and then incubated with a WST-8 solution for 4 h in a CO_2 incubator at 37 °C. To examine the proliferation of cells cultured on bare and rGO-patterned substrates, the absorbance was measured at 450 nm using an ELISA reader (SpectraMax® 340, Molecular Device Co., Sunnyvale, CA). Each cell proliferation profile was determined as the percentage ratio of the optical density in the cells (incubated on rGO-patterned substrates at each time point) to that on bare glass substrates at day 1.

Analysis of L-929 Cell Responses on Micropatterned rGO by Fluorescence Staining: After pre-determined incubation period, the cell responses of L-929 fibroblasts cultured on the patterned arrays of rGO rings were imaged with a fluorescence microscope (Olympus IX81-F72) and images were post-processed by ImageJ software (National Institutes of Health, Bethesda, MD). Briefly, the cells were washed three times with DPBS and then fixed by 75% ethanol for 20 min, and further washed twice with DPBS. After washing, the cytoplasm of cells was stained with 5 μM of 5(6)-carboxyfluorescein diacetate (cFDA, Sigma-Aldrich) before 4 h of observation, followed by counterstaining for the nuclei with 1 μM of 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 20 min prior to imaging.

Analysis of Alignment and Myogenic Differentiation of C2C12 Cells on Substrate with rGO Micropatterns by Immunocytochemistry: To examine the alignment of myotubes, C2C12 myoblasts were cultured on the substrate with patterned rGO arrays for 5 days. After incubation, the cells were fixed with a 4% paraformaldehyde (Sigma-Aldrich) in DPBS for 10 min and immersed in 0.1% Triton X-100 (Sigma-Aldrich) for 5 min to increase the permeability of cell membranes. Subsequently, the cells were blocked with a 2% bovine serum albumin (BSA, GenDEPOT, Barker, TX) solution in DPBS for 30 min. A mouse monoclonal antibody (Ab) to α-smooth muscle actin (SMA), conjugated with FITC isomer I, (clone 1A4, 1:240 in DPBS; Sigma-Aldrich) was added to the cells overnight at 4 °C. The nuclei were counterstained with 1 μM propidium iodide (PI, Sigma-Aldrich) for 20 min prior to observation. The immunofluorescence images of anti-SMA staining were analyzed under 40 and 200 magnifications and measured by ImageJ software (5 × 20 random measurements per image).[3] For myotube orientation, the value of 0° denoted parallel alignment from the axis of the rGO pattern and 90° represented perpendicular alignment. After 7 days of culture on the substrate with rGO micropatterns, the myogenic differentiation of C2C12 myoblasts was examined by immunocytochemistry. In brief, the cells were fixed and blocked with the same method as mentioned above. A mouse monoclonal Ab to myosin heavy chain (MHC), conjugated with Alexa Fluor 488, (clone MF20, 1:200 in 1% BSA in DPBS; eBioscience Inc., San Diego, CA) was added to the cells overnight at 4 °C. The nuclei were counterstained with 1 μM PI for 30 min prior to observation. The quantitative analysis of myogenic differentiation was conducted by analyzing the fusion and maturation indices. The fusion index was obtained by calculating the percentage of the number of nuclei within the multinucleate myotubes containing more than two nuclei to the total number of nuclei. The maturation index was calculated as the percentage of myotubes containing more than five nuclei to the total number of myotubes.[4] On the other hand, the cellular responses and behaviors of C2C12 myoblasts on the substrate with patterned rGO arrays were evaluated at 1 and 3

days of culture by immunofluorescence staining with FITC-conjugated anti-SMA and PI as mentioned above.

Western Blotting: After incubation on the substrate with patterned rGO arrays for 7 days, C2C12 myoblasts were washed twice with cold PBS (10 mM, pH 7.4) and ice-cold RIPA lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was then added. After 5 min, the cells were scraped, and the lysate was centrifuged at 14,000 × g for 20 min at 4 °C. The proteins were extracted from the total lysate, and the protein concentration was determined using a BCA™ protein assay according to the manufacturer's protocol (Pierce, Rockford, IL). For immunoblot analysis, equal amounts of protein (40 μg) were run on a 4/20 polyacrylamide–SDS gel (Daiichi Pure Chemicals Co, Ltd, Tokyo, Japan) for 1 h at 30 mA and blotted onto a PVDF membrane for 50 min at 35 mA. The blots were blocked in a blocking buffer (Nacalai Tesque Inc., Kyoto, Japan) for 1 h at room temperature and incubated with rabbit polyclonal anti-MyoD and mouse monoclonal anti-myogenin Abs at a 1:500 dilution. As a reference, a mouse monoclonal Ab to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, MA) was used at a 1:5,000 dilution overnight at 4 °C. The blots were incubated with either goat anti-rabbit (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or goat anti-mouse (Amersham Biosciences, Buckinghamshire, England) secondary Ab, horseradish peroxidase-conjugated at 1:2,000 dilution. Protein expression was detected using a Chemilumi-one chemiluminescent kit (Nacalai Tesque Inc.) and X-ray film (Fujifilm, Tokyo, Japan).

Analysis of HT-22 Cell Responses to rGO Micropatterns by Immunofluorescence Staining: For observing neuronal cell responses to the rGO micropatterns, HT-22 cells (low density of initial seeding, 1 × 10^4 cells mL^{-1}) were fixed and blocked with the same method as mentioned above. The cells were incubated with a rabbit polyclonal anti-neurofilament M (NF) Ab (1:200 in DPBS; Chemicon, Temecula, CA), followed by incubation with a fluorescein anti-rabbit IgG (1:250; Vector

Laboratories, Burlingame, CA) for 1 h in the dark at room temperature.[5] The nuclei were counterstained with 1 µM PI for 30 min prior to imaging.

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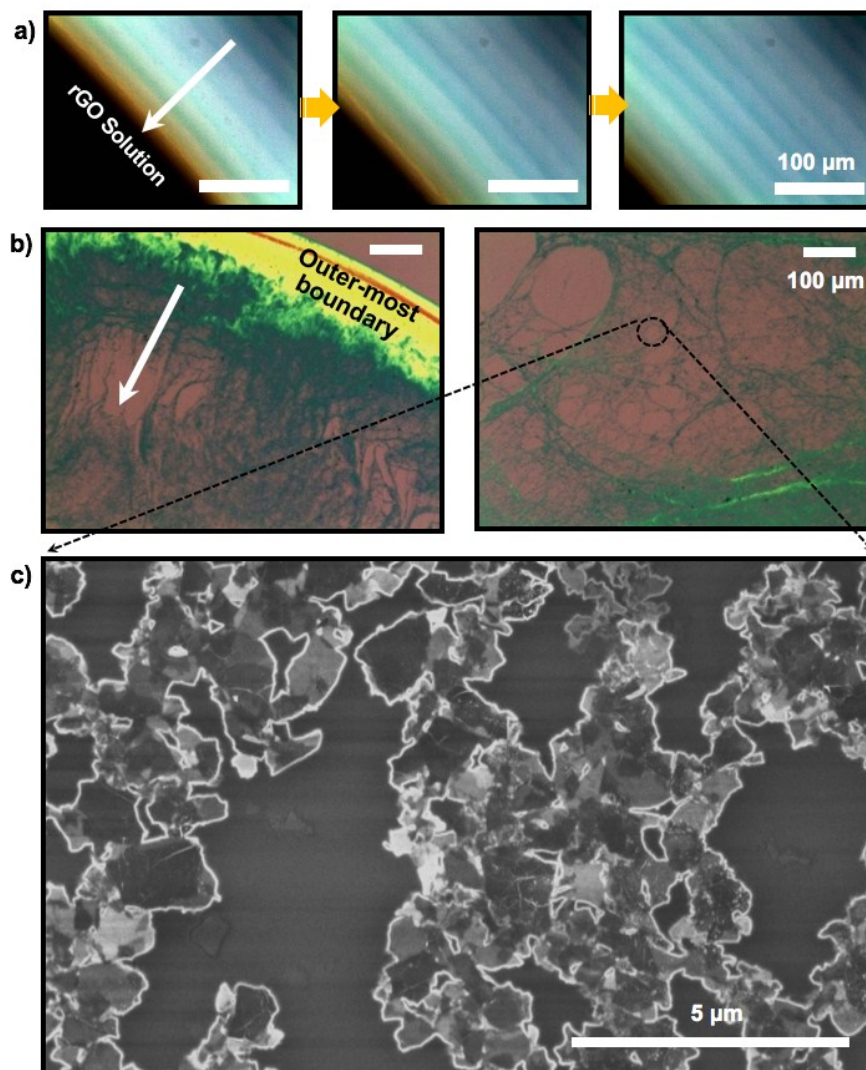
Fig. S1. (a) Real time monitoring of the formation of rGO ring arrays: stepwise optical micrographs from the in-situ observation represent the consecutive stick-slip motions of rGO solution in a sphere-on-flat confined geometry. (b) Optical micrographs from the result of evaporated rGO solution on a single substrate (Si/SiO$_2$) without the use of confined geometry (a white arrow indicates the direction of moving meniscus): highly concentrated rGO solutes migrated to the outer-most region forming a thick ring-like structure (left), and the randomly distributed aggregates of rGO sheets at the inside of a droplet (right). (c) Magnified SEM image corresponding to the marked area.
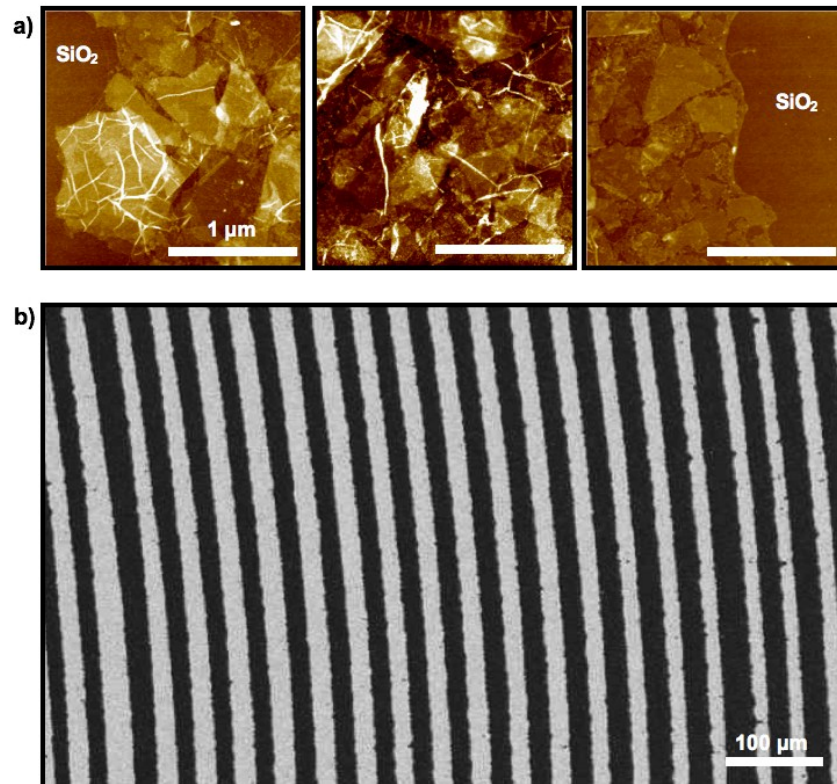
Fig. S2. (a) The magnified AFM images of a singular rGO stripe at the different regions corresponding to the marked area shown in Fig. 2h. (b) SEM image of radially patterned of highly regular rGO ring arrays over large surface coverage with excellent uniformity.
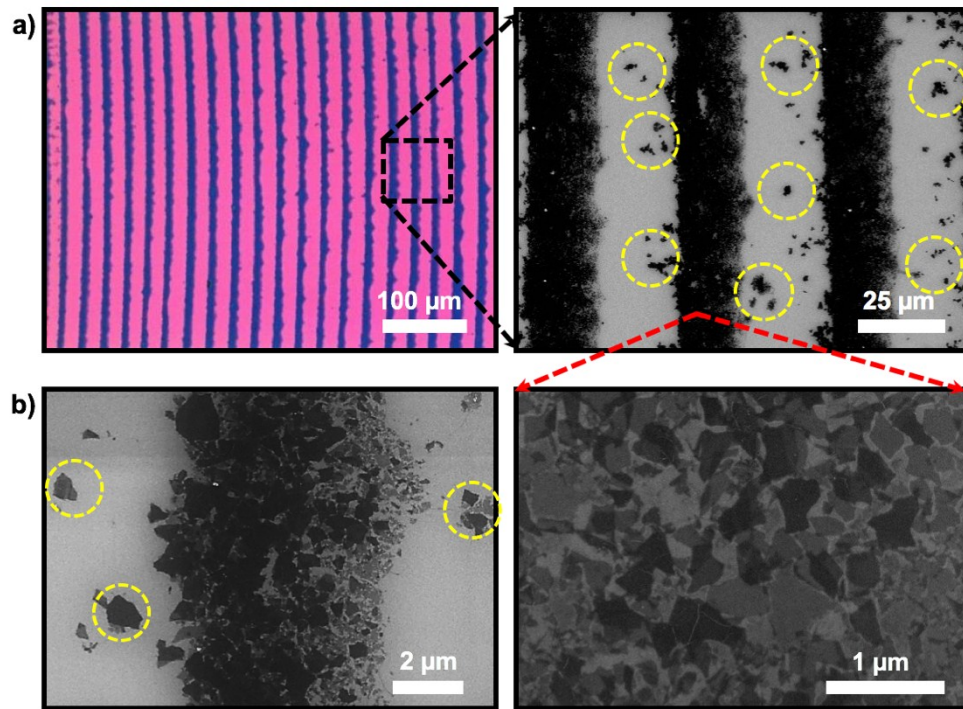
Fig. S3. Evaporation of rGO solution (c = 25 μg/ml) at room temperature in a sphere-on-flat confined geometry. (a) Optical and SEM micrographs of rGO ring arrays formed on a Si/SiO$_2$ substrate with some residues of rGO sheets (dotted-yellow circles) in-between the adjacent rGO stripes. (b) The magnified SEM micrographs of rGO residues with a rGO stripe pattern (left), and the overlapped rGO mosaics at the center region of a rGO stripe (right).
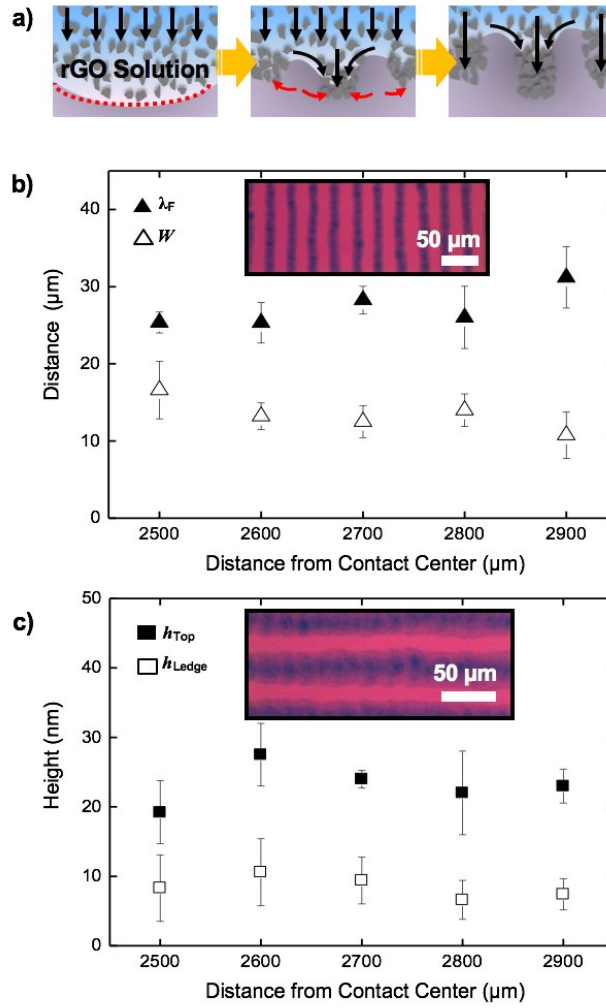
Fig. S4. Geometrical configuration of the rGO-spokes. (a) Stepwise schematic illustration of spoke pattern formation of rGO resulted from the fingering instabilities at the edge of drying solution front. (b) The graph related to the distance between adjacent rGO spokes (λ_F, center-to-center distance) and the width (W) of spokes plotted as a distance from the contact center. Inset: a representative optical micrograph of rGO spokes at the intermediate region. (c) The graph of heights plotted as different region of spokes as a distance from the contact center. Each blue and green arrow shown in Fig. 3f indicates h_{Top} and h_{Ledge}, respectively. Inset: a representative optical micrograph of rGO spokes at the outer-most region.
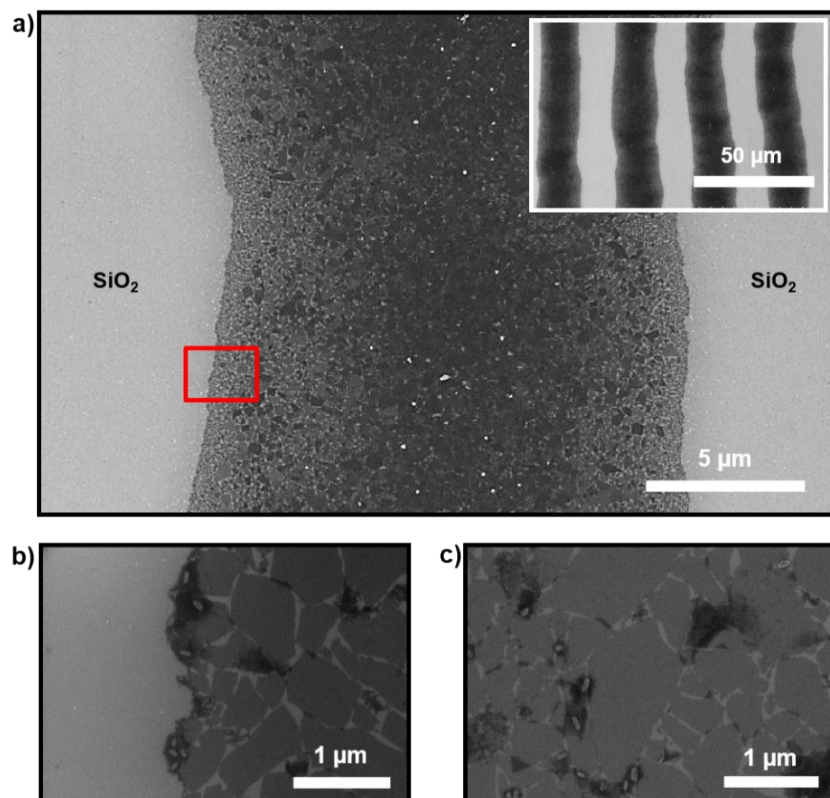
Fig. S5. SEM images of the rGO spokes. (a) a singular rGO spoke formed on Si/SiO$_2$ surface that magnified from the inset SEM image that locally shows regularly arrayed rGO stripe patterns. (b, c) Highly magnified SEM images of individual rGO sheets showing slightly overlapped thin film in a face-to-face mode corresponding to the boxed area shown (a).
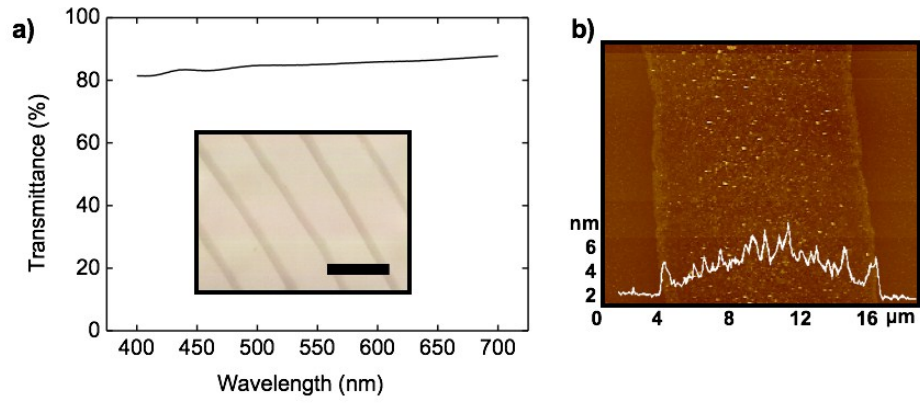
Fig. S6. (a) Transparency and optical micrograph of stripe patterned rGO arrays on a grass coverslip (inset). Scale bar is 100 μm. (b) Typical AFM image of a rGO patterned stripe with height profile.
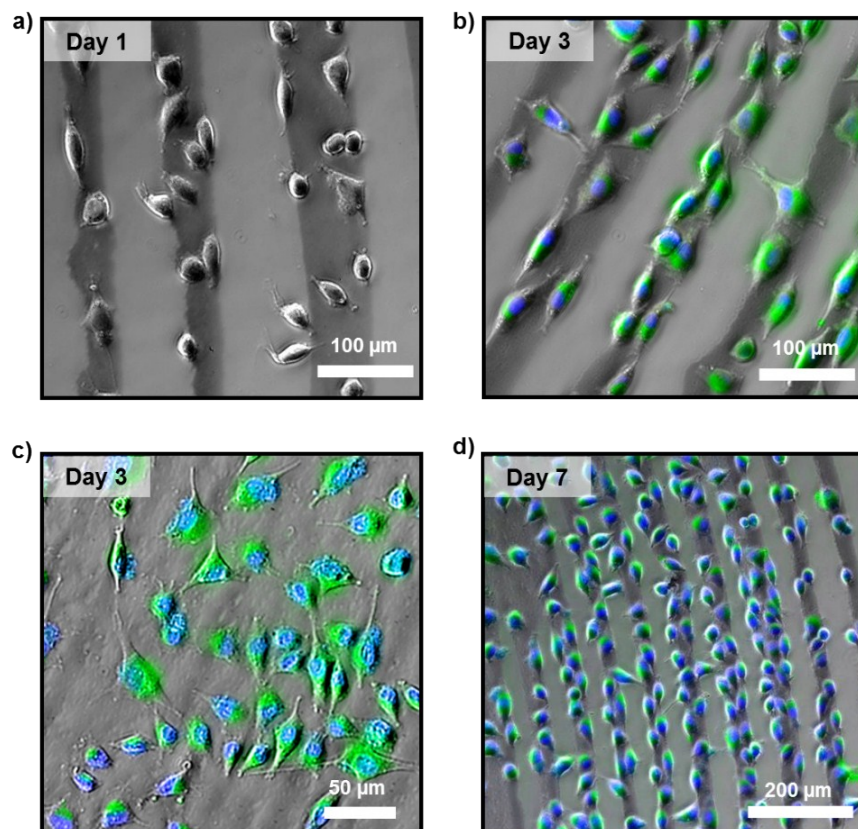
Fig. S7. Fibroblast behaviors on the patterned rGO ring arrays on a glass coverslip substratum that displays a high pattern fidelity and topological integrity of assembled rGO rings. (a) Representative optical micrograph of L-929 fibroblasts on the patterned rGO stripes at day 1 of culture. (b, c) Fluorescent merged images of L-929 fibroblasts on (b) the patterned rGO arrays and (c) the continuous thin film of rGO on a glass coverslip substratum at day 3 of culture. (d) Fluorescent merged images of L-929 fibroblasts on the patterned rGO stripes at day 7 of culture. The cytoplasm of cells was stained with cFDA (green), and cell nucleus was counterstained with DAPI (blue).
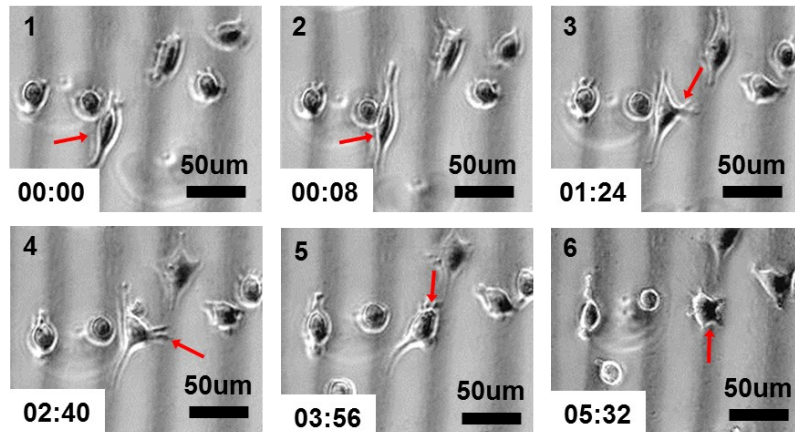
Fig. S8. Consecutive time-lapse recorded images of L-929 fibroblasts on the rGO patterned stripes (time span of 30 min). A single motile cell (marked by the red arrow) jumped around adjacent rGO stripes: (left to right) from the second (1-4) to the third rGO stripe (5, 6) by projecting its cytoskeletal actins riding on the rGO stripes.
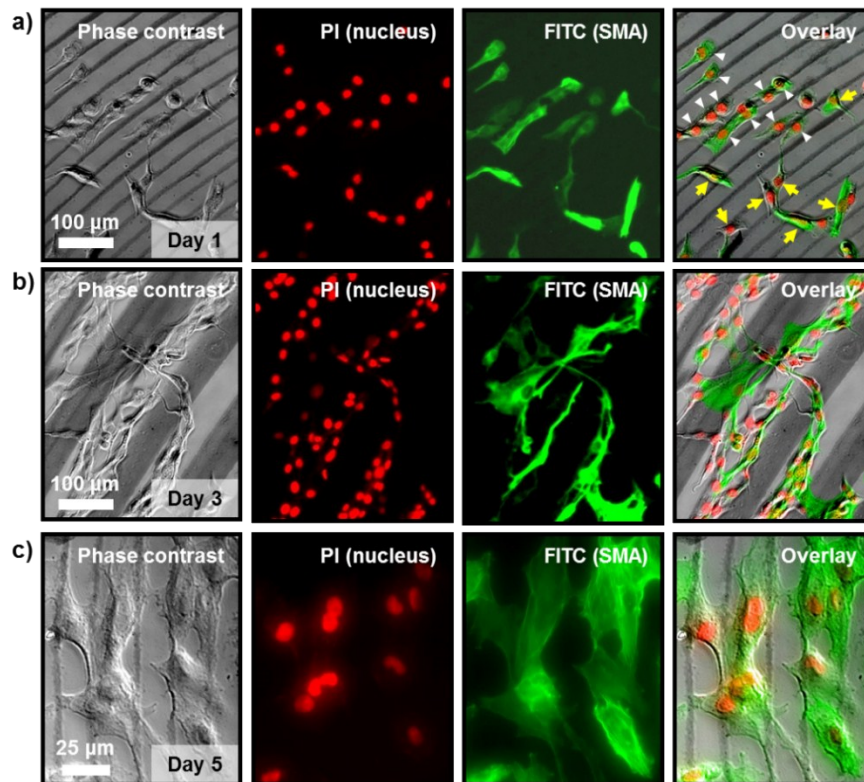
Fig. S9. Time-course analysis of skeletal muscle cell behaviors (i.e., arrangement) on the rGO patterned arrays. Fluorescent images of C2C12 skeletal muscle cells on the rGO patterned arrays at (a) day 1, (b) day 3 and (c) day 5 of culture. Alpha-smooth muscle actin (SMA) was stained with FITC-conjugated anti-SMA antibody (green), and cell nucleus was counterstained with PI (red). White triangles in (a) indicate the individually aligned myoblasts, while yellow arrows indicate randomly positioned myoblasts.
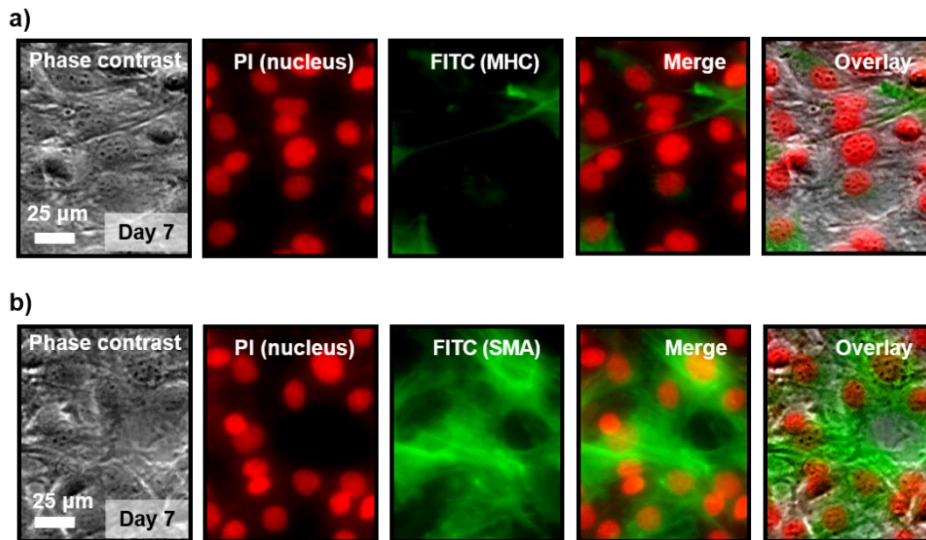
Fig. S10. Skeletal muscle cell behaviors on bare glass coverslips. (a) Myogenic differentiation of C2C12 myoblasts on a bare glass coverslip. Myosin heavy chain (MHC) was stained with Alexa Fluor 488-conjugated anti-MHC antibody (green), and cell nucleus was counterstained with PI (red). Only partially weak green fluorescence of MHC was detected, and a fusion between adjacent cells was not appeared. (b) Growth of C2C12 skeletal muscle cells on a bare glass coverslip. Alpha-SMA was stained with FITC-conjugated anti-SMA antibody (green), and cell nucleus was counterstained with PI (red). Myoblasts were favorably grown with well-organized SMAs.
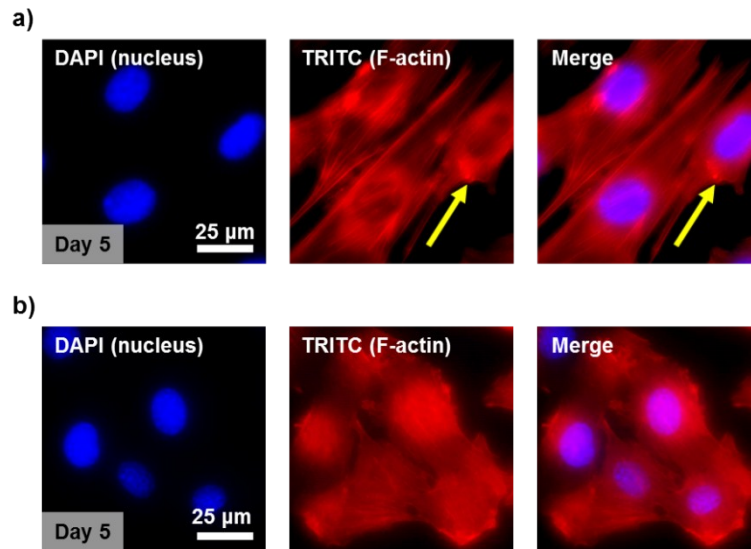
Fig. S11. Cytoskeleton alignment of C2C12 skeletal muscle cells. Fluorescent images of C2C12 cells on (a) the rGO stripe pattern and (b) the bare glass coverslip at day 5 of culture. The F-actins (cytoskeletons) were stained with TRITC-labelled phalloidin (red) and cell nuclei were counterstained with DAPI (blue). A yellow arrow in (a) indicates the direction of patterns.
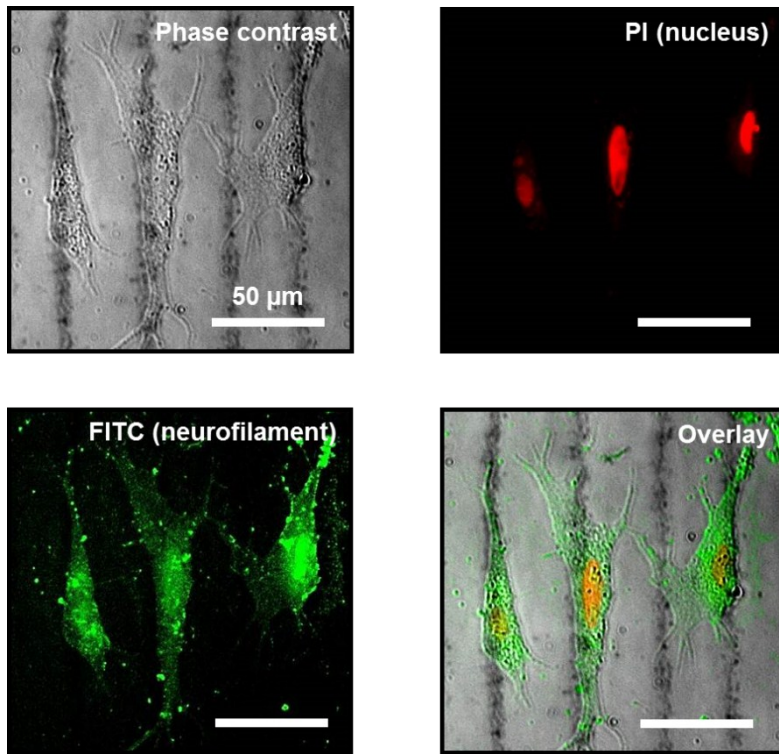
Fig. S12. Cell guidance effect of the rGO patterned arrays on neuronal cells. Fluorescent images of HT-22 hippocampal neuronal cell on the rGO patterned arrays at day 5 of culture. Neurofilament (NF) was stained with fluorescein-conjugated anti-NF antibody (green), and cell nucleus was counterstained with PI (red).
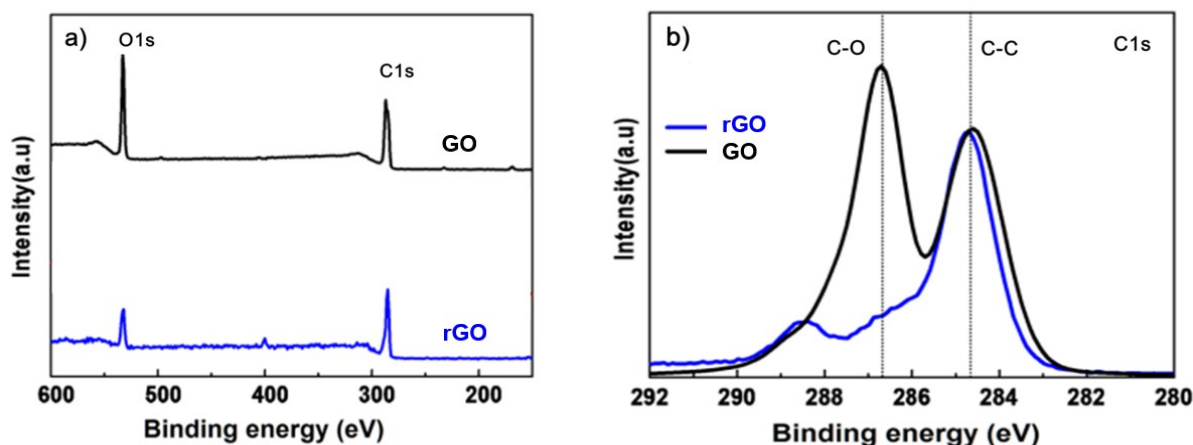
Fig. S13. X-ray photoelectron spectroscopy (XPS) was analyzed to examine the change of functional groups before and after the solvothermal reduction. (a, b) As shown in the XPS survey spectra and C_{1s} spectra of GO and rGO, the intensity ratio between C_{1s} and O_{1s} (C/O) increased from 0.6 to 1.6 and the intensity of C-O related peaks decreased tremendously after reduction, which indicate the reduction of oxygen related functional groups in GO during solvothermal reduction.

Caption for Supporting Movie

Movie S1: In-situ observation for the effective movement of L-929 fibroblasts on the rGO patterned stripes. A single motile cell (marked by the arrow) jumped into the adjacent rGO stripe.