Supporting Information for

Enhanced Neurite Outgrowth by Intracellular Stimulation

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Cell culture and differentiation.

Before cell seeding, the VNEA was sterilized using 70% ethanol and a UV light. The PC-12 cells were seeded with a cell density of 1×10^5 per cm^2. Seeded samples were incubated in original media (85% RPMI 1640; Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% Penicillin-Streptomycin (Gibco, Grand Island, NY) under 5% CO_2 at 37°C. When the PC-12 cells reached 70−80% confluence, they were trypsinized with 0.5% trypsin/EDTA (Gibco, Grand Island, NY) and expanded in a 75-cm^2 flask. The medium was replaced every 3−4 days with fresh subculture. Two days after seeding, the medium was changed to RPMI 1640 (Gibco, Grand Island, NY), 1% Penicillin-Streptomycin (Gibco, Grand Island, NY) and 100 ng/ml recombinant rat beta-nerve growth factor (rβ-NGF; R&D Systems, Minneapolis, MN). After the seeding period, electrical stimulation was performed for 4 days. A 2-day stabilization period followed the 4 days of stimulation. For comparison, the other group was cultured for 6 days under standard culture conditions, and the medium was replaced every 2 days.

Immunocytochemistry and neurite length analysis.

To study phenotypic changes following neurite outgrowth induction with or without electrical stimulation, or with a general differentiation protocol, PC-12 cells of each sample were fixed with 4% paraformaldehyde, and washed two times with 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO) in 1× PBS (Gibco, Grand Island, NY). These samples were then incubated with 10% normal donkey serum (Jackson, Bar Harbor, ME) and 0.3% Triton® X-100 (Sigma, St. Louis, MO) in 1× PBS. Primary antibodies against Tuj-1 (Abcam; Burlingame, CA) and neurofilament-66 (NF-66, Abcam; Burlingame, CA) were allowed to react overnight at 4°C. Subsequently, each sample was rinsed three times with PBS containing 0.1% BSA. Next, the samples were treated with secondary antibodies (Cy3 for NF-66 and FITC for Tuj-1) at room temperature for 1 hr. For additional nuclear staining, the cells were mounted with 4′,6-diamidino-2-phenylindole (DAPI)-conjugated mounting medium. PC-12 cells were imaged using fluorescent optical microscopy and statistical analysis of neurite length distribution was performed using ImageJ (NIH, USA).

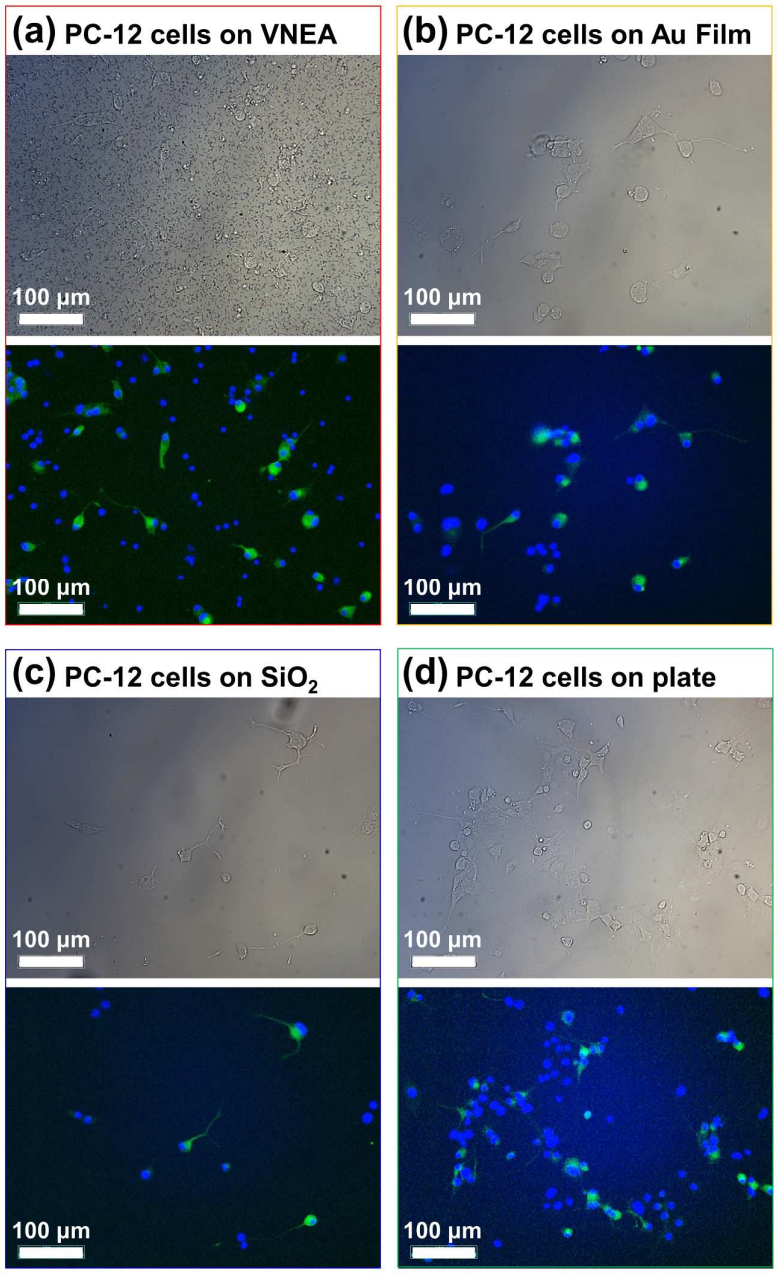

Figure S1. Optical and fluorescence microscope images of PC-12 cells grown on various substrates. (a) PC-12 cells on the VNEA (b) PC-12 cells on Au film. (c) PC-12 cells on SiO_2 substrate. (d) PC-12 cells on general culture plate. Same culture condition applied to all samples. The PC-12 cells on the VNEA grew well (about four times more densely) relative to the planar-type Au film or SiO_2 substrates, and similarly to the density on standard cell culture plate.

Vertical nanowire electrode-cell interface.

To conform where the vertical nanowire electrodes are located inside the cytoplasm of cells, we fixed the cells on VNEA device and cross-sectioned the cells by a high-resolution Cross Beam Focused Ion Beam - Field Effect SEM (FIB-FESEM). PC-12 cells on the VNEA device were firstly fixed with 2.5% glutaraldehyde in distilled water at room temperature for 30 minutes, and then were rinsed three times with PBS (Gibco, Grand Island, NY) for 5 minutes each. Subsequently, post-fixation of PC-12 cells was carried out with 1% osmium tetroxide in distilled water at room temperature for 30 minutes, and then cells were rinsed three times with PBS (Gibco, Grand Island, NY) for 5 minutes each. The cells were dehydrated in graded ethanols (25, 50, 70, 80, and 95%) at room temperature for 10 minutes. The last dehydration step was in 100% ethanol with two changes within 10 minutes at room temperature. Finally, the cells were critical point dried and coated with a 20 nm-thickness of gold film by a sputtering process. After the cell fixation, the cells were cross-sectioned at almost 2 μm height from the substrate by a high-resolution Cross Beam FIB-FESEM and vertical nanowire electrode-cell interfaces observed by SEM.

The cross section SEM image in Figure S2 shows a nanowire penetrating a cell. The image also shows a clean interface between the nanowire and the interior of the cell, which safely excludes the possibility of the nanowire being wrapped up by membrane. We investigated several vertical nanowire-cell interfaces and found that nanowires with lengths of > 3 μm typically penetrated PC-12 cells. This supports the fact that nanowire electrodes penetrated into the cytoplasms of cells.

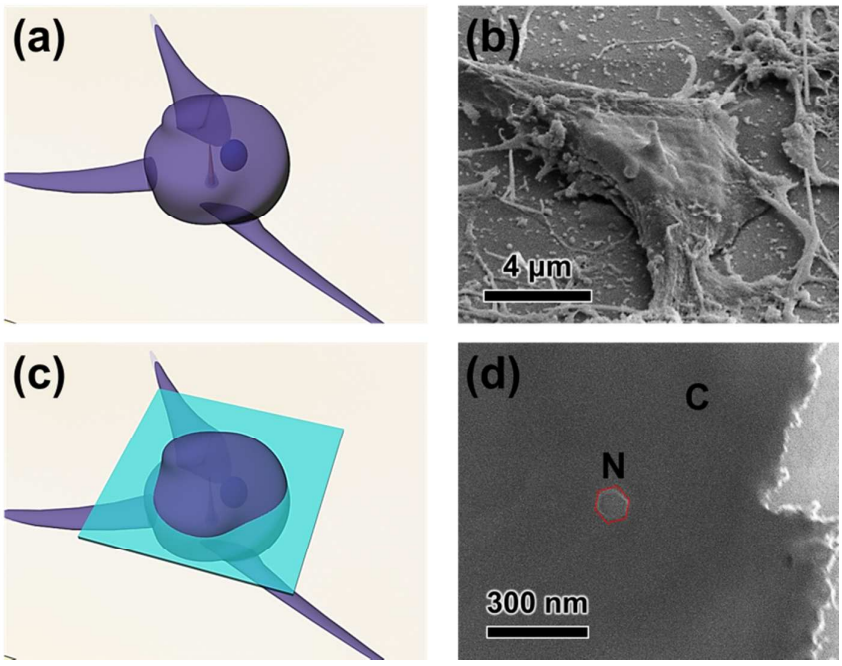

Figure S2. Schematic and cross-sectional SEM images of PC-12 cells on the VNEA. (a, b) Cell images cultured on a vertical nanowire electrode. (c, d) Schematic and SEM images of a cross section in the vertical Z direction with height of c.a. 2 μm. The image shows a nanowire (denoted N) and a cell (denoted C), and indicates the penetration of the nanowire into the cell through its membrane. Clean interfaces between the nanowire and cell can also be observed. The images indicate that nanowires did not just invaginate cells through enwrapped portions of the cell membrane, but that these wires both perforated the membrane and penetrated into cells.

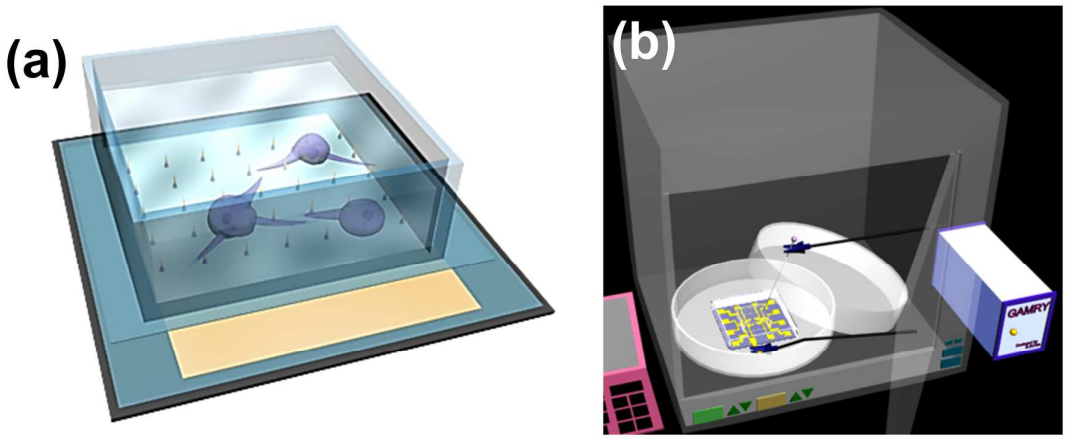

Figure S3. An intracellular electrical stimulation (ES) system using VNEA device. (a) Schematic of PC-12 cells on VNEA device. (b) Schematic of long-term and real-time intracellular ES system of living cells using VNEA device.

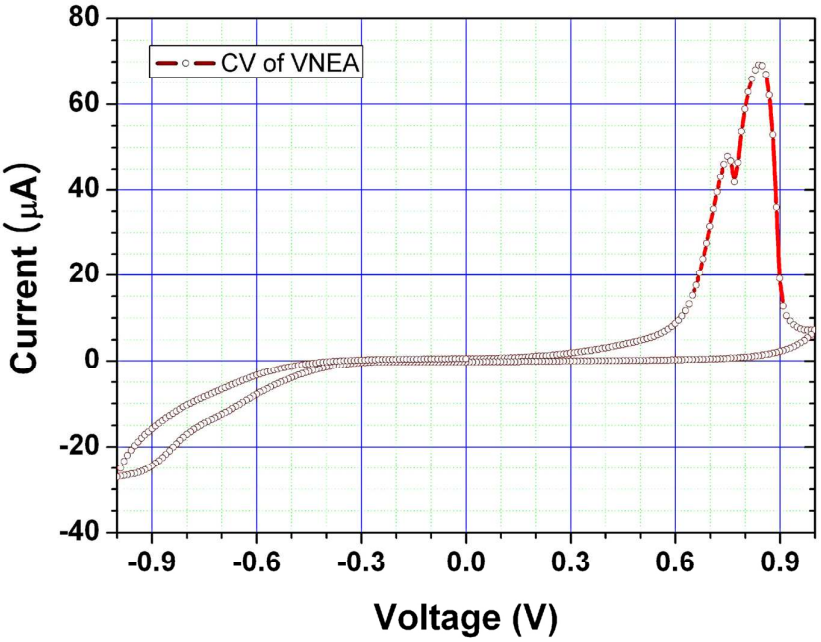

Figure S4. Cyclic voltamogram of VNEA in cell culture medium. Three-electrode configuration was used in CV measurement system with Au coated VNEA as a working electrode. The CV measurement is performed at a scan rate of 20 mV/s.

The device circuit with vertical nanowire electrode array.

In the device circuit with the vertical nanowire electrode array (VNEA), each nanowire electrode was connected in parallel, as shown in figure S5. Therefore, the induced voltage at each nanowire electrode was identical to the electrical stimulation voltage applied by the controlled potentiostat in the VNEA device.

$\Delta V_{VNEA} = \Delta V_{NW1} = \Delta V_{NW2} = \Delta V_{NW3} = \ldots \ldots$

In order to ensure that the extracellular stimulation via the close free nanowire electrodes actually influences cell potential, it must be transferred through the crosstalk capacitance (C_c) and resistance (R_c) by the membrane capacitance (C_m), resistance (R_m), outside resting potential (V_{rest}), etc. Since electrical stimulations were controlled with direct current (DC) voltages of -50, 50, and 100 mV in our experiment, all capacitance terms (C_c as well as C_m) in the device circuit were ignored via the DC bias voltage at steady state conditions. R_c and R_m were then main contributors of the *parasitic* stimulation and could be estimated with an actual set of conditions. For example, an actual set of stimulation conditions could be assumed as follows:

- The number of total nanowire electrodes :

  (5 EA/400 μm^2) × 1.2 mm^2 = 15,000 EA

  (Calculated from SEM images of VNEA device)

- Electrical properties of the cells in general case :

  $C_M = 10^{-2}$ F/m2 = 1 $\mu F/cm^2$

  C_m = 50 pF (a spherical cell which is 20 microns in diameter)

  R_M = 10,000 Ωcm^2

  R_m = 198 MΩ (a spherical cell which is 20 microns in diameter)

Based on these conditions, the current can be estimated as 250 pA when the applied voltage and membrane resistance are 50 mV and 200 MΩ, respectively. Using an estimated 0.1 %

voltage difference between intracellular and extracellular stimulations at 50 mV, the current through R_c would be 0.25 pA. Notably, the actual voltage difference would be lower than this estimated value because of the difference between intracellular and extracellular stimulations being close to zero. In our VNEA device, because the total current measured through the nanowire electrodes was almost 300 nA, and the number of total nanowire electrodes was 15,000 EA, the circuit current of each nanowire was almost 20 pA. Therefore, the current effect through R_c could be safely ignored when compared to the nanowire circuit current. This means that the extracellular stimulation occurring by free nanowire electrodes can be safely excluded, and that only intracellular stimulation needs to be considered for the stimulation in the VNEA device.

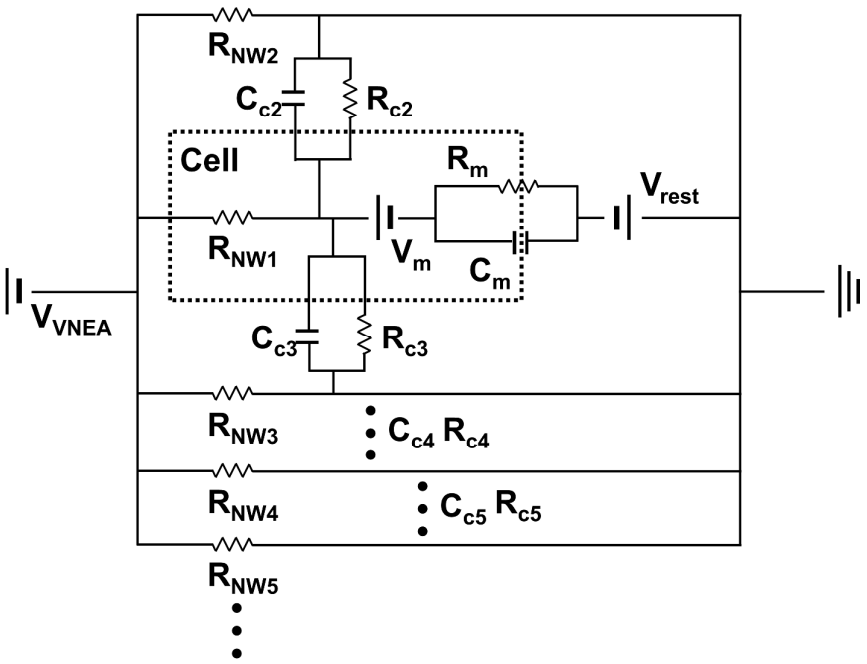

Figure S5. The simplified equivalent circuit model during electrical stimulation by the VNEA. In the device circuit with the VNEA, each nanowire electrode was connected in parallel, and the induced direct current (DC) voltage at each nanowire electrode was identical with V_{VNEA} (V_{VNEA} : applied voltage in VNEA, V_m : membrane potential, V_{rest} : resting potential, R_{NW} : nanowire electrode resistance, R_m : membrane resistance, R_c : crosstalk resistance by free nanowire electrode, C_m : membrane capacitance, C_c : crosstalk capacitance by free nanowire electrode). In this circuit model, the extracellular stimulation was transferred into the cell through the crosstalk capacitance (C_c) and resistance (R_c).

Electrical stimulation through two regions of the same substrate.

Electrical stimulation on the VNEA was performed partly through two regions of the same substrate: the electrically conducted VNEA part (right part seen in Figure S6a) and the electrically unconducted vertical SiNWs part (left part seen in Figure S6a). The nanowires of the two regions of the substrate in the controlled experiment were selectively exposed without the isolating SiO_2. Therefore, the two regions had almost identical surface conditions including the surface topology/roughness, with the exception that the Au film on the surface of the SiNW was in the electrically conductive region. In our comparisons between Au film and SiO_2, we determined that the Au metal itself had no influence on cell adhesion and neurite outgrowth (Figure 3 and 4). The controlled experiment observed active neurite outgrowth in only the electrical stimulated region and suggests that intracellular ES intensively enhances the cellular outgrowth.

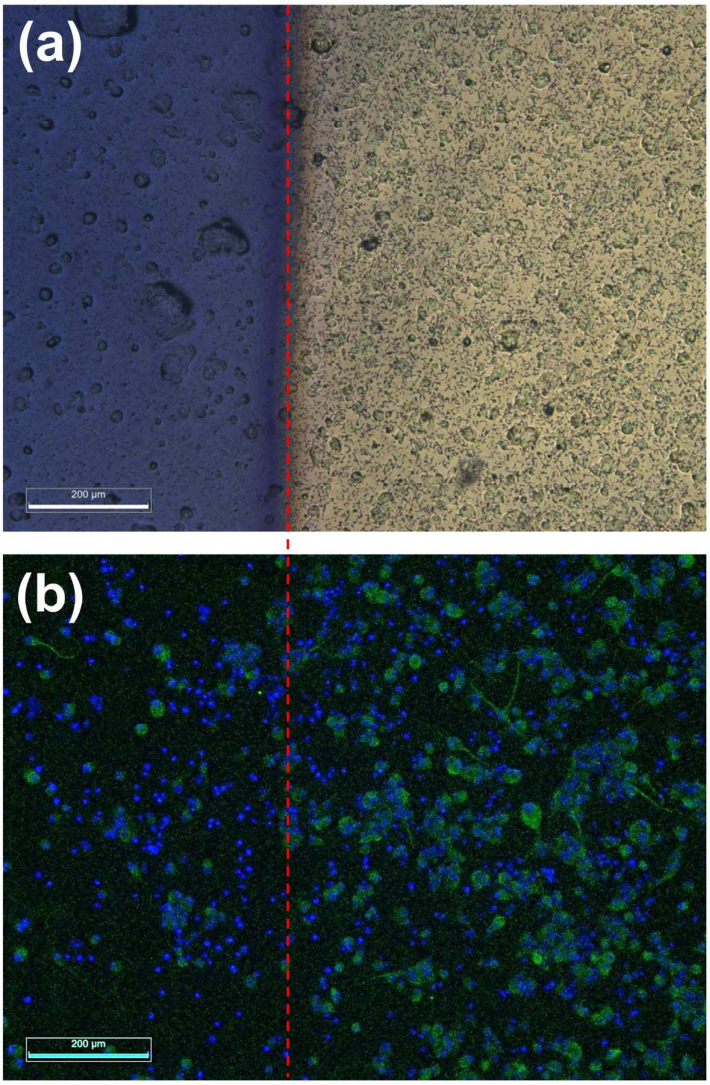

Figure S6. Microscope images of PC-12 cells in two regions of the same substrate. (a) Optical and (b) fluorescence microscope images of PC-12 cells grown on two regions of the same VNEA substrate. The right side of the red line is the Au coated VNEA region with intracellular ES. The left side of the red line is the vertical Si NWs region without ES.

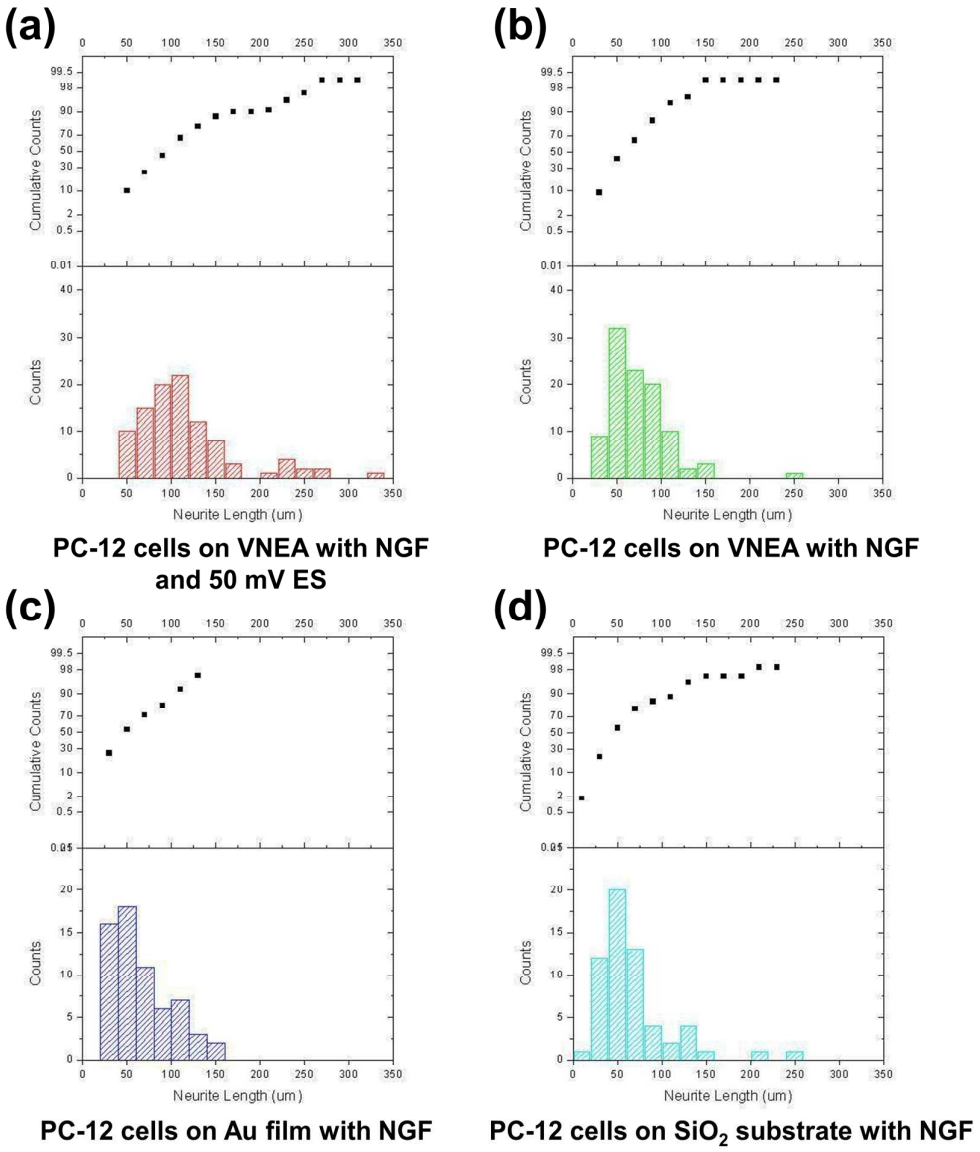

Figure S7. The distribution data of the neurite length of PC-12 cells in different conditions. (a) PC-12 cells on the VNEA with 50 mV intracellular ES. (b) PC-12 cells on the VNEA without ES. (c) PC-12 cells on Au film with 50 mV ES. (d) PC-12 cells on SiO_2 substrate without ES. (Distribution data obtained by 100 samples of neurite length in each condition)

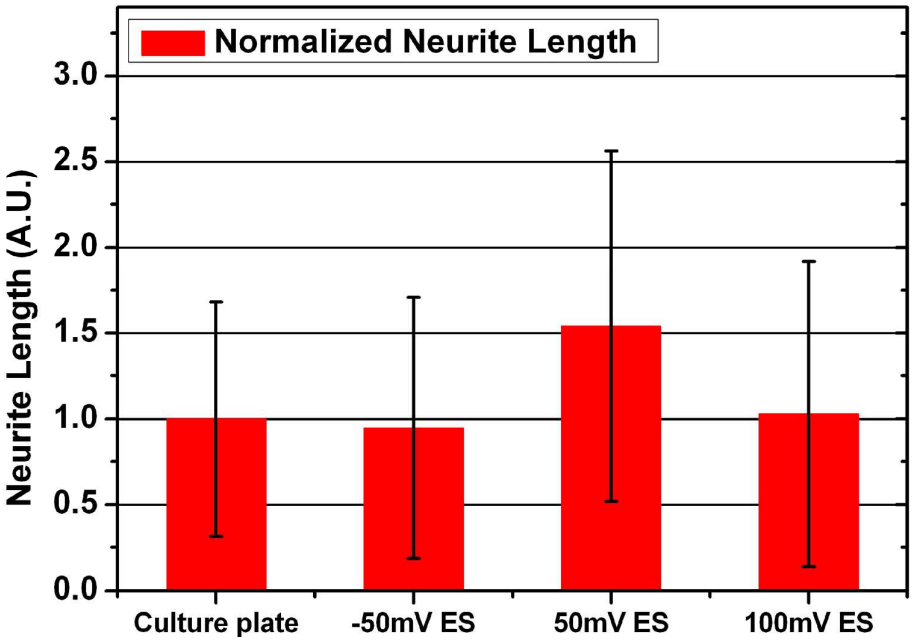

Figure S8. Statistical analysis of the neurite length of PC-12 cells with different intracellular ES conditions. Strong ES (100 mV), or negatively charged ES (-50 mV), did not enhance differentiation against 50 mV ES. (Statistical analysis performed by 100 samples of neurite length in each condition)