

Supplementary Information

Polarization-Controlled Differentiation of Human Neural Stem Cells Using Synergistic Cues from the Patterns of Carbon Nanotube Monolayer Coating

*Sung Young Park,¹ Dong Shin Choi,¹ Hye Jun Jin,² Juhun Park,² Kyung-Eun Byun,² Ki-Bum
Lee,^{*,3} and Seunghun Hong^{*,1,2,4}*

¹Interdisciplinary Program in Nano-Science and Technology, Seoul 151-747, Korea

²Department of Physics and Astronomy, Seoul 151-747, Korea

³Department of Chemistry and Chemical Biology, Rutgers, The State University of New
Jersey, Piscataway, New Jersey 08854

⁴Department of Biophysics and Chemical Biology, Seoul National University, Seoul 151-747,
Korea

Supplementary Method

Fabrication of CNT monolayer pattern. Polycrystalline Au films were prepared by thermally depositing 5~6 nm thick Ti layer followed by 10~20 nm Au deposition on cover glass substrates under a high vacuum condition (base pressure $\sim 5 \times 10^{-6}$ torr). For micro contact printing, polydimethylsiloxane (PDMS) stamps were fabricated using photoresist (AZ5214) patterns as a template. 1-octadecanethiol (ODT, Sigma, MO, USA) solution (5 mM in acetonitrile) was utilized as an ink. For CNT assembly, typical concentration of multi-walled CNT (Nanolab Inc, 98% purified) suspension of 0.2 mg/ml was prepared, and the ODT patterned surface by microcontact printing was placed in CNT suspension usually for 10~30 sec and rinsed thoroughly with 1,2-dichlorobenzene. PI (VTECTM Polyimide 1388, Richard Blaine International, Inc., PA, USA) in solution was coated on a cover glass by spin coating at 1,000 rpm for 1 min. After the PI on cover glass was cured on a hot plate at 110 °C for 30 min in N₂ gas environment, the temperature of the hot plate was increased from 110 to 220 °C with a temperature ramping rate of 5 °C/min, and then maintained at 220 °C for 2 hours. Afterwards, Ti (5~6 nm) and Au (10~20 nm) were thermally deposited on the PI surface.

Supplementary Figures

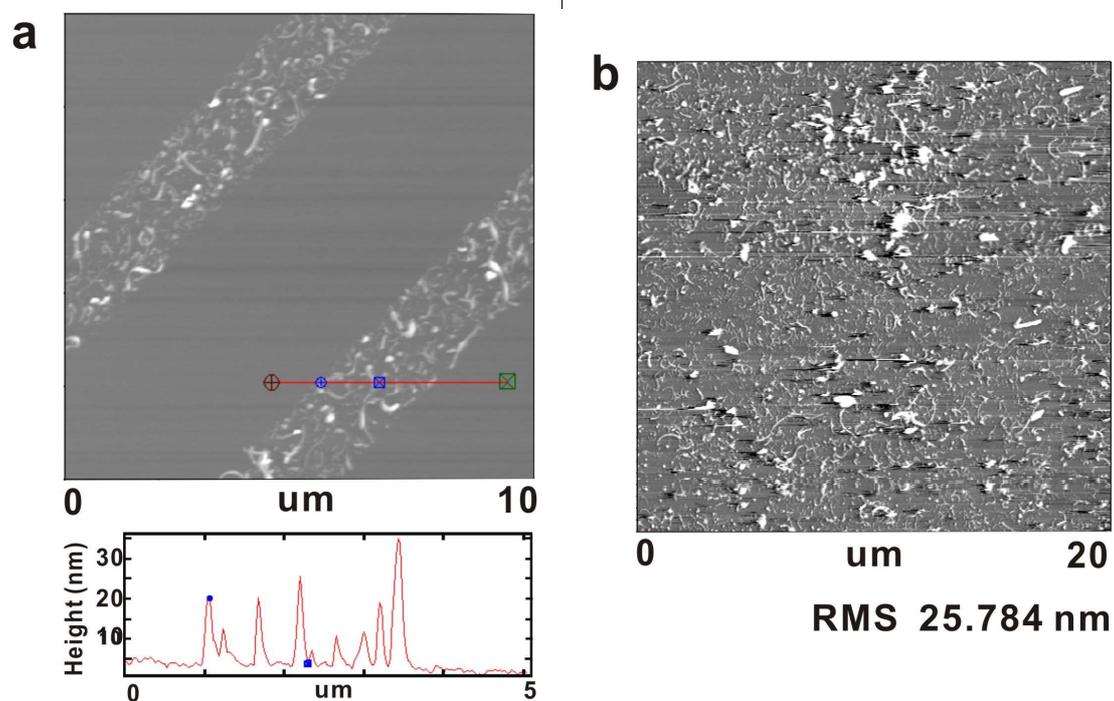


Figure S1. **a.** AFM topography image of multi-walled CNT monolayer patterns created on the Au substrate. As indicated in the height profile (lower), the height of the CNT monolayer nanostructure ranged from 10 to 40 nm. **b.** Topography of laminin-coated CNT monolayer structures in PBS. The average surface roughness of the laminin-coated CNT networks measured by AFM was 25.784 nm.

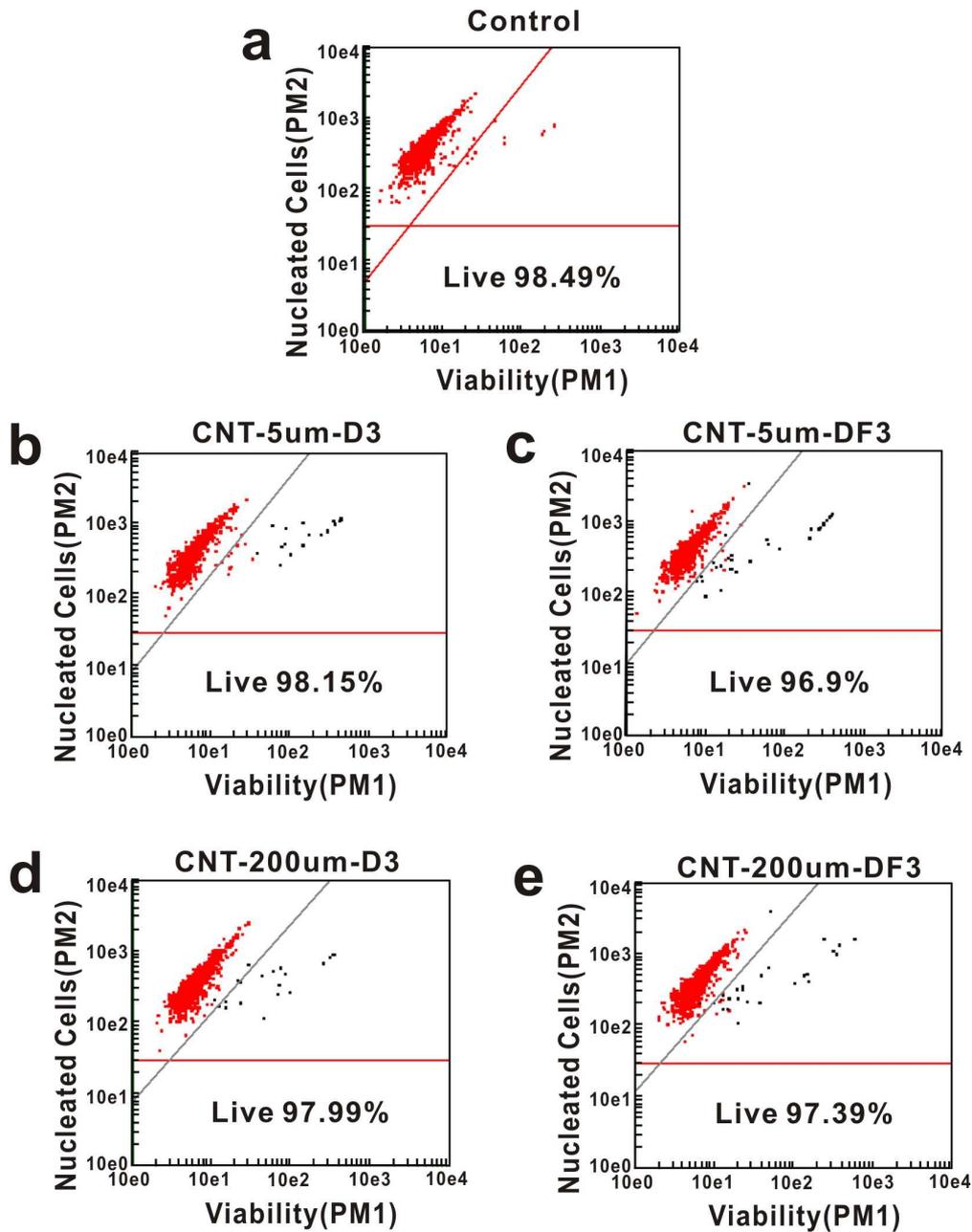


Figure S2. Cell viability assay by flow cytometry of hNSCs on CNT pattern: three-day proliferation and three-differentiation, respectively. Cell viability assay was carried out by flow cytometry. It was measured by the calculations performed automatically (Guava ViaCount, Millipore). The acquired data are displayed in dot plot: viability (PM1) and nucleated cells (PM2). Live cells appear on the left side of the plot. Dead cells are on the right side of the plot. Apoptotic cells are shown in between the live and dead cell populations. Viability data on **a.** Control group grown on laminin-coated Au surface for three days (Control), **b.** CNT pattern (5 μ m \times 5 μ m, 5- μ m spacing) for three day proliferation (CNT-5 μ m-D3), **c.** CNT pattern (5 μ m \times 5 μ m, 5- μ m spacing) for three day differentiation (CNT-5 μ m-DF3), **d.** CNT pattern (200 μ m \times 200 μ m, 200- μ m spacing) for three day proliferation (CNT-200 μ m-D3), **e.** CNT pattern (200 μ m \times 200 μ m, 200- μ m spacing) for three day differentiation (CNT-200 μ m-DF3).

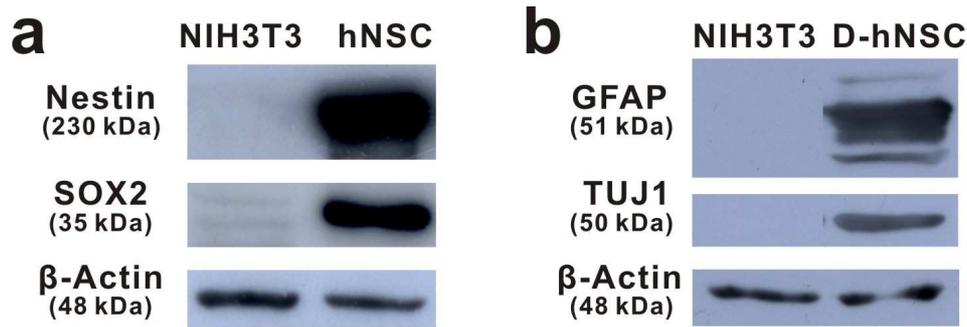


Figure S3. Western Blot analysis of hNSCs. Nestin (monoclonal, 1:5000, Millipore, Temecula, CA, USA) and SOX2 (monoclonal, 1:2500, Millipore, Temecula, CA, USA) were used to confirm undifferentiated human neural stem cells (hNSCs). GFAP (monoclonal, 1:2500, Millipore, Temecula, CA, USA) and TUJ1 (monoclonal, 1:1000, Millipore, Temecula, CA, USA) were used to confirm the differentiation into glial cells and neurons, respectively. NIH3T3 cells were used as a negative control. hNSCs were cultured and differentiated on laminin-coated cell culture petri dishes. **(a)** Western blot results of hNSCs grown in culture media with the growth factors (EGF and bFGF). The cells exhibited positive for nestin and SOX2, indicating undifferentiated hNSCs. **(b)** Western blot results of hNSCs grown in culture media without the growth factors. The cells were positive for GFAP and TUJ1, indicating the differentiation of hNSCs (D-hNSC) into neuronal cells.

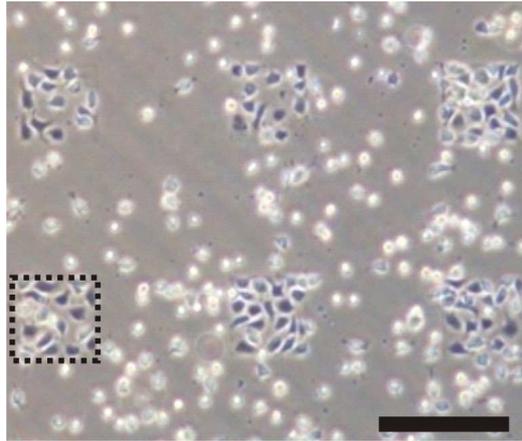


Figure S4. Phase contrast image of the hNSC seeded on CNT square pattern (100- μm -width, 200- μm -spacing). The rapidly attached hNSCs on the CNT-coated regions (black dotted square) were observed within 30 min after cell seeding as shown in dark gray color, while those on ODT SAM area in white circular shapes were not adhered yet. Scale bar represents 200 μm .

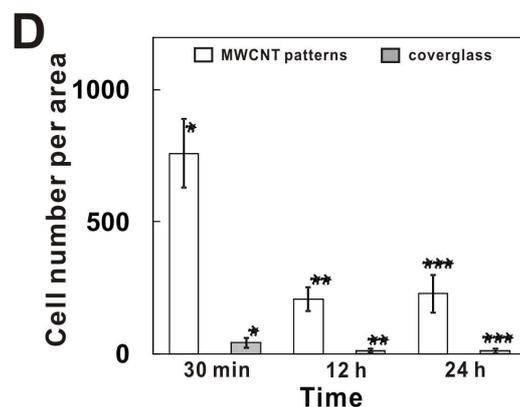
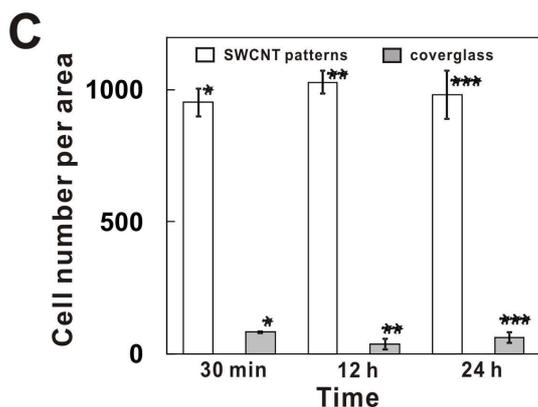
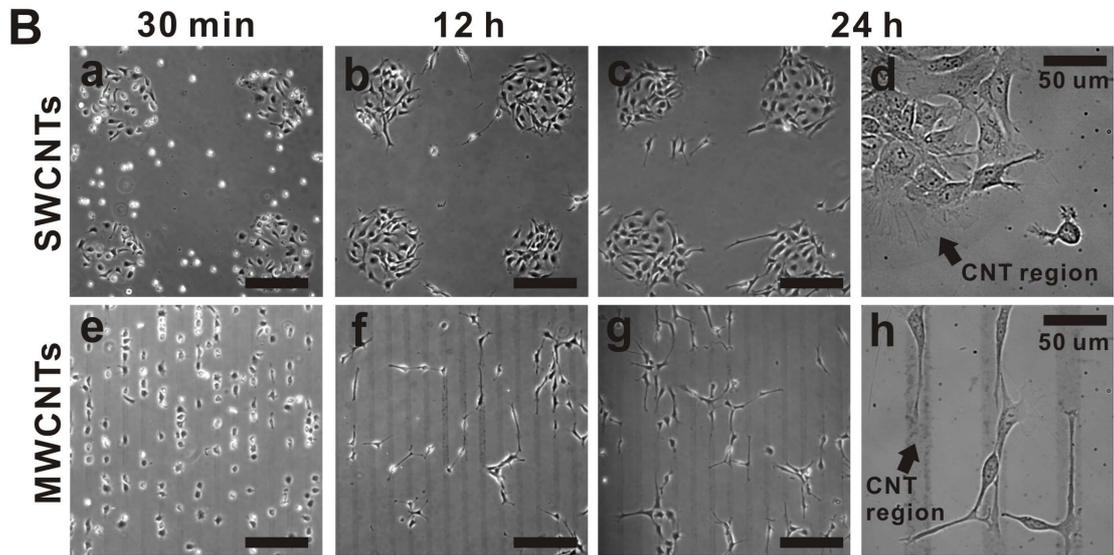
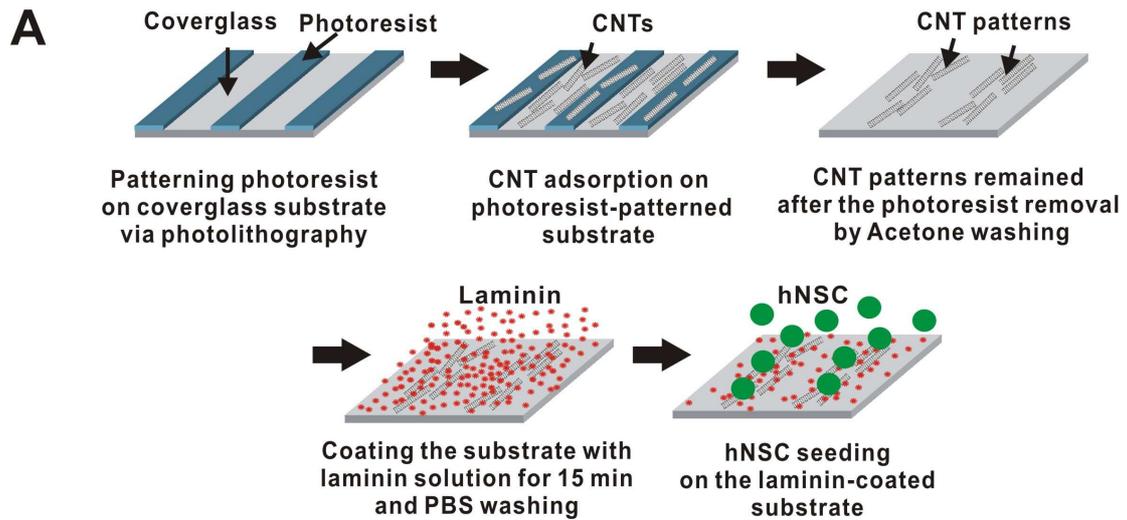


Figure S5. hNSC growth on CNT patterns compared with that on bare glass surface. (A) A schematic diagram depicting the procedure to prepare CNT patterns on bare glass substrates and to culture hNSCs on them. (B) Bright field images showing selective hNSC growth on the patterns of single wall CNTs (marked by SWCNTs, upper images) and multi-wall CNTs (marked by MWCNTs, lower images) after different growth time periods. Scale bars represent 200 μm unless it is specified differently. Within 30 min after cell seeding, the hNSCs adhered selectively on the

regions of SWCNTs (a) and MWCNTs (e). We observed that the hNSCs grew selectively on the CNT regions after 12 h (b and f) and 24 h (c, d, g and h). The darker area with black arrows (d and h) represents the CNT regions, while the brighter area represents the bare glass regions. (C) Cell number per area (0.64 mm^2) in *SWCNT-coated* or *bare* cover glass regions on the circular-shaped patterns of SWCNT networks in Figure S5B (a)-(d) ($n = 3$, *: $p < 0.001$, after 30 min, **: $p < 0.001$, after 12 h, **: $p < 0.005$, after 24 h). (D) Cell number per area (0.64 mm^2) in *MWCNT-coated* or *bare* cover glass regions on the line-shaped patterns of SWCNT networks in Figure S5B (e)-(h) ($n = 3$, *: $p < 0.005$, after 30 min, **: $p < 0.01$, after 12 h, **: $p < 0.05$, after 24 h).

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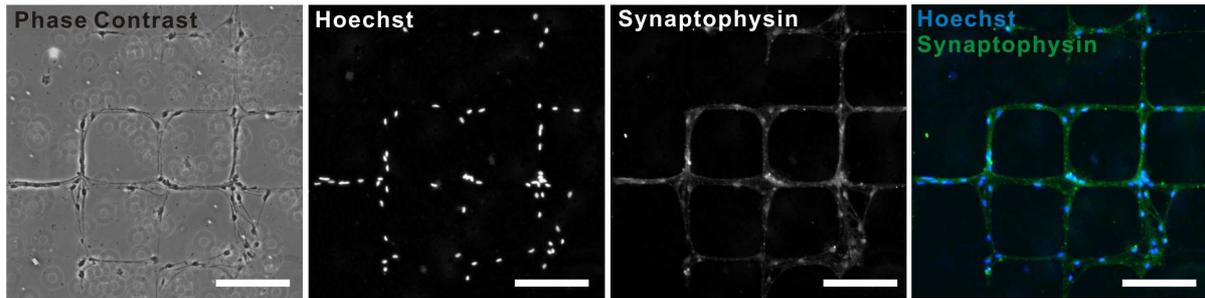


Figure S6. Synapse formation of hNSCs differentiated on the CNT patterns.

Phase contrast image (left first) and immunofluorescence images of the differentiated hNSCs on CNT patterns. Scale bars represent 200 μm . The CNT patterns are comprised of square-shape patterns connected by narrow line-shape patterns. Hoechst and synaptophysin were used as immunostaining markers for nuclei and neuronal presynaptic vesicles, respectively. Note that the differentiated hNSCs formed neural network on the CNT patterns with synapse formation (green color from the merged image).

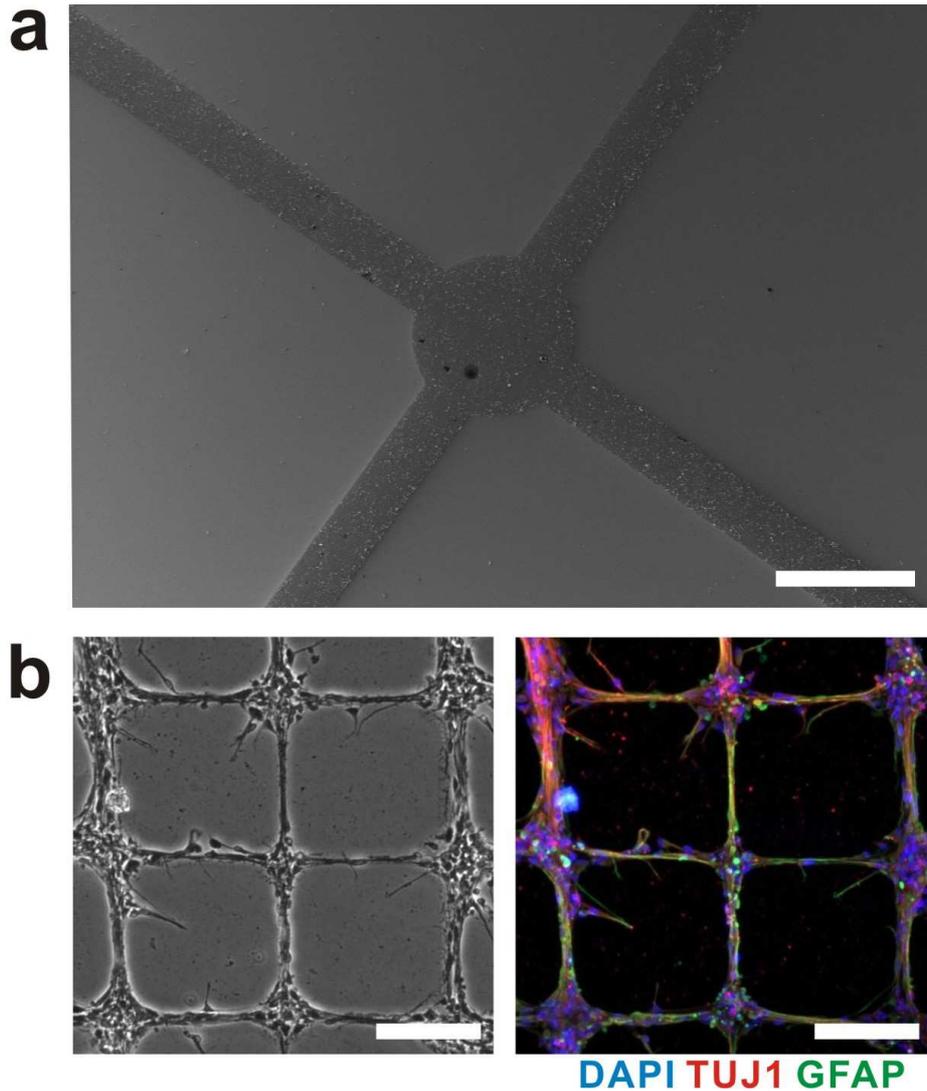


Figure S7. The CNT patterns on flexible polyimide (PI) substrate and hNSC differentiation for three weeks. **a**, SEM image of CNT patterns fabricated on PI substrate. Scale bar represents 50 μm . **b**, Phase contrast image (left) and immunofluorescence image (right) of the differentiated hNSCs after three-week differentiation. Immunofluorescence images are DAPI for nucleus (blue), GFAP for astrocytes (green) and TUJ1 for neuron (red). Scale bar represents 200 μm .

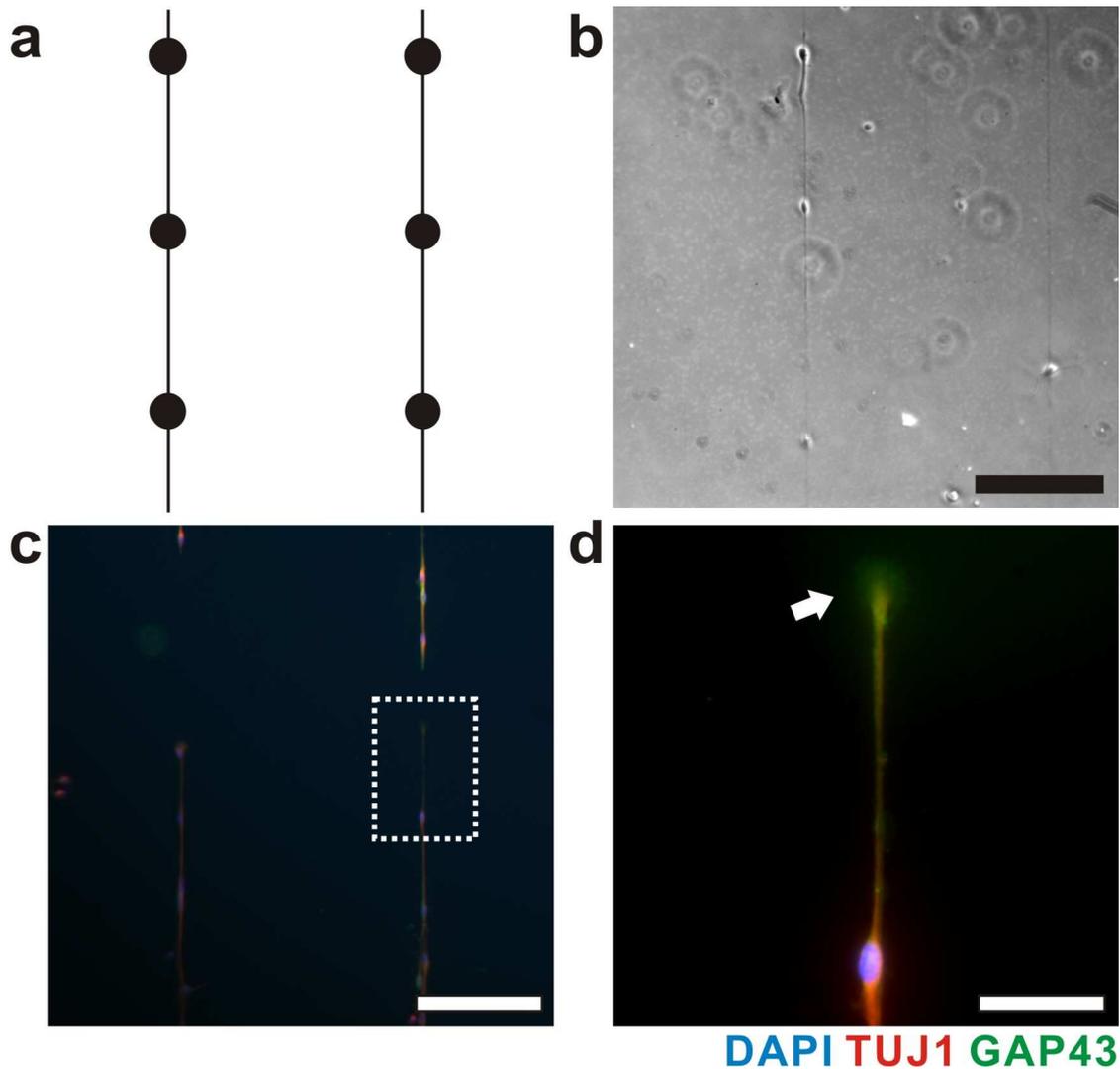


Figure S8. CNT bipolar patterns for axonal guidance and arborization. The markers are TUJ1 (red) for neuronal marker, GAP43 (green) for growth cone marker and Hoechst (blue) for nucleus in the immunostaining images. **a**, Schematic of CNT bipolar patterns composed of circle array connected with narrow strips, which can enclose only single cell body within a circle. **b**, Phase contrast image of hNSCs with the axon-like parts growing along the predefined CNT strip pattern. Scale bar represents 200 μm . **c**, Immunofluorescence image of the differentiated hNSCs on the bipolar pattern. Scale bar represents 200 μm . **d**, Magnified image of immunofluorescence image of highlighted in (c), which showing clearly positive for the growth cone marker, GAP 43 (green). Growth cone of hNSC is clearly shown to be arborizing on the CNT circle pattern region after outgrowing along the protruding CNT strip pattern. Scale bar represents 50 μm .

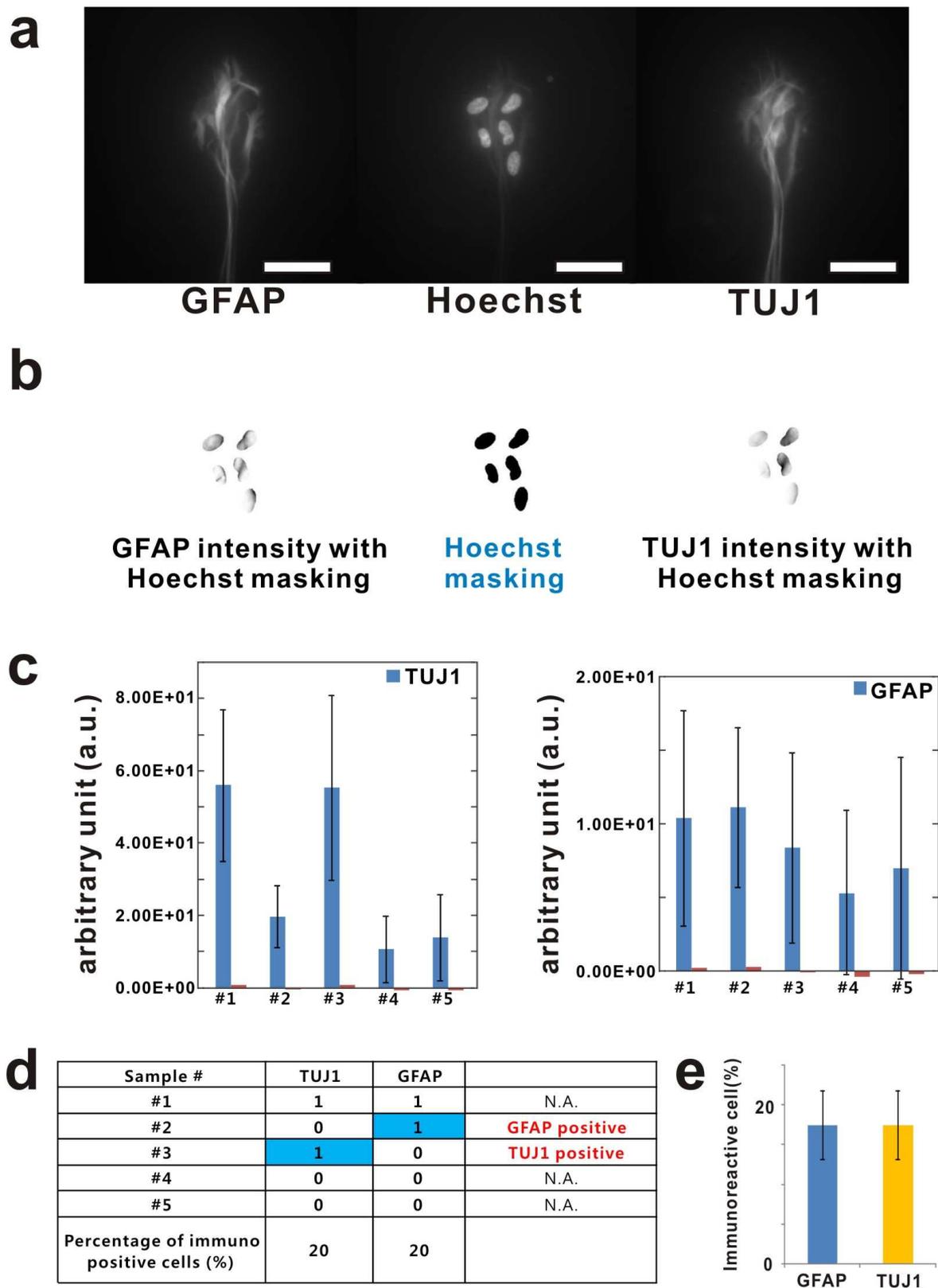


Figure S9. Calculation of a neural-lineage percentage of the differentiated hNSCs on CNT polarization-control pattern. The number of immunoreactive cells for GFAP (astroglia) and TUJ1 (neurons) were analyzed and calculated using ImageJ (NIH). Each numbered sample indicates each nucleus region of individual

cell from the immunofluorescence image. **a**, Original fluorescence images of GFAP, TUJ1 and Hoechst (nuclei). The scale bar represents 50 μm . **b**, Hoechst masking to quantify the corresponding nuclear regions of each marker of GFAP and TUJ1. The background signal of each image was subtracted before the masking processes. **c**, Fluorescence signal intensities were presented as arbitrary unit and the intensities above a mean value in each marker were counted as '1'. **d**, The table summarizing the image analysis. Only when one marker is positive and the other marker is negative, we counted the marker as immunoreactive. For example, if TUJ1=1 and GFAP=0, this is TUJ1 positive; if TUJ1=0, and GFAP=1, this is GFAP positive; if TUJ1=1, and GFAP=1, this might be possibly called as 'astron' but it cannot be confirmed conclusively from this data; if TUJ1=0, and GFAP=0, this cell is not immunoreactive for the both markers at all. **e**, The calculation result showed that 20% of the five hNSCs on the CNT polarization-control pattern were differentiated to neural cells and 20% of them to astroglial cells. The data was obtained from three different samples.