Supporting Information

Localized Committed Differentiation of Neural Stem Cells based on Topographical Regulation Effect of TiO_2 Nanostructured Ceramics

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Experimental Section

Preparation of TiO_2 ceramics with different topography

To prepare PorousT ceramic, a TiO_2 nanobelt was prepared firstly as described in a previous study,[1] and was then compacted under an axial pressure of 75 MPa into a piece of pellet. After the resultant pellet was sintered at 1000°C for 2 h in a muffle furnace, the PorousT ceramic was obtained. For preparing the FlatT ceramic, the P25 nano-powders were uniaxially pressed at 50 MPa into pellets, followed by a cool isostatic pressure at 200 MPa, and then sintered at 1100°C for 6 hours. After natural annealing and polishing, the FlatT ceramics were obtained.

Characterization of the two TiO_2 ceramics

The topographies of the TiO_2 ceramics were characterized by SEM (SU8020, Hitachi, Japan), and the wettability of the ceramics was detected by Drop Shape Analysis System (Kruess DSA100). To eliminate the influce of drop evaporation, pictures were captured in 3 seconds after droping.

Fabrication of micropattern

The synthesized TiO_2 ceramics were cut into 0.8 mm wide strips by a wire cutting machine. The TiO_2 ceramic strips were stuck on the capton tightly in a FlatT/PorousT sequence, and then, PDMS was infused. After PDMS solidification, the capton was taken off of the ceramics, and we obtained a micropattern on the PDMS substrates.

Cell Culture

NSCs were isolated from the cerebral cortex of a Sprague Dawley pregnant rat E13.5 [2] and characterized by Nestin staining. For suspension culture, the NSCs were passaged at a cell density of 10^5 per ml in DMEM/F12 supplemented with 1% N2, 2% B27, 1% GlutaMax, 1% NEAA, 20 ng/ml bFGF and 20 ng/ml EGF. All of the NSCs used in the experiments were at

passages 3-5. For adhesion culture, titanium dioxide materials were first incubated in 20 ng/ml PDL overnight at 4°C and then washed with PBS twice. The next day, the materials were incubated in a 20 ng/ml laminin solution for more than 1 h at 37°C and then washed twice with PBS. Neural differentiation was initiated by removing bFGF and EGF from the culture medium.

Cell viability and proliferation assay

To investigate the cytocompatibility of the ceramics, a cell viability assay was performed using the LIVE/DEAD Cell Imaging kit for mammalian cells (Life Technology) according to the manufacturer's instructions. A cell count kit-8 (Dojindo Molecular Technology) was also used to evaluate the cell proliferation on the different TiO_2 topographies after 1, 3 and 5 days of culture according to the manufacturer's instructions.

Immunofluorescence

After culturing and differentiation on the TiO_2 ceramics, immunofluorescence was performed to detect the neural-specific marker expression. Briefly, the cells were fixed with 4% paraformaldehyde, permeablized with 0.1% Triton X-100, and blocked with 10% goat serum solution at room temperature. The cells were incubated with the primary antibodies Alexa Fluor 488 conjugated Nestin (1:200, Millipore), GFAP (1:1000, abcam) and Tuj1 (1:1000, Abcam). The secondary antibodies are Alexa Fluor 488-conjugated goat anti-mouse IgG and Cy3 conjucted goat anti rabbit IgG (1:100; Jackson ImmunoResearch). The nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI, Life Technology). Then, the samples were observed under confocal laser scanning microscopy (Leica, SP8).

qPCR

The total RNA was extracted from the cells using the RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's instructions. The RNA samples were reverse-transcribed into cDNA for qPCR, using the PrimeScriptTM reagent Kit with gDNA Eraser (Takara). qPCR was performed with SYBR Premix Ex TaqTM with ROX (Takara) according to manufacturer's instructions. The signals were detected with the ABI 7500 Fast Real Time PCR system (Applied Biosystems) for analyzing the expression of Nestin, Tuj1, MAP2 and GFAP. The gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal standard. The information on primers is provided in the Supplementary Information Table S1.

Cell morphology

The NSCs differentiated on the ceramics were fixed with 4% paraformaldehyde in PBS for 2 h at room temperature. After removing the fixative, the samples were gently washed with PBS

and immersed in 1% w/V osmic acid for 2 h. After rinsing with PBS twice, these samples were subjected to sequential dehydration for 15 min twice each with an ethanol series (30%, 50%, 70%, 80%, 90%, 95%, 98% and 100%). After carbon dioxide supercritical drying and coating with gold, the cells were observed under the SEM

Calcium Imaging

To record the spontaneous calcium activity of the NSCs-derived neurons, calcium imaging was performed with Fluo-4 AM (Dojindo Molecular Technology) according to the manufacturer's instructions. The fluorescence intensity was recorded with a Leica SP8 confocal laser scanning microscope (CLSM) in time-lapse mode at a speed of 1600HZ.

Statistical Analysis

The results are shown as the mean ± standard error of the mean. The statistical significance of the differences was determined by a one-way ANOVA. Values with $P < 0.05$ were considered statistically significant.

S1. Preparation and characterization of the two ceramics

S2. Characterization of isolated NSCs derived from E13.5 embryo

S3. SEM images of NSCs differentiation on three substrates for 15 days

S4. MAP2 and GFAP staining of NSCs differentiation for 21 days

S5. Fabrication of titanium dioxide ceramics micropattern

S6. NSCs localized committed differentiation on micropattern for 15 days

S7. Protein absorption of three substrates

S8. Primers sequence of qPCR. Table S1

S9. Spontaneous calcium dynamics in differentiated neurons on FlatT. Movie S1

S1. Preparation and characterization of the two ceramics

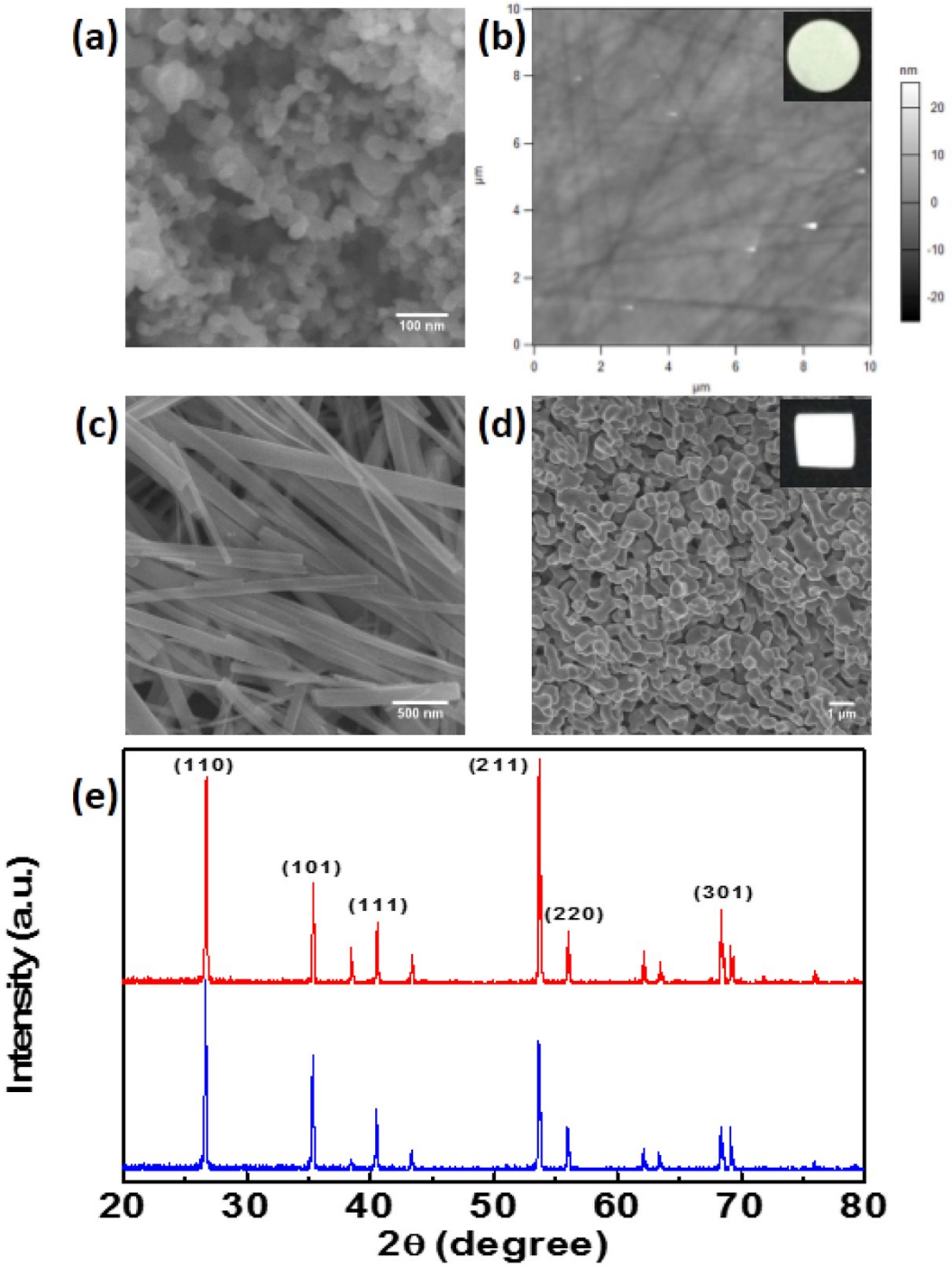

Figure S1. SEM images and XRD patterns of the TiO_2 ceramics. (a) SEM image shows the morphology of P25 nanoparticles (scale bar, 100 nm). (b) AFM image of FlatT to detect the roughness. (c) SEM image of synthesized TiO_2 nanobelts (scale bar, 500 nm). (d) SEM image of cut- surface of PorousT (scale bar, 1 um). The insets show digital camera images of two ceramics (b-FlatT, d-PorousT). (e) XRD patterns of the ceramics parallel to the sample stage. Red color shows FlatT (Up), and blue color shows PorousT (Down).

The procedure of FlatT is as follows: (1) Preparation of TiO_2 pellets by dry-press method: Degussa P25 was purchased from EVONIK Industries (Figure S1a). P25 powders were into stainless steel die 10mm in diameter, and a 50 MPa axial pressure was applied. After pressure released, TiO_2 pellet can be obtained. (2) The pellets were sintered in muffle furnace at 1100 °C for 6 hours to get dense TiO_2 ceramic. (3) The surface of the ceramic was grinded and polished to get flat surface. This sample is FlatT. Atomic force microscope (AFM) image shows the the roughness of FlatT is 1.471 nm (Figure S1b). The Procedure of PorousT is as follows: (1) Synthesis of TiO_2 nanobelts. A hydrothermal method based synthesis approach was used to synthesis of TiO_2 nanobelts. The detailed experimental procedure can be found in our previous paper. Briefly, 0.8g P25 and 80 mL of 10 M NaOH aqueous solution were mixed and stirred for 1 h before transferred to a 100 mL Teflon-lined stainless steel autoclave and heated at 180 °C for 72 h. After naturally cooling down to room temperature, the $Na_2Ti_3O_7$ product was washed with deionized water to remove the excess NaOH, followed by immersing in 100 mL of 0.1 M HCl for 24 h to produce $H_2Ti_3O_7$ nanobelts. Then the obtained product was isolated from the solution by centrifugation, which was washed thoroughly with deionized water for several times, and dried at 70 °C for 10 h. Finally, anatase TiO_2 nanobelts with coarsened surface were acquired by thermal annealing at 600 °C for 2 h. The morphology of TiO_2 nanobelts is shown in Figure S1 c. From Figure S1c, we can find that TiO_2 nanobelts are 20-30 nm in thickness, 100-15nm in width, and the length of the nanobelts are up 100 μm. (2) Preparation of pressed pallets: TiO_2 nanobelts were filled in metal die with 8 mm in diameter. A 75 MPa axial pressure was applied, and dry-pressed TiO_2 pellet with 8 mm in diameter and 1 mm in thickness can be obtained. (3) Preparation of TiO_2 nanostructured ceramic: Dry-pressed TiO_2 pellets were placed a muffle furnace, and heated to 1000 °C for 2 hours. After cooling down, nanostructured porous TiO_2 ceramic (PorousT) can be obtained.

SEM of fracture surface of PorousT is shown in Figure S1d. After sintering, the nanoblets connect together and form uniform nanostructured ceramic. The both the wall and the diameter of the nanoporous ceramc are from 300 to 500nm, because the solid diffusion occurred during the sintering process. The digital images round-shaped FlatT pellet and the square-shaped PorousT are in Inset in Figure S1b, d. To identify the ceramics crystalline phase, the X-ray diffraction (XRD) pattern was recorded with TiO_2 ceramics parallel to the sample stage. Both XRD patterns have almost same diffraction peaks, which can be indexed as rutile TiO_2 (PDF-72-1148). The results indicates that FlatT and PorousT share same crystal

structure. The only difference is their surface morphology, which is a good model for investigation on the effect of surface topology on fate of stem cells.

S2. Characterization of isolated NSCs derived from E13.5 embryo

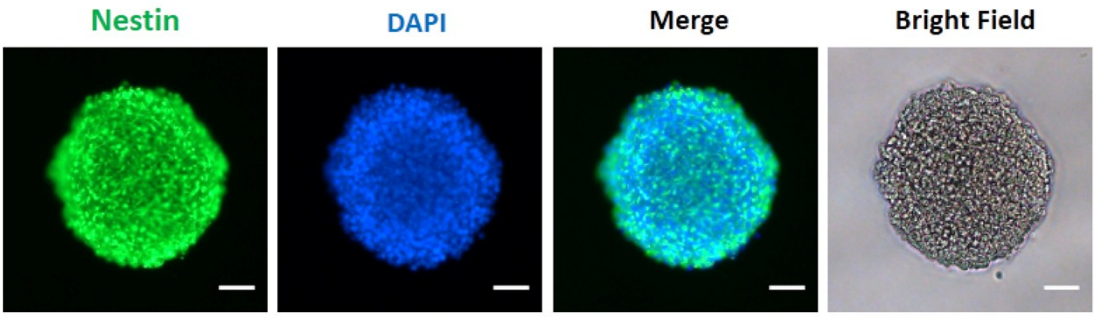

Figure S2. Nestin staining of the isolated NSCs. The NSCs were stained with Nestin-488 (green), the nuclei were labeled with DAPI (blue) and the morphology of fixed NSCs by phase contract microscopy in suspension culture. Scale bars, 50 μm.

In order to identify the characterization of isolated NSCs, we used immunofluorescence of Nestin staining to check the NSCs. For suspension staining, the process as follows: (1) Collect and rinse the cells with Phosphate Buffered Saline (PBS) once; (2) Fixe the cells with 4% paraformaldehyde at 4℃ for 15 minutes; (3) Wash the cells twice with PBS containing 0.5% bovine serum albumin (BSA) and 0.1% saponin (Buffer A) twice; (4) Resuspend the cells in small volume of Buffer A, add 1ug Nestin-488 antibody and incubate for 1 hour at 4℃; (5) Wash the cells twice with Buffer A, and add DAPI to incubate for 15min at room temperature; (6) Wash the cells twice and store the cells in PBS containing 0.5% BSA (Buffer B) at 4℃ in dark. Nestin is an intermediate filament protein that is typically expressed in NSCs. Our results showed that almost NSCs cultured both in suspension and adherence expressed Nestin positive.

S3. SEM images of NSCs differentiation on three substrates for 15 days

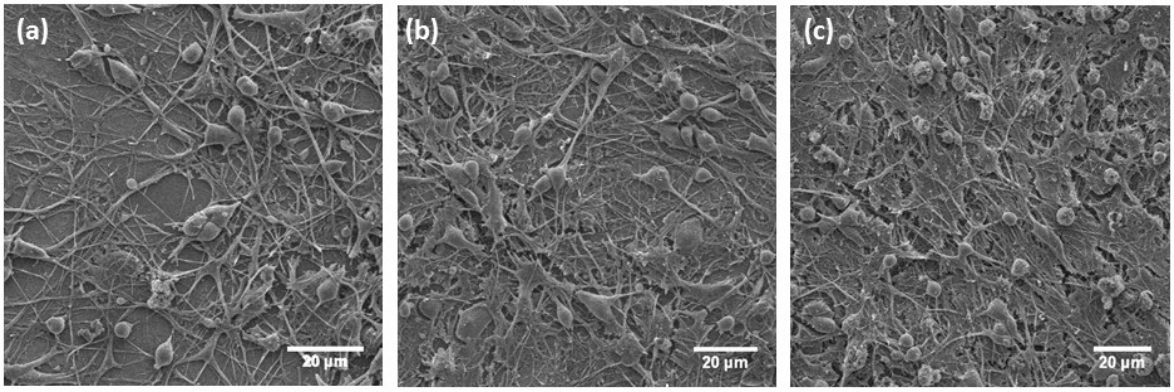

Figure S3. SEM images of NSCs differentiation for 15 days. TCP (a), FlatT (b) and Porous T(c). Scale bars, 20 μm.

The morphology of differentiated cells for 15 days on three substrates was also characterized by SEM images. Astrocytes are flat and neurons are round cell body with long neurite and dendrite. The morphology of cells on FlatT is almost same as TCP, but a little more cell number than TCP (Figure S3a, b). For cells on PorousT, almost astrocytes have covered the surface of PorousT, and neurons adhere on astrocytes to grow and spread (Figure S3C), and the number of cells is more than that on TCP (Figure S3a).

S4. MAP2 and GFAP staining of NSCs differentiation for 21 days.

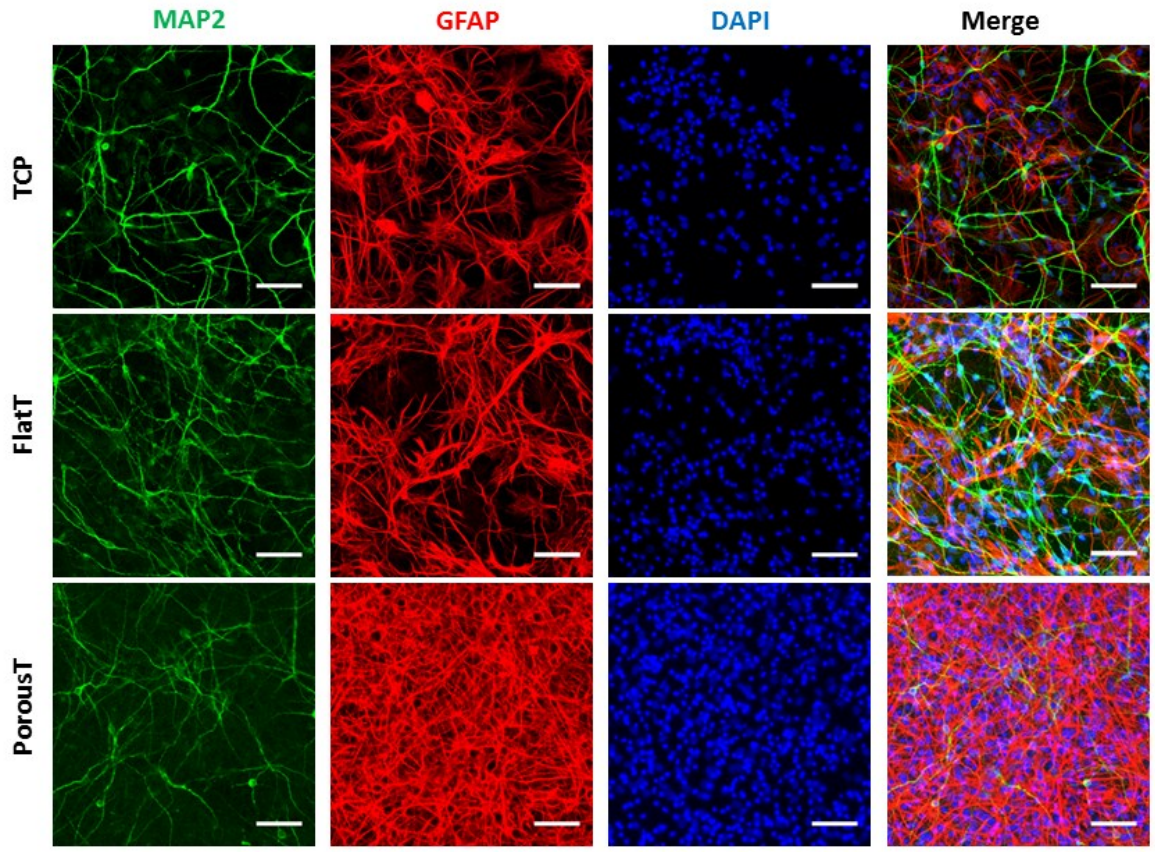

Figure S4. MAP2 and GFAP staining of NSCs differentiation on three substrates for 21 days. Scale bars, 50 μm.

Tuj1 is an early stage marker for neurons, and MAP2 (Microtubule-Associated Protein 2) is a strict marker. For 21 days differentiation, MAP2 expression was also checked by immunofluorescence. Compared to TCP, there is no significant difference of neurons and astrocytes differentiation on FlatT. But for PorousT, there is a significant increase of astrocytes number, and a little decrease of neurons differentiation. These results also confirm that FlatT can induce neurons differentiation and PorousT promote astrocytes differentiation.

S5. Fabrication of titanium dioxide ceramics micropattern.

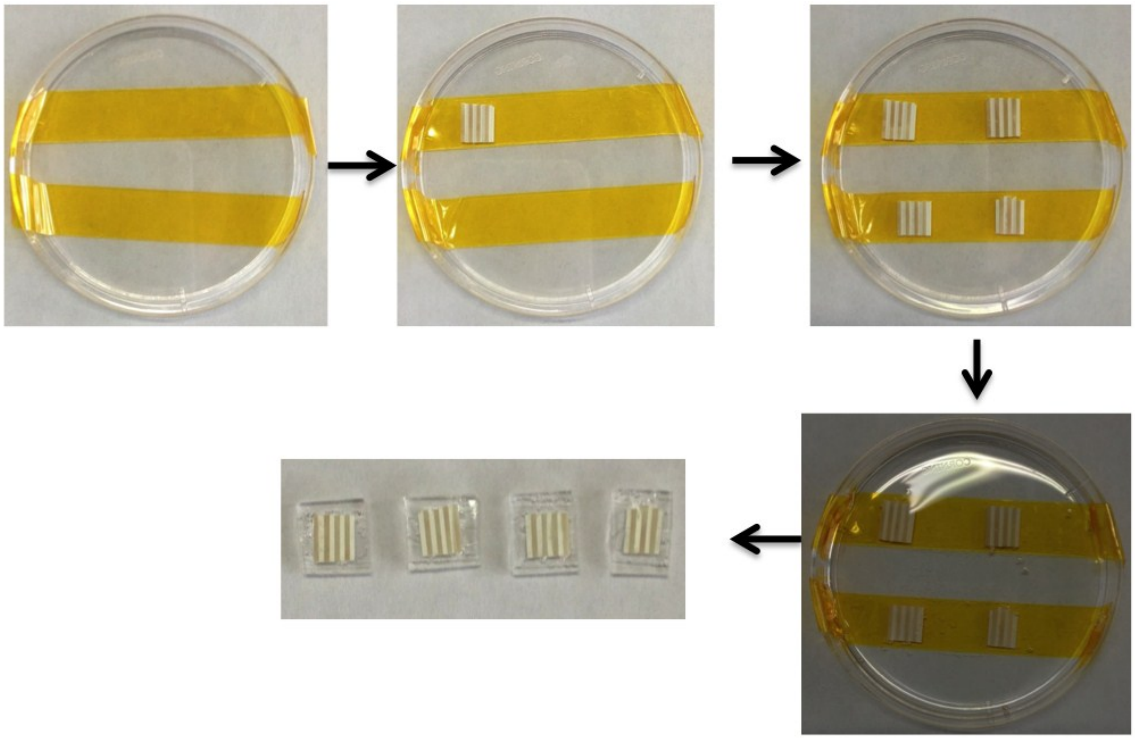

Figure S5. Fabrication of different topography micropattern. The images captured by digital camera show the process of micropattern fabrication, and the detail information can be found in Materials and Methods.

S6. NSCs localized committed differentiation on micropattern for 15 days

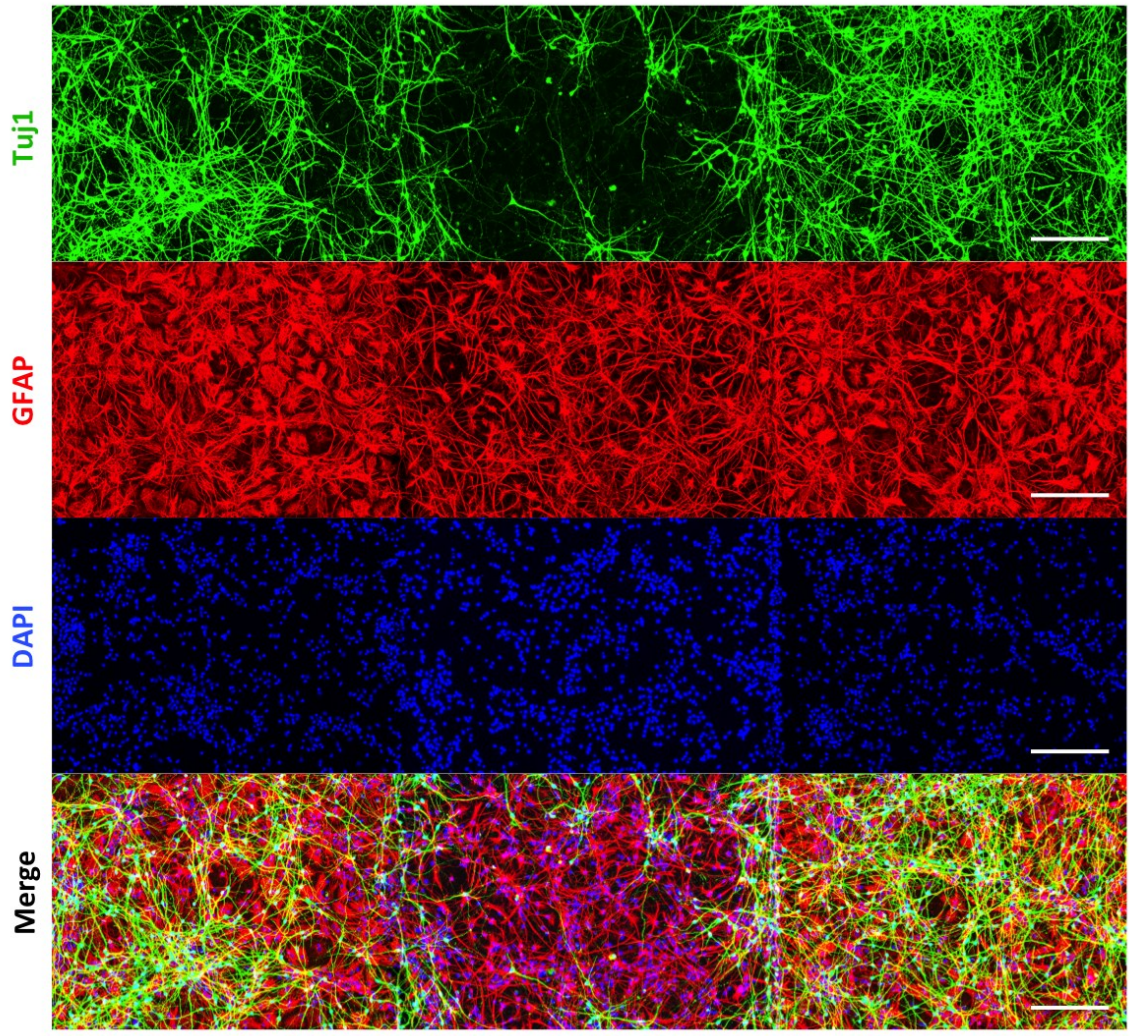

Figure S6. Localized committed differentiation of NSCs on micropattern for 15 days-Figure 3b4. The differentiated NSCs were immunostained with Tuj1 (green) for neurons, GFAP (red) for astrocytes, and DAPI (blue) for neuclei. Scale bars, 200 μm.

For astrocytes differentiation, there is a significant difference of cell morphology between PorousT and FlatT. For astrocytes on PorousT (media) have a fibroblast-like morphology- few and thin branches, and have a good proliferation in the culture (Figure S4). Astrocytes on FlatT and TCP have a process- bearing morphology-many and small branches, and infrequently divide (15 days-Figure 2a, 21 days Figure S4). It seems that NSCs on PorousT can differentiate into fibrous astrocytes, and NSCs on TCP and FlatT can differentiate into protoplasmic astrocytes. For neurons differentiation, there is a significant decrease on PorousT, compared to TCP and FlatT.

S7. Protein absorption of three substrates

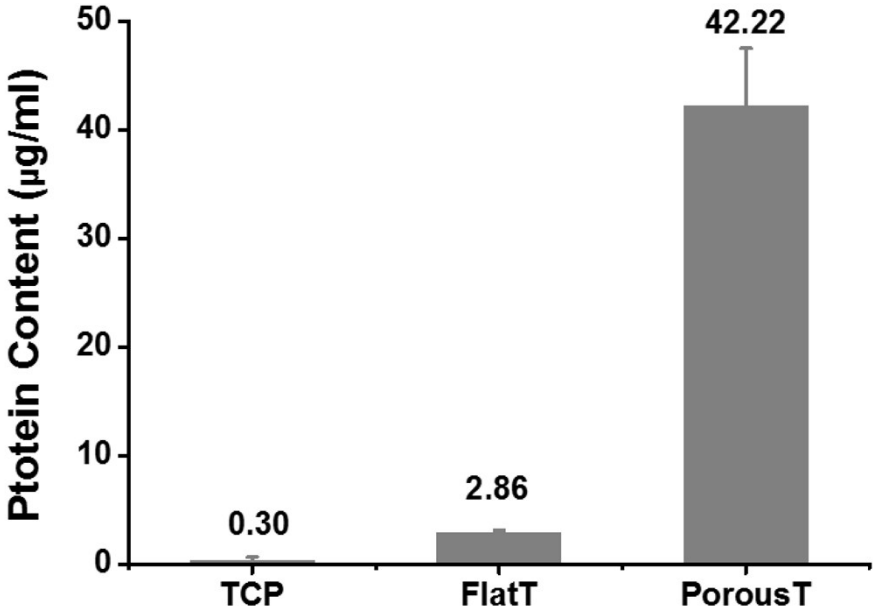

Figure S7. Evaluation of protein content on three substrates absorption by BCA assay. All data represented the mean ± standard deviation (n=3, statistics by ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

To evaluate the protein absorption of three substrates, we designed the experiment to test the capability of protein absorption on three substrates as follows:

(1) Soak the substrates in NSCs culture medium, and incubate for 72 hours at 37°C; (2) Transfer the substrates to a new 24 well plate, and wash three time with DPBS; (3) Aspirate DPBS, and add 400 ul 2% SDS to incubate for 1 hour at 37°C; (4) Collect the samples and do BCA assay, according to the manufacturer's instructions. The results show that the protein content of TCP, FlatT and PorousT absorption is 0.29 μg /cm^2, 2.86 μg /cm^2 and 42.21 μg/cm^2, respectively. There is a significant difference between the TiO_2 ceramics and TCP, and especially an obvious increase of protein absorption on PorousT.

S8. Primers sequences of qPCR

Table S1

Sequence of Real-Time PCR Primers			
Gene	5'-3'	Sequence	Size (bp)
GAPDH	F	AGGTCGGTGTGAACGGATTTG	123
	R	TGTAGACCATGTAGTTGAGGTCA	
Nestin	F	AGAGTCAGATCGCTCAGATC	93
	R	GCAGAGTCCTGTATGTAGCCAC	
Tuj1	F	TAGACCCCAGCGGCAACTAT	127
	R	GTTCCAGGCTCCAGGTCCACC	
MAP2	F	GCCAGCATCAGAACAAACAG	146
	R	AAGGTCTTGGGAGGGAAGAAC	
GFAP	F	CGGAGACGTATCACCTCTG	123
	R	TGGAGGCGTCATTCGAGACAA	

S9. Spontaneous calcium dynamics in differentiated neurons on FlatT. Movie S1

References:

1. Y. Wang, G. Du, H. Liu, D. Liu, S. Qin, N. Wang, C. Hu, X. Tao, J. Jiao and J. Wang, *Advanced Functional Materials*, 2008, **18**, 1131-1137.
2. T. Itoh, T. Satou, S. Hashimoto and H. Ito, *Neuroreport*, 2005, **16**, 1687-1691.