Dimensionality of Rolled-up Nanomembranes Controls Neural Stem Cell Migration

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Supporting Methods

Microtube Substrates for Cell Culture

Transparent silicon monoxide/ silicon dioxide (SiO/ SiO$_2$) microtube samples were fabricated as previously described.$^{1-3}$ Activation of the surface in an oxygen plasma (3 ppm O$_2$, 3 min, 50 W; Femto plasma system, Diener electronic, Ebhausen, Germany), silanization of the surface by incubation overnight in a 2% carboxyethylsilanetriol solution (ABCR, Karlsruhe, Germany) in sterilized water and subsequent functionalization with 0.02 mg/mL fibronectin (Sigma-Aldrich) in DPBS in the presence of 0.1 M N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (Sigma-Aldrich) and 0.025 M N-hydroxysulfosuccinimide (Sigma-Aldrich) at 37 °C for 3 h rendered the glass microtube samples suitable for cell culture. The samples were carefully rinsed with DPBS solution (Gibco®/ Invitrogen Carlsbad, USA) and stored at 4 °C until used.

Micropatterned Trenches for Cell Culture

16 mm x 16 mm glass cover slides (0.16 mm thickness, VWR, Germany) were cleaned with acetone, isopropanol and oxygen plasma and dried for 5 min at 120 °C on a hotplate. A 20 μm thick layer of negative photoresist (SU8-10, MicroChem Corp., USA) was spin-coated at 500 rpm for 10 s and 1650 rpm for 30 s onto the cleaned substrates, which were then pre-baked on a hotplate at 65 °C for 6 min and then soft-baked at 98 °C for another 10 min. The trench patterns were structured into the photoresist by gray scale lithography with a maskless aligner system (μPG 501, Heidelberg Instruments, Germany). Plateau areas were exposed with 80 % lamp intensity ($\lambda = 390 \pm 2$ nm) for 1.5 s per pixel. 16 μm wide and 6 μm deep trenches were created by exposing stripes with a transversal intensity variation (stripe pattern width 20 μm and
illumination at 8 % lamp intensity with an increase to 64 % at the rims starting 5 μm away from the sides). The exposure was followed by a two-step post-baking process of 2 min at 65 °C and 12 min at 98 °C, before the samples were immersed in a bath of SU-8 Developer (MicroChem Corp., USA). Soaking in a bath of isopropanol stopped the development process. The coating of the samples with Al₂O₃ and subsequent surface functionalization was achieved according to the same protocol as for the microtube substrate preparation. The trench profiles of the ALD-coated samples were investigated with a VK-X210 confocal microscope (Keyence Deutschland GmbH, Germany).

**NSC Derivation and Maintenance**

Male and female Nestin-GFP mice⁴ were housed, bred and treated according to the guidelines of the European Community (86/609/EEC). The local animal rights committee approved all experiments. NSCs from the cortex region of E14 mouse embryonic brains were isolated according to Meyer et al.⁵ Dissociated cells were maintained in a 2:1 mixture of high glucose DMEM (Sigma-Aldrich) and Ham's F-12 Nutrient Mixture (Life Technologies GmbH, Germany), complemented with 2 % B-27 supplement (50x, Life Technologies GmbH), 1 % penicillin/ streptomycin (10000 U/mL, Life Technologies GmbH) and 20 ng/ml each of Egf and Fgf-2 (Sigma-Aldrich). Adherent cell monolayers formed spontaneously when plating the cells onto 15 μg/mL poly-L-ornithine (Sigma-Aldrich)/ 4 μg/mL fibronectin-coated 25 cm²-tissue culture flasks (Greiner Bio-One GmbH, Germany). Cultures were kept in a humidified incubator at 37 °C and 5 % CO₂. Fresh growth factors were provided every two to three days and the subconfluent cell monolayers were passaged every 5-7 days using Accutase (PAA Laboratories GmbH, Austria).
**Immunocytochemistry**

For the immunofluorescent staining of the in vitro grown NSCs, the samples were washed once with DPBS and fixed with ice-cold ethanol (Sigma-Aldrich) for 12 min at -20 °C. After washing with DPBS and permeabilization with 0.1 % Triton X-100 in DPBS for 15 min, the samples were immersed for 45 min in a 1 % BSA solution (Sigma-Aldrich) in DPBS to block unspecific bindings. Cells were then incubated overnight at 4 °C in the 1 % BSA solution containing a primary antibody against fascin (mouse, 1:500, EMD Millipore Corporation, USA). The samples were carefully washed three times for 5 min each with DPBS and then incubated for 1 h at room temperature with the 1 % BSA solution containing an Alexa647-conjugated secondary antibody (donkey anti-mouse, 1:400, Molecular Probes, USA), phalloidin-rhodamine (1:200, Life Technologies GmbH) to stain for actin filaments and 4′,6-diamindino-2-phenylindole (DAPI; 1:1000, Life Technologies GmbH) to stain the DNA. After another DPBS washing step, the samples were mounted and stored at 4 °C until being imaged. Since the microtube and control glass slide samples were glued to 3.5 cm Petri dishes, a small drop of mounting medium (Vector Laboratories, UK) was applied to the circular region of interest and a 13 mm round coverslip (VWR) was carefully placed on top of the substrates. The edges were sealed with conventional nail polish. Optical images of the fixed samples were taken with a Zeiss LSM 700 inverse confocal laser scanning microscope.

**Immunohistochemistry**

Fixed Tbr2 (eomes)-GFP mice brains were a generous gift of W.B. Huttner. 40 μm sections of the brains were obtained at a cryotom (Reichert Jung Frigocut 2800, Reichert Jung, USA) and mounted on glass slides. Mounted sections were preincubated with 3 % blocking serum containing 0.2 % Triton X-100 in PBS for 90 min at room temperature and then incubated
overnight at 4 °C with primary antibodies followed by secondary fluorescence conjugated antibodies for 1 hour at room temperature. The following primary antibodies were used: fascin (mouse, 1:500, EMD Millipore Corporation, USA) and phalloidin-rhodamine (1:200, Life Technologies GmbH) to stain for actin filaments. Alexa647-conjugated secondary antibody (donkey anti-mouse, 1:400, Molecular Probes) was used to visualize fascin, and DAPI (1:1000, Life Technologies GmbH) to stain the DNA. Samples were mounted with Vectashield (Vector Laboratories, CA) under a glass cover slip. Images were taken at a Zeiss LSM-780 confocal microscope of the light microscopy facility BIOTEC/CRTD (Dresden, Germany). Cells were imaged that had already acquired Tbr2-GFP autoimmunofluorescence but were still migrating towards the bulk of Tbr2-GFP positive cells in the subventricular zone.

**Motility Experiments**

5x10^5 NSCs were seeded on fibronectin-functionalized microtube samples. Alternatively, 5x10^4 NSCs were seeded on Al_2 O_3-coated and fibronectin-functionalized control glass cover slides. The cell density was adjusted to enable the observation of single cell motility either inside the microtubes or on the planar substrates one to two days after seeding. Live-cell imaging was performed using a Zeiss Axio Observer Z1 inverse microscope equipped with a 37 °C heated stage and CO_2 chamber. The software Axio Vision Rel. 4.8 (Carl Zeiss, Inc.) was used for the image acquisition. NSC motility was observed with differential interference contrast (DIC) imaging every 2 min for 4 h.

**Inhibitor Experiments**

Samples were prepared and imaged according to the protocol for motility experiments. Additionally, the respective inhibitor solution in DMSO (Merck, Germany) was added to the cell culture medium after the equilibration of the samples in the heated microscope stage inside the
CO₂ chamber. The cells were treated with the Cdc42 inhibitor ML 141 (40 μM, Sigma-Aldrich), the Arp2/3 complex inhibitor CK-636 (200 μM, Sigma-Aldrich), the actin polymerization inhibitor latruncutlin A (0.02 μg/mL, Life Technologies GmbH), the myosin II inhibitor blebbistatin (5 μM, Sigma-Aldrich) or pure DMSO as a control. The ML 141 and CK-636 inhibitor concentrations were optimized to impede the respective cell protrusions while still maintaining NSC motility on a planar surface. The final DMSO concentration in the cell culture medium did not exceed 0.1 % v/v.

**Data Analysis**

The acquired fluorescent images and DIC time series were processed and analyzed with Fiji (distribution of ImageJ 1.48q, 64 bit).

For the analysis of the cell shape descriptors, Nestin-GFP fluorescence for the *in vitro*-grown cells or Tbr2-GFP fluorescence of the *in vivo* cells in fixed brain slices was recorded. For the planar glass and microtube substrates, images of the NSC circumferences were taken (Zeiss Axio Observer Z1, Zeiss HXP 120 UV lamp). For the *in vivo* grown cells, where the cell orientation not necessarily laid within the focal plane of the microscope, image stacks of different focal planes (z- stacks) at a slice distance of 0.5 μm were taken of fixed brain slices (Zeiss LSM 780). Afterwards, maximum intensity projections of the z-stacks were generated. A Gaussian blur filter (0.4 μm scaled units) was applied to the images. They were thresholded (Fiji default settings, “dark background” active) and the cell “footprints” were characterized with the particle analyzer plugin. The cell spreading area ($A$), the perimeter ($P$) of the spreading area, the circularity ($\text{Circ}$) defined as:

$$
\text{Circ} = \frac{4\pi A}{P^2}
$$
and the aspect ratio as the ratio of major to minor cell axis were evaluated.

For the quantification of the z-height and volume of the cell bodies, z-stacks at a slice distance of 0.5 μm were acquired of fixed NSCs stained for actin (in vitro-grown cells) or of fixed Tbr2-GFP NSCs (brain slices). To assess the z-height of the cells, a cut view of the z-stacks was generated and the z-dimension was measured from the thresholded (Fiji default settings, “dark background” active) images. For the quantification of the cell volume, the cell area on each slice of the z-stack was measured according to the cell spreading area evaluation procedure, and then multiplied with the slice distance of 0.5 μm. For the z-height and cell volume evaluation only microtubes with dimensions larger than the cell diameter were considered so that the increase in cell dimensionality inside the glass tubes was not restricted by the microtube walls. Thereby NSCs of different sizes were all included in the quantification and a bias due to a potential cell size selection through the microtube diameter was avoided.

Cell trajectories were recorded with the MTrackJ plug-in. Every 2 min the cell position was marked by manually clicking on the cell center. From the x and y-positions of the cells, the average velocity for each track (length of cell trajectory divided by length of time) and the arrest coefficient of the cells were calculated. The arrest coefficient is the percentage of time that a cell moved slower than a certain value, which we set as the average velocity of 15 resting cells without a noticeable displacement, namely 0.27 μm/min.

The mean squared displacement (MSD(τ)) is defined as the average squared Euclidian distance a cell has covered after a certain time interval τ and was calculated by using the equation:

$$MSD(τ) = \langle [x(t + τ) - x(t)]^2 + [y(t + τ) - y(t)]^2 \rangle$$
The resulting $MSD(\tau)$ curve characterizes the area that a cell has explored in the system and therefore is a measure of migration efficiency. On longer timescales, the slope of the curve indicates the type of motion involved, e.g. random walk or anomalous diffusion.

**Statistics**

For each condition, a minimum of three independent experiments were conducted. Data are presented as mean ± standard deviation. For the Tukey box plots, the box frames data between the 25$^{th}$ and 75$^{th}$ percentile (interquartile range IQR); the line shows the median, the square the mean value and the whiskers indicate the upper and lower fence of the data set (1.5 x IQR). Statistical analysis was performed employing the Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn’s multiple comparisons post test using GraphPad InStat 3 (Version 3.10, 32bit, GraphPad Software, Inc., La Jolla, USA). A value of $p < 0.05$ was considered as statistically significant.
Supporting Figure

**Figure S1.** Suitability of functionalized substrates for NSC cell culture. a) Cultivation of NSCs on a fibronectin-functionalized sample for 7 d. Arrowheads point to round cells on 2D. b) DIC images of a dividing NSC at different time points that are indicated as h:min. c) Incubation with 12 μM propidium iodide helps to distinguish apoptotic cells stained in red from intact GFP-Nestin expressing cells (green). The scale bar in a) equals 50 μm, in b) and c) 10 μm each.
Supporting Videos

**Video S1.** Reversibility of microtube-triggered NSC morphology change (compare Fig. 1d).

**Video S2.** Elongated morphology of an NSC moving inside a 10 μm wide and 6 μm deep trench. Cells moving on the planar surface around the trench appear out of focus.

**Video S3.** Comparison of *in vitro* cell shapes with *in vivo* morphology. Shown are 3D-reconstructed confocal stacks of an NSC on a planar substrate (2D, left), in a microtube (middle) and in the developing brain *in vivo* (3D, right) (compare Fig. 2a).

**Video S4.** NSC changes its direction of movement after filopodia reorganization (compare Fig. 3a).

**Video S5.** Elongation of a round NSC inside a microtube upon Cdc42 inhibition (start: 2 h after treatment; compare Fig. 3b).

**Video S6.** Preservation of round NSC morphology and occurrence of blebbing upon Arp2/3 complex inhibitor treatment inside a microtube (start: 2 h after treatment; compare Fig. 3c).

**Video S7.** NSC track on a planar (2D) substrate (compare Fig. 4a).

**Video S8.** NSC track inside a microtube (compare Fig. 4b).

**Video S9.** Appearance of blebs at both cell ends in a microtube with a diameter of 6 μm.

**Video S10.** Inhibition of actin polymerization with a low dose of latrunculin A leads to a reduced NSC motility and spreading of the cell when moving (compare Fig. 4g).
**Video S11.** Inhibition of cell contractility with blebbistatin does not interfere with filopodia-based migration, but leads to a prolongation of cell attachment to the microtube walls (compare Fig. 4h).

**Supporting References**

1. Harazim, S. M.; Xi, W.; Schmidt, C. K.; Sanchez, S.; Schmidt, O. G. *J. Mater. Chem.* **2012**, 22, 2878-2884.

2. Koch, B.; Sanchez, S.; Schmidt, C. K.; Swiersy, A.; Jackson, S. P.; Schmidt, O. G. *Adv Healthcare Mater* **2014**, 3, 1753-8.

3. Xi, W.; Schmidt, C. K.; Sanchez, S.; Gracias, D. H.; Carazo-Salas, R. E.; Jackson, S. P.; Schmidt, O. G. *Nano Lett.* **2014**, 14, 4197-204.

4. Mignone, J. L.; Kukekov, V.; Chiang, A. S.; Steindler, D.; Enikolopov, G. *J. Comp. Neurol.** **2004**, 469, 311-24.

5. Meyer, A. K.; Jarosch, A.; Schurig, K.; Nuesslein, I.; Kissenkotter, S.; Storch, A. *Brain Res.** **2012**, 1474, 8-18.

6. Meijering, E.; Dzyubachyk, O.; Smal, I. *Methods Enzymol.* **2012**, 504, 183-200.

7. Boissonnas, A.; Fetler, L.; Zeelenberg, I. S.; Hugues, S.; Amigorena, S. *J. Exp. Med.* **2007**, 204, 345-56.