Neuronal networks *in vitro*: formation and organization on biofunctionalized surfaces

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Receptor-mediated recognition of substrate molecules is a prerequisite for nerve cells in order to find their target structures *in vivo* and leads to formation of neuronal connections and networks. In order to study these mechanisms under *in vitro* conditions, we cultured embryonic hippocampal neurons or neuronal cell lines, SH-SY5Y and PCC7-PCC7-Mz1, onto biofunctionalized surfaces. Micropatterning on polymer surfaces, glass- and silicone-oxide-based chip materials was performed in a micrometer range by microcontact printing using polydimethylsiloxane (PDMS) stamps. Hippocampal neurons were found to form networks on chip surfaces under serum-free conditions and remained functional for more than a week. Human neuroblastoma cells SH-SY5Y as well as PCC7-Mz1 stem cells were found to follow microcontact printed pattern on polystyrene surfaces. Both cell lines showed neuronal marker expression and were cultured for up to 7 days with serum containing culture medium. Widths of 3–5 μm of coating lines were found to enhance single cell spreading along the pattern. The techniques described in this study may be useful in promoting nerve cell regeneration and organization following transection due to trauma or surgery. The neuronal alignment and network formation *in vitro* may furthermore serve as a model system in the field of biosensors.

1. Introduction

A variety of applications have been described for the immobilization of cellular adhesion molecules or ligands to surfaces of biomedical relevant materials. In general, the goal of such approaches is to render the surfaces of synthetic materials bioactive for a particular application by enhancing favorable cellular reactions or interactions. Thus, a biomaterial can control cell attachment, differentiation, and tissue organization [1, 2]. The extracellular matrix (ECM) of nerves contains cell-attractive as well as cell-repulsive cues which act to direct regenerative processes [3]. Of the ECM the 600–800 kD protein laminin in particular has been shown to guide neuronal growth cones. It is leading neurite outgrowth and directing cells to form network structures [4, 5]. Laminin contains different cell binding domains including IKVAV at 2116–2120 in the A chain.

In order to study these mechanisms under *in vitro* conditions, we cultured embryonic hippocampal neurons and neuronal cell lines, SH-SY5Y and PCC7-Mz1 onto biofunctionalized surfaces. Laminin or binding sequences containing peptides were patterned onto different substrates, polystyrene, tissue culture polystyrene, glass and silicone-oxide-based chip surfaces. Cell adhesion, growth, differentiation and neurite outgrowth were examined in order to describe possible neuronal alignment and network formation *in vitro* as a model system in the field of biosensors and neuroscience.

2. Materials and methods

2.1. Reagents and materials

A synthetic oligopeptide, including the IKVAV-containing laminin binding sequence, PA22-2 (Sigma, Germany) was covalently bound to a silicon-oxide-based material surface via aminosilanization and cross-linker chemistry as described previously [6]. In other experiments PA-22-2 or laminin (Boehringer, Germany) in a concentration of 10 μg/ml or 20 μg/ml, respectively was contact printed and physisorbed on either glass coverslips, tissue culture polystyrene (TCPS) or polystyrene (Falcon, Greiner, Germany) surfaces. Micropatterning was achieved by using microcontact printing. Stamps were fabricated by casting polydimethylsiloxane (PDMS) on a master pattern formed by photoresist on glass. The grid consisted of 3, 5, 8 and 10 μm lines either with or without crossing at 50 μm in one and at 100 μm in the other direction. In addition a pattern of 50 μm per 50 μm for a width of 5 μm was used. Surface modifications and pattern formation were confirmed by several means, including immunofluorescence and interference contrast microscopy. Atomic force microscopy (AFM), time of flight secondary ion mass spectroscopy

(ToF-SIMS) and X-ray photoelectron spectroscopy (XPS) was performed as described earlier [6].

2.2. Cell culture

Primary hippocampal neurons were isolated from embryonic rats as described in [4], and SH-SY5Y human neuroblastoma cells were cultured as described in [7]. PCC7-Mz1 mouse embryonal carcinoma cells were cultured and differentiated in [8]. Cells were cultured in Dulbecco's modified minimal essential medium (DMEM; Gibco, Germany) with additional L-glutamine, 4.5 g l^{-1} glucose and 12.5% fetal calf serum in a humidified atmosphere containing 5% CO_2. Tissue culture was supplied by Falcon, Becton Dickinson, Heidelberg, FRG.

2.3. Preparation of cell cultures

In order to prepare test surfaces, cells were detached from the culture and resuspended as a single cell suspension in culture medium. SH-SY5Y were detached using trypsin and ethylenediamine tetra-acetic acid (EDTA). The cells were washed, counted and seeded at a density of 20 000 cm^{-2}. Induction of differentiation of PCC7-Mz1 was performed by addition of all-trans retinoic acid (RA) to a final concentration of 0.1 μM 24 h after cell seeding on the surfaces. Cells were washed with prewarmed medium at days 1 and 2. At day 3 samples were washed twice in 37 °C warmed cytoskeleton (CS) buffer pH 6.9 containing 0.1 M PIPES, 1 mM egtazic acid (EGTA), 4% polyethylene glycol (PEG) and 0.1 M NaOH, followed by a 15 min fixation step in 3.7% paraformaldehyde (PFA) in CS buffer. Cells were washed twice and stored overnight for immunofluorescence staining. Cellular differentiation was analyzed by immunocytochemical staining for the marker Tau using a rabbit-anti-tau antibody (Sigma T6402, Germany) 1 : 100. Rat anti-EX1 was used as cell supernatant. As secondary antibodies $F(ab')_2$-fragments with either fluoroscein isothiocyanate (FITC) or Cy-3 were used.

2.4. Microscopical evaluation

Cells have been characterized by using light microscopy, phase contrast microscopy as well as fluorescence microscopy and graded for cell loss, cell death or morphological changes. Dual-label fluorescence microscopy was performed by the use of a DM-IRBE microscope (Leica, Bensheim, Germany). The development of a neuronal cell alignment and network was recorded by using time-lapse video microscopy. Photographs as well as digital images were taken.

3. Results and discussion

We demonstrate that the surface modification performed resulted in patterned biofunctionalization of the surface which induced the hippocampal neurons to form a neuronal network on the coated surface areas, as shown in Fig. 1. Cells expressed the tau-protein that was immunostained using an FITC-labeled antibody, demonstrated in Fig. 1. The dimensions of the adhesive pattern were found to be a critical factor in setting directional motility, axon targeting and the formation of neuronal connections. Most of the cell bodies were positioned on the cross points of the pattern within 24 h of plating. Migration of cells plated in between two cross points in the direction to the nodes was observed for patterns of 5, 8 and 10 μm diameter, whereas on patterns of 3 μm diameter, the cells did not migrate (data not shown). Immunocytochemistry and patch clamp recordings revealed that the patterned neurons expressed neuronal markers and were fully functional with respect to electrochemical properties correlating to earlier findings [6].

SH-SY5Y cells as well as PCC7-Mz1 did adhere and proliferate on TCPS, silicone-oxide-based chip surfaces and glass and covered the surface irrespective of the pattern. In addition, for RA-differentiated PCC7-Mz1 the formation of typical cell clusters consisting of glial cells, neurons and fibroblast phenotypic cells was observed. This is in accordance with earlier findings [9].

SH-SY5Y cells showed elongated morphology and succeeded in forming extensions along the pattern lines. For a width of 20 μm a doubled line of SH-SY5Y was found, as shown in Fig. 2. Some cells formed small aggregates and entered the space between the lines by branches. For a width of 10 and 5 μm mostly single cells were found on the lines forming long cellular extensions. Fig. 2 demonstrates that for a width of 3 and 5 μm, respectively, differences in the cell pattern morphology were not observed. Tau was clearly expressed by SH-SY5Y at this state. A diffuse cytoplasmatic expression including part of the extensions were found for cells on the pattern, as shown in Fig. 3.

For the PCC7-Mz1 cell culture model one has to take into account that these cells succeed in *de novo* acquisition of neuronal polarity [5]. This is in contrast to most other cell lines of neuronal phenotype. In addition, PCC7-Mz1 derived differentiated cells are of ectodermal origin, exclusively, whereas e.g. F9, NG2 or P19 include mesodermal and endodermal cells. For a laminin concentration of 20 μg ml^{-1} on a width of 20 μm pattern cells followed the directions of the pattern clearly as shown in Fig. 4a. However, the distinct observation of single cells and cellular interconnections by, for example, extended gate electrode array-based biosensors appears to be critical. In contrast for a line width of 3 μm and a reduced laminin concentration of 10 μg ml^{-1} PCC7-Mz1 cells formed distinct connections with long extensions between smaller cell clusters, as shown in Fig. 4b. After a 7 day culture and 6 days after RA-induced induction of cell differentiation cells showed expression of tau in the cytoplasm as well as in parts of distal branches, as demonstrated in Fig. 5. EX1 staining was less intensive and stained the total cell including extensions (data not shown). The differentiation progress is in accordance with earlier findings on laminin adsorbed surfaces without a pattern [9]. Other authors describe B104 neuroblastoma cells that were cultured on PDMS-stamped polylysine [10] on glass. Besides laminin further neuroinductive proteins or peptides are used, including heparin-binding peptides ATIII, NCAM and PF4, that were shown to significantly increase neurite extensions in the dorsal root ganglia model [11].

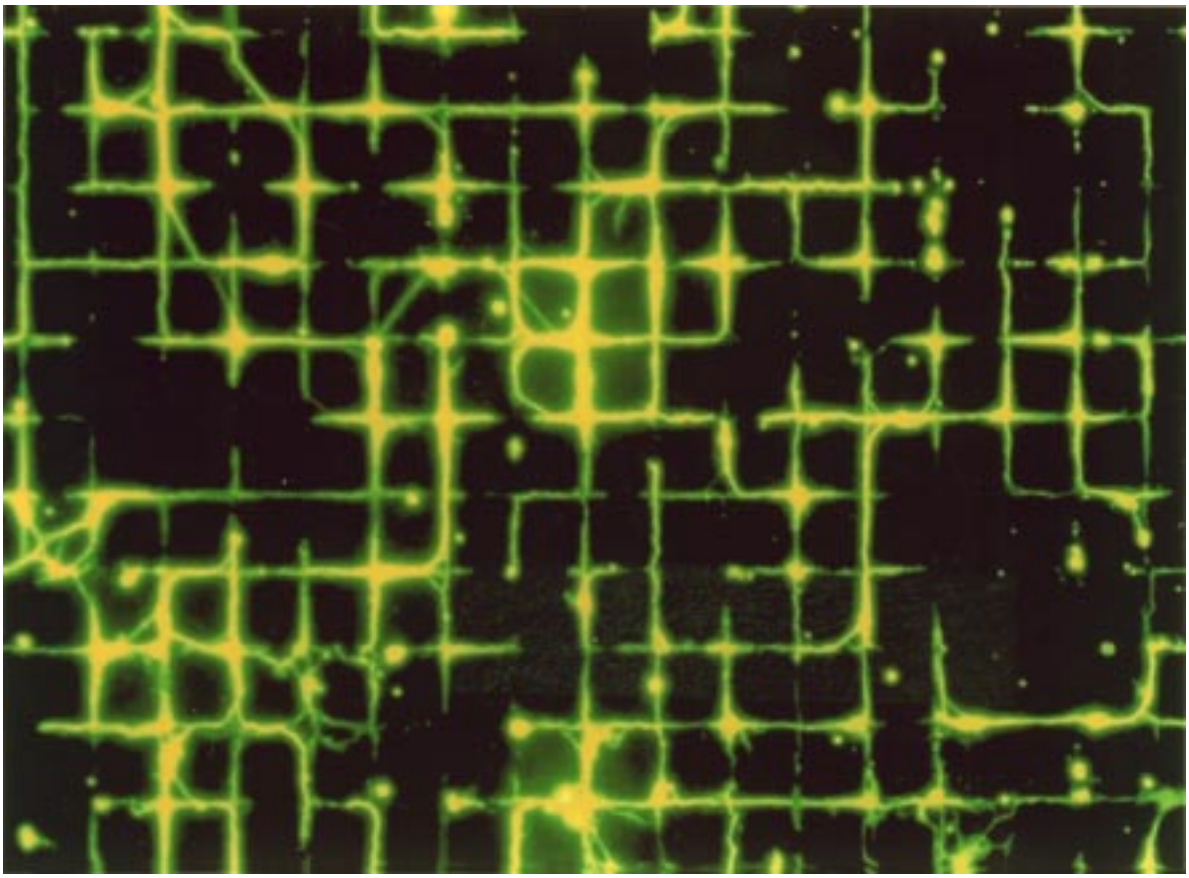

Figure 1 Hippocampal neuronal cells on a PA22-2 micropatterned surface, fluorescence micrograph of FITC staining of the tau-protein, bar = 100 μm.

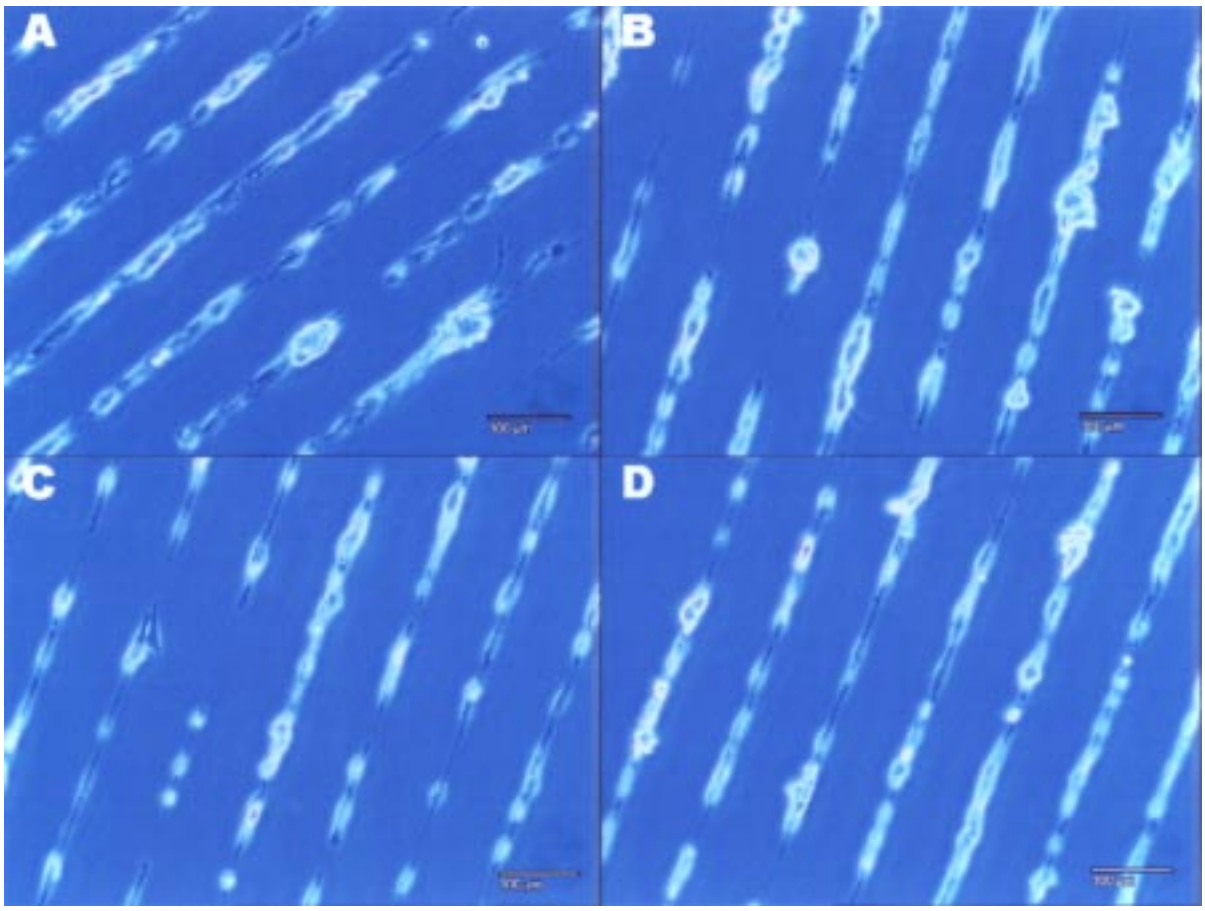

Figure 2 Human neuroblastoma SH-SY5Y on line patterned laminin width (a) 20 μm (b) 10 μm (c) 5 μm or (d) 3 μm on polystyrene after 3 days in culture: phase contrast micrographs.

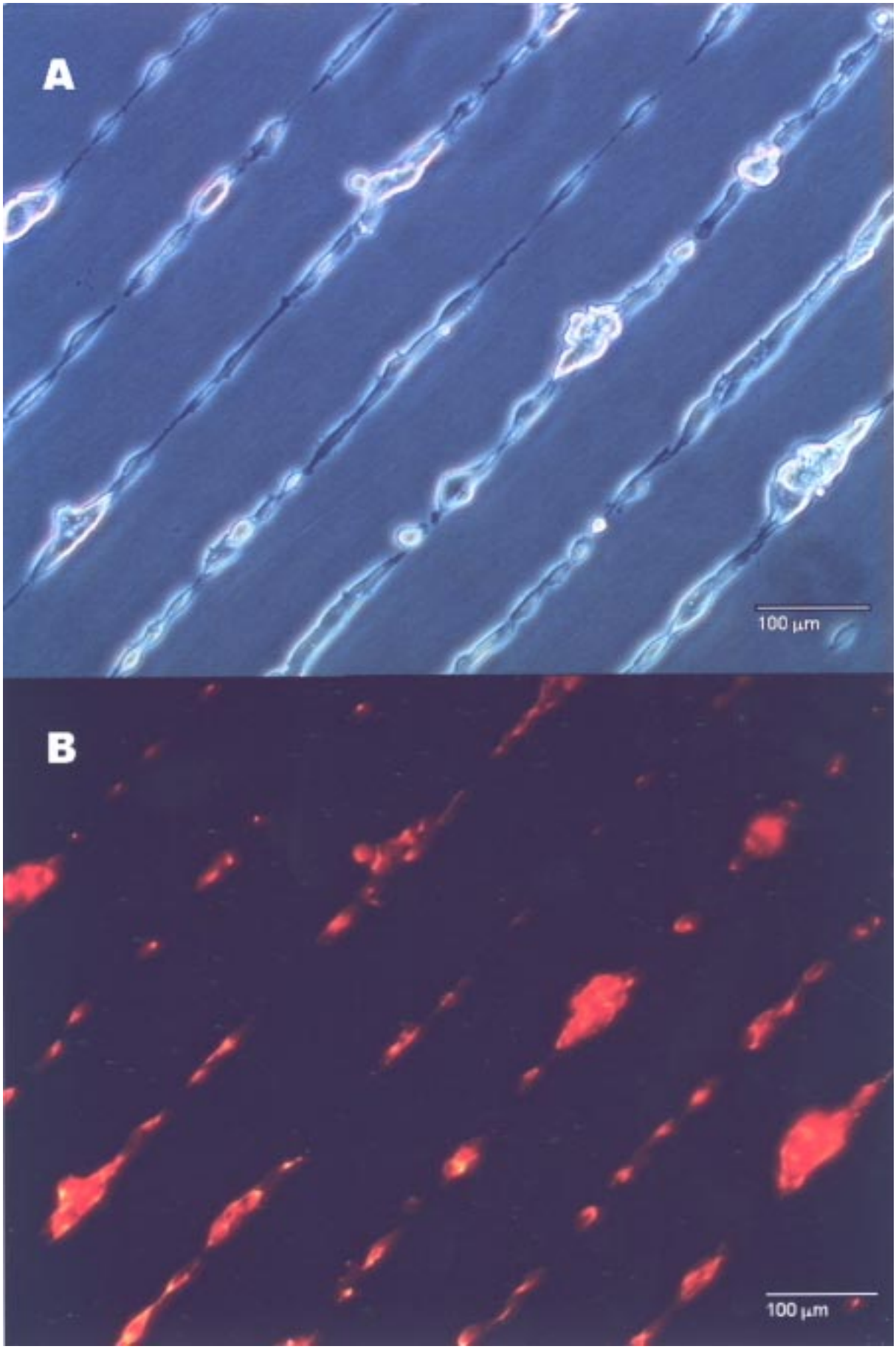

Figure 3 Human neuroblastoma SH-SY5Y on line patterned laminin width 5 μm on polystyrene after 3 days in culture: (a) phase contrast micrograph (b) fluorescence micrograph of immunofluorescence staining for tau-protein.

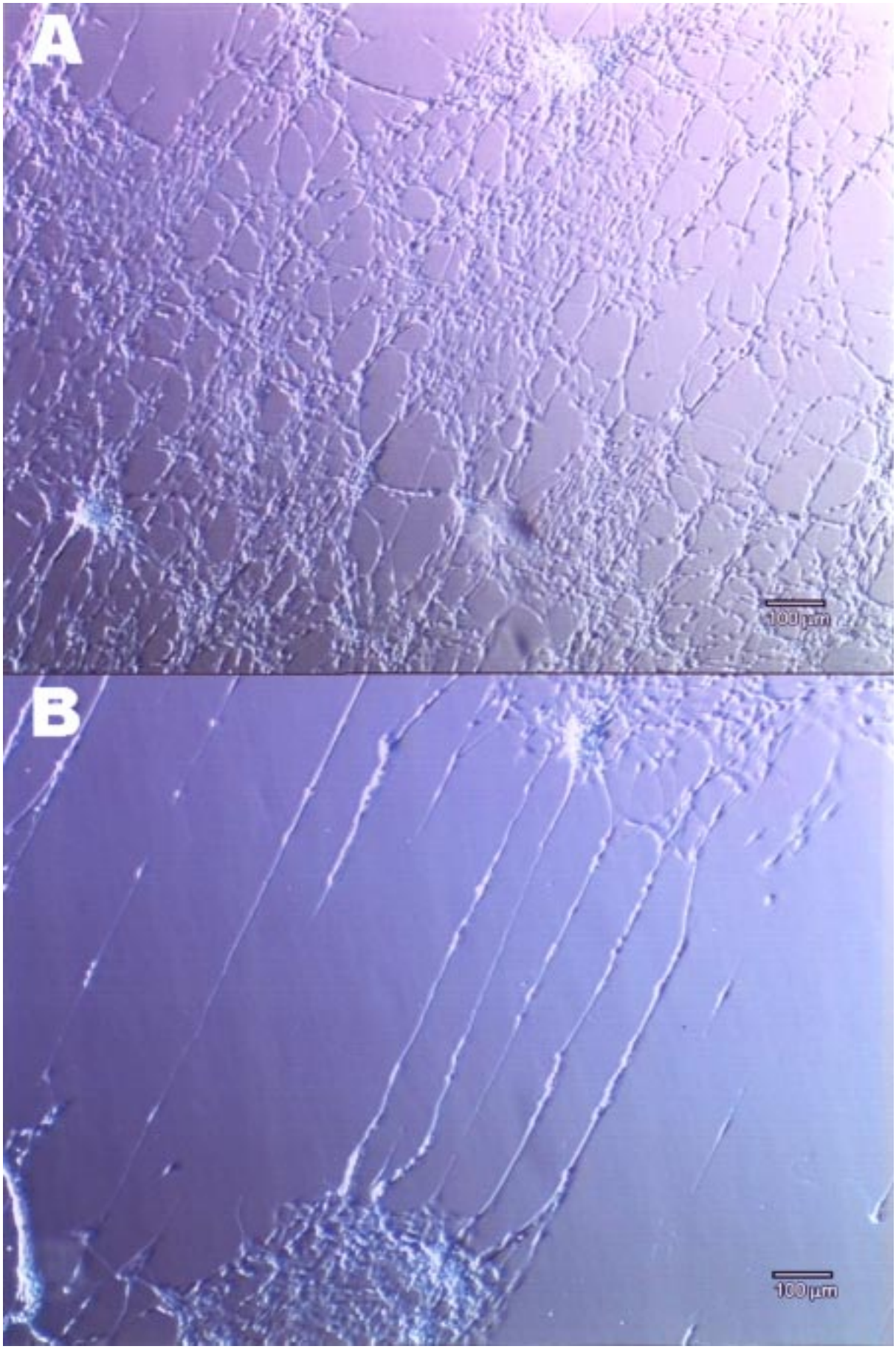

Figure 4 PCC7-Mz1 mouse embryonal carcinoma cells on line patterned laminin width (a) 20 μm (b) 5 μm on polystyrene after 7 days in culture, 6 days after RA-induced differentiation: difference interference contrast micrographs (DIC).

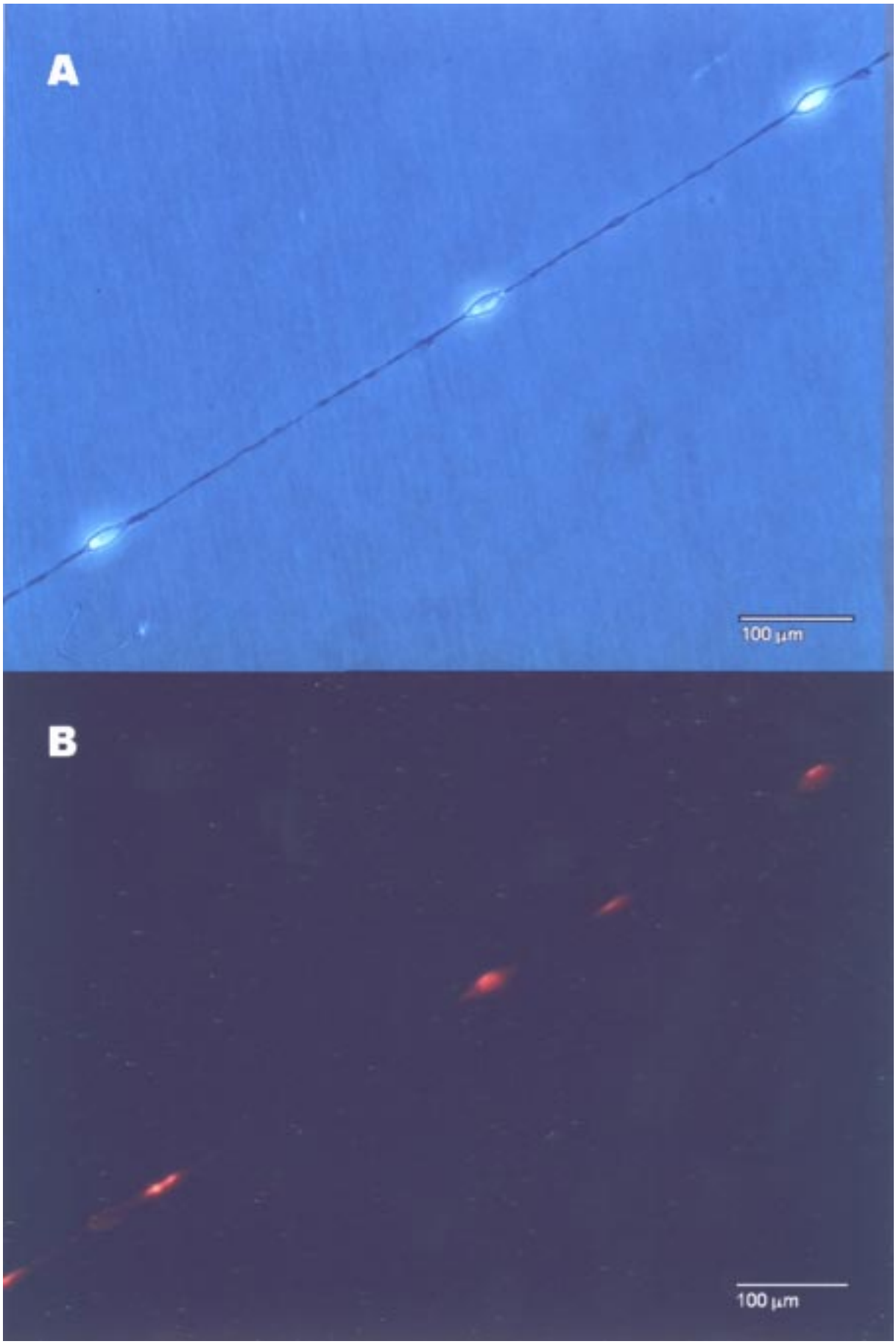

Figure 5 PCC7-Mz1 mouse embryonal carcinoma cells on line patterned laminin width 3 μm on polystyrene after 7 days in culture, 6 days after RA-induced differentiation: (a) phase contrast micrograph (b) fluorescence micrograph of immunofluorescence staining for tau-protein.

4. Conclusions

Micropatterning was performed successfully by microcontact printing using PDMS stamps. Hippocampal neurons form alignments and networks on chip surfaces. Two cell lines, human neuroblastoma cells SH-SY5Y as well as PCC7-Mz1 stem cells were found to follow the microcontact printed pattern. Both cell lines showed neuronal marker expression and neuronal phenotype. The model systems described in this study may be useful in investigation of nerve cell regeneration and organization on biomaterials. The neuronal alignment and network formation *in vitro* may furthermore serve as a model system in the field of biosensors.

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