

## Investigation of Nanofibrillar Influence on Cell-Cell Interactions of Astrocytes by Epi-fluorescence and Atomic Force Microscopies

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### ABSTRACT

Long distance intercellular communication between astrocytes on nanofibrillar and planar surfaces was investigated by epi-fluorescence microscopy and atomic force microscopy. We found that astrocytes on nanofibrillar surfaces and astrocytes on planar surfaces diverged in apparent cell-cell contact structures. Astrocytes on nanofibrillar surfaces exhibited a “single cellular process” response, while astrocytes on planar surfaces exhibited a filopodial network response. The possibility that astrocytes can sense their geometrical environment and form different cell-to-cell contacts on nanofibrillar versus planar surfaces, with activation of different signaling pathways, is discussed.

### INTRODUCTION

Cell-cell interactions via direct connective bridges over distances that are much larger than traditional cell membrane-to-cell membrane junction distances are currently of great interest. These interactions are potentially overlooked vectors for the exchange of genetic information or for viral transmission [1]. In the present work, epi-fluorescence microscopy and atomic force microscopy (AFM) were used to investigate the possible long distance (> 50  $\mu\text{m}$ ) cell-cell interactions of astrocytes in detail. Astrocytes are key cellular components of the central nervous system that function as cellular intermediaries between capillaries and neurons and also provide a mechanical support environment [2]. In a wound-healing situation, astrocytes form a continuous layer that seals off the wound site. Intercellular communication, initiated prior to cell body contact, may therefore play a role in wound healing.

Cultured astrocytes have been shown by our group to support enhanced neurite outgrowth in co-culture with cerebellar granule neurons when they are plated on synthetic polyamide nanofibrillar surfaces compared with planar plastic (ACLAR) surfaces [3]. This may indicate a more physiologically relevant function for astrocytes cultured on nanofibrillar versus planar surfaces, as a critical role of astrocytes *in vivo* is to support neuronal outgrowth during development and following injury. Nanofibrillar meshworks

in turn structurally resemble the basement membrane that separates astrocytes from capillary endothelial cells in the brain and the spinal cord. In the present work, we therefore explored the hypothesis that astrocytes sense their geometrical environment, forming different cell-to-cell contacts on nanofibrillar versus planar surfaces. This in turn likely activates different signaling pathways and influences cellular function.

## **EXPERIMENTAL PROCEDURES**

### **Preparation of samples**

ACLAR coverslips (Ted Pella, Redding, CA) were used as planar cell culture surfaces. In some cases, coverslips were coated with synthetic polyamide (proprietary composition) nanofibers by Donaldson Co., Inc. (Minneapolis, MN). The nanofibers were electrospun in a controlled thickness ( $\sim 2 \mu\text{m}$  thick) from a blend of two polymers,  $(\text{C}_{28}\text{O}_4\text{N}_4\text{H}_{47})_n$  and  $(\text{C}_{27}\text{O}_{4.4}\text{N}_4\text{H}_{50})_n$ , as previously described [4]. The coverslips and the nanofibers employed in the present study were not chemically modified.

Rat cerebral cortical astrocytes were prepared from postnatal day P1 (P1) Sprague Dawley rats as previously described [3]. The astrocytes were grown to confluence in  $75 \text{ cm}^2$  tissue culture flasks in astrocyte medium. The astrocyte medium comprised Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) plus 10% calf serum (Invitrogen). After reaching confluence, flasks were shaken overnight on a rotary shaker at  $37^\circ\text{C}$  to remove any loosely adherent top cells such as oligodendrocytes, neurons, or macrophages. Astrocytes were then subcultured in astrocyte medium (0.5 ml) at a density of 50,000 cells/well onto 12 mm ACLAR coverslips or coverslips coated with nanofibers in 24 well trays. The astrocytes were maintained for 24 h and then fixed with paraformaldehyde (4%). Phalloidin was used to stain actin in the fixed astrocytes, as previously described [5].

### **Analytical techniques**

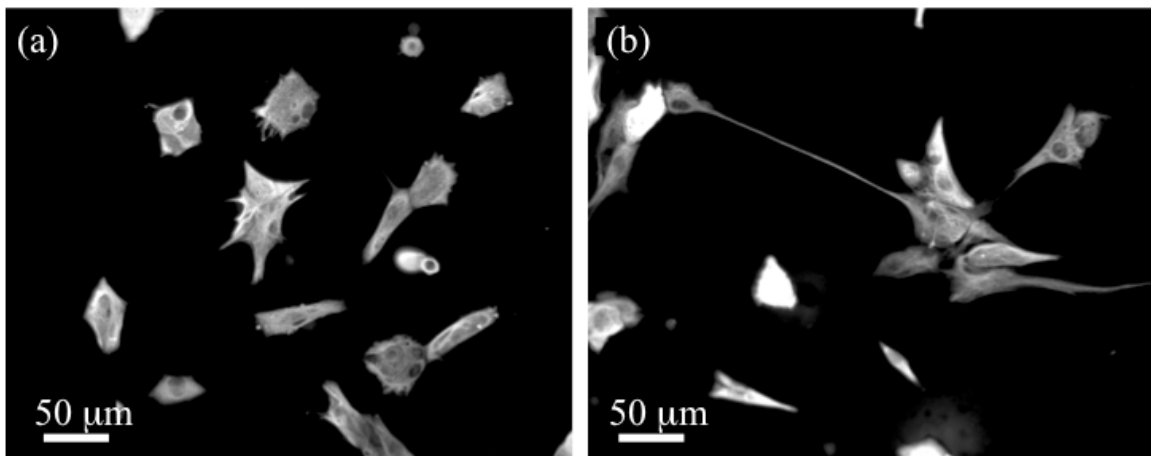
Fluorescence images of the astrocyte cultures at 24 h were captured using a Zeiss Axioplan microscope (Carl Zeiss Microimaging GmbH, Germany) equipped with an epi-fluorescence illuminator. To ensure that the results were representative for the cultures as a whole, at least five images were captured from different regions of three coverslips with and without the nanofibrillar layer. Moreover, these experiments were repeated using at least three separate astrocyte cultures (data not shown).

AFM images of the astrocyte cultures at 24 h were taken using a Veeco Instruments Nanoscope IIIA (Veeco Instruments, Woodbury, NY) operated in ambient air, using a J scanner with a maximum scan range of  $125 \times 125 \mu\text{m}^2$  x-y range and  $\pm 2.774 \mu\text{m}$  z range or an E scanner with a maximum scan range of  $13.5 \times 13.5 \mu\text{m}^2$  x-y range and  $\pm 1.54 \mu\text{m}$  z range. Contact mode and tapping mode images were acquired, using silicon nitride tips with a nominal tip radius of 20 nm and cantilever spring constant  $k = 0.58 \text{ N/m}$ , and silicon tips with a nominal tip radius of 10 nm and drive frequency of  $\sim 320 \text{ kHz}$ , respectively. The relevant operating parameters for individual images are given in the figure captions.

## EXPERIMENTAL RESULTS

### Epi-Fluorescence Microscopy

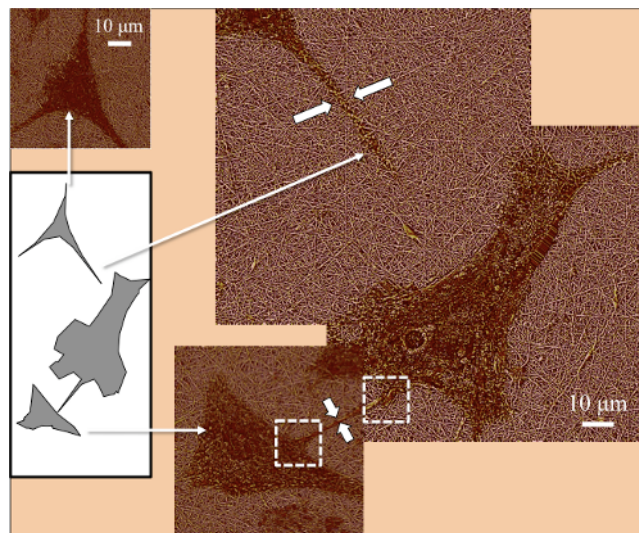
Representative epi-fluorescence microscopy images of astrocytes cultured for 24 h on (a) nanofibrillar surfaces and on (b) planar surfaces are shown in Figure 1. Astrocytes on the nanofibrillar surfaces were largely isolated, with cells separated by  $> 50 \mu\text{m}$  distances. They also demonstrated linear bundles of actin, as previously reported for fibroblasts cultured on nanofibers [5]. Even at 24 h, the astrocytes were beginning to adopt a stellate morphology, as is observed *in vivo* and has been shown by us in earlier work [3]. Astrocytes cultured on the planar culture surfaces showed small groups of confluent cells as well as isolated cells separated by  $> 50 \mu\text{m}$  distances. Figure 1 (b) shows a  $> 150 \mu\text{m}$  process between two cells in adjacent cell groups.



**Figure 1.** Epi-fluorescence microscopy images of 24 h astrocytes cultured on (a) nanofibrillar surfaces and on (b) ACLAR coverslips

### Astrocytes on Nanofibrillar Surfaces

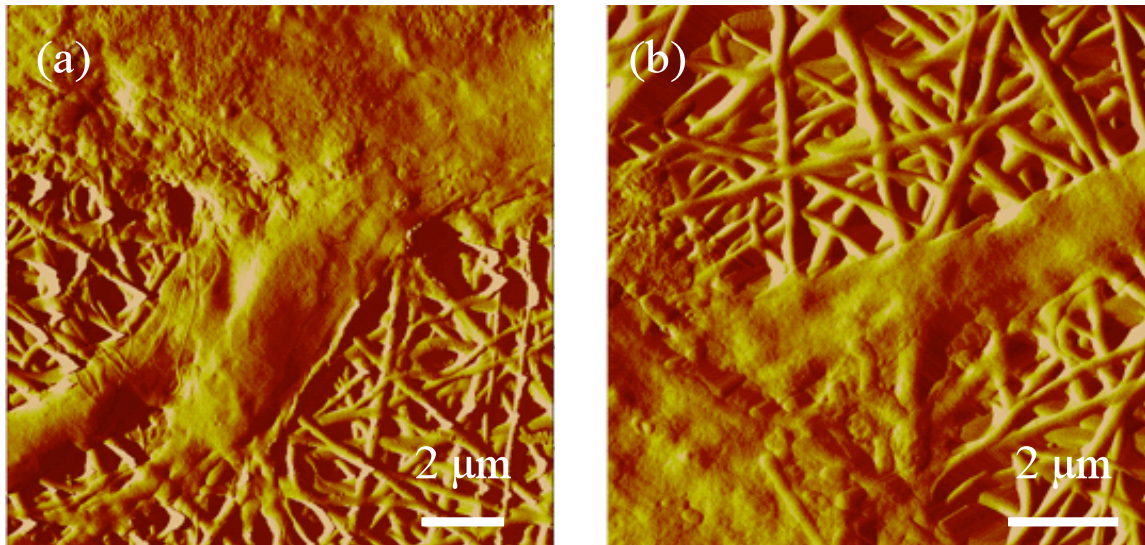
AFM investigation of astrocytes cultured for 24 h on nanofibers revealed cell-cell interactions between most of the astrocytes via  $\sim 50 \mu\text{m}$  extensions. These extensions were not observable in the phalloidin-stained epi-fluorescence microscopy images (Figure 1 (a)). Two extensions, one which has partially and one which has totally traversed the distances between neighboring cells, are shown in the composite AFM image of Figure 2. By AFM section measurement, the partial extension was about  $\sim 5 \mu\text{m}$  wide and



**Figure 2.** Composite AFM image of astrocyte cell-cell interactions on nanofibrillar surface (J scanner, contact mode)

~350 nm tall at the point halfway along its length identified by arrows, with a tapered advancing edge about ~1  $\mu\text{m}$  wide. The completed extension was about ~3  $\mu\text{m}$  wide and ~260 nm tall halfway along its length (arrows).

Close-ups of the completed extension at its emanation and termination points (boxed areas in Figure 2) are shown in Figure 3. The extension grew from an originating cell as shown in Figure 3 (a), and traversed the inter-cell distance as a single extension (no branches). It developed a ~8  $\mu\text{m}$  broadened tip where it met the neighboring cell, as shown in Figure 3 (b).



**Figure 3.** Close-up AFM images of process shown in Figure 2 (boxes) at its (a) emanation and (b) termination points. (Amplitude images in (a) tapping mode, E scanner, (b) contact mode, J scanner).

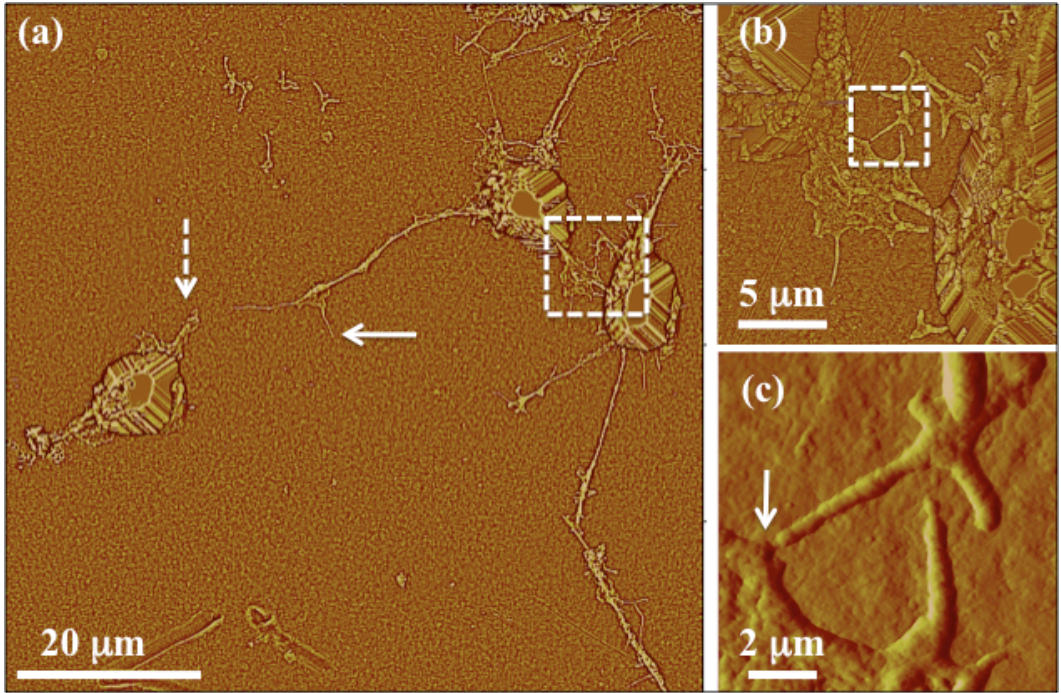
### **Astrocytes on Planar Surfaces**

AFM images of astrocytes cultured on planar surfaces demonstrated the presence of a putative filopodial network, which was more branched and extensive than observable in the epi-fluorescence microscopy images.

A long branched ~50  $\mu\text{m}$  cellular process is observable in Figure 4 (a). It appears to be initially targeted to a distant cell at the point marked by the dashed arrow. However, it has developed a second exploratory short branch angled with respect to the long branch, as shown by solid arrow. The second branch is not targeted toward the cell.

The network shown within the dashed box in Figure 4 (a) appears to bridge the short inter-cellular distance (5  $\mu\text{m}$ ) between two astrocytes. Close-up AFM images, shown in Figures 4 (b) and (c), demonstrate that direct contact with the targeted cell was incomplete at 24 h, despite proximity. Regions such as the one identified by an arrow in Figure 4 (c) actually terminated in small (~50 nm) gaps. The AFM images of Figures 4 (a) and (b) also showed artifacts that indicated the cell somata exceeded 3  $\mu\text{m}$  in height and had steep sides.

Quantitative information for the investigation of twenty-eight astrocytes cultured on the nanofibrillar surfaces and thirty-seven astrocytes cultured on the planar surfaces are given in Table 1. As cell-cell interactions on the planar surfaces involved extensive filopodia networks, individual processes were not counted for these cells.



**Figure 4.** (a) Composite spatial highpass filtered AFM height image of astrocytes on ACLAR coverslip show extensive filopodia networks. (b-c) Close-up of dashed box regions shown in (a-b) (Contact mode, J scanner).

**Table 1.** Quantitative information for astrocytes on nanofibrillar and planar surfaces.

Group	Nanofibrillar surfaces		Planar surfaces	
	Number of cells in the group	Number of extensions in the group		
		Complete		Partial
1	3	1	1	3
2	2	1	0	10
3	6	3	0	8
4	3	2	0	16
5	4	1	0	-
6	7	3	0	-
7	3	2	0	-
Average	4			~9

**DISCUSSION**

In the present work, we used epi-fluorescence microscopy and atomic force microscopy to explore the hypothesis that astrocytes sense their geometrical environment,

forming different cell-cell contacts on nanofibrillar versus planar surfaces. We found that astrocytes on nanofibrillar surfaces and planar surfaces diverged in apparent cell-cell contact structures.

Epi-fluorescence microscopy images showed that astrocytes cultured on nanofibrillar surfaces had linear actin arrangements within their cell bodies, that correlated with a more physiologically biomimetic stellate morphology. AFM images revealed that, for astrocytes cultured on nanofibrillar surfaces, wide, low processes, one originating per cell, appeared to form a single direct physical contact to a neighboring cell. In contrast, astrocytes cultured on the planar surfaces had extensive branched networks of filopodia processes in the intercellular regions. The long processes were observed in the epi-fluorescence microscopy images but the short angled branches were not observed. The present investigation indicated that the filopodia processes may not form physical contacts either to other filopodia or to neighboring cell bodies down to nanoscale separation distances.

Quantitative data indicated that the astrocytes cultured on planar surfaces had a more exploratory response than the astrocytes on nanofibrillar surfaces, because the number of astrocytes in a group on the planar surfaces was relatively higher than the number of astrocytes per group on the nanofibrillar surfaces.

We have previously shown that astrocytes cultured on nanofibrillar surfaces promote enhanced neurite outgrowth from cerebellar granule neurons in co-cultures compared with astrocytes cultured on planar surfaces [3]. The present work showed that astrocytes cultured on these two different surfaces exhibited different patterns of cell-cell contact. Potentially different intercellular signaling mechanisms may account for the structural observations. This hypothesis remains to be explored in future work and has clear implications for tissue engineering studies that employ nanofibrillar surfaces as cellular transplant vehicles.

## CONCLUSIONS

Astrocytes cultured on nanofibrillar versus planar surfaces diverged in apparent cell-cell contact structures. Astrocytes on nanofibrillar surfaces exhibited a single process response while astrocytes on planar surfaces exhibited a filopodia network response. This indicates the possibility that astrocytes can sense their geometrical environment and form different cell-to-cell contacts on nanofibrillar versus planar surfaces, through and with activation of different signaling pathways. This has implications for tissue engineering work that employs nanofibrillar surfaces as cellular transplant vehicles.

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