New Atomic Force Microscopy Based Astrocyte Cell Shape Index

Volkan Mujdat Tiryaki[1], Usienemfon Adia-Nimuwa[1], Steven Allen Hartz[1], Kan Xie[1], Virginia M. Ayres[1], Ijaz Ahmed[2], and David I. Shreiber [2]

[1]Department of Electrical and Computer Engineering, Michigan State University, East Lansing, MI 48824, USA

[2]Department of Biomedical Engineering, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

ABSTRACT

A new three-dimensional cell shape index using the AFM height images of cells cultured on cell substrates was defined. The new cell shape index revealed quantitative cell spreading information of cells that is not included in the conventional cell shape index. The quantitative morphometry of rat cerebral cortical astrocytes cultured on four different kinds of cell substrates were investigated using the conventional and the new cell shape index, and the results were compared. The new cell shape index showed the quantitative astrocyte spreading and stellation behavior that agrees with the AFM height images of astrocytes.

INTRODUCTION

In vivo, cells have different morphologies depending on their type, e.g., astrocytes have a stellate morphology in the central nervous system (CNS) [1], and endothelial cells are elongated in the circulatory system [2]. When cells are cultured on substrates, they may adopt a similar morphology to their shape *in vivo*. A cell shape index (CSI) is a measurement of the morphological differentiation that is induced by the biochemical and physical environment of the cell. It is a quantitative cell morphology evaluation method that can be used to compare the morphology of cells *in vitro* and *in vivo*.

The field of measuring cell morphology changes is known as quantitative morphometry [3]. Stellation is an important process for astrocytes, and CSI can be used to quantify the astrocyte stellation response. Conventional CSI is the ratio of perimeter squared to the cell projection area [1]:

$$CSI = P^2 / (4 \pi A) \tag{1}$$

where P is cell perimeter, and A is the cell projection area. This equation describes stellation as a cell's departure from a circular cross section since:

$$(2 \pi r)^2 / 4 \pi (\pi r^2) = 1 \tag{2}$$

This definition for CSI was created for use with two-dimensional optical microscopy images and does not include three-dimensional aspects such as cell spreading. Our recent work indicates that cell spreading behavior may be an important part of an astrocyte's response to its nanophysical environment, and that it can vary as much as 50% as a function of culture surface

properties [4]. In this study, the cell spreading behavior was observed as a result of an atomic force microscopy (AFM) investigation of astrocyte morphological responses to four culture environments that supplied the cells with different nanophysical cues. We are especially interested in astrocyte responses to polyamide nanofibrillar scaffolds because they have demonstrated promise for CNS repair in *in vivo* and *in vitro* investigations [5,6]. As only external cues were used to trigger cell responses, astrocyte responses to the nanofibrillar surfaces were studied in comparison with their responses to three additional culture surfaces: poly-L-lysine-functionalized planar glass (PLL glass), unfunctionalized planar Aclar (Aclar), and PLL-functionalized planar Aclar (PLL Aclar). PLL glass is a standard astrocyte culture surface, and astrocyte responses to it are well characterized, making it useful for identifying differences in astrocyte responses to other surfaces. The polyamide nanofibrillar scaffolds were electrospun on Aclar substrates; therefore, astrocyte responses to Aclar surfaces were investigated to distinguish responses to the nanofibrillar scaffolds from possible responses to the underlying Aclar substrate. Astrocyte responses to PLL Aclar surfaces were studied to clarify the role of the underlying substrate versus surface functionalization. AFM investigation produces volumetric information that should be included in describing the quantitative morphological response. A three-dimensional confocal cell morphometry was defined by Chvátal et al. [7], but a CSI that incorporates volume information acquired from high-resolution AFM height images of cells on culture surfaces has not been reported yet. The new CSI reported in this work is therefore a new cell morphometry tool that reveals quantitative information about cell spreading as well as stellation.

EXPERIMENTAL PROCEDURES

Preparation of samples

Four culture surfaces were investigated in this work: PLL glass, PLL Aclar, Aclar and polyamide nanofibrillar scaffolds. Glass coverslips (12 mm, No. 1 coverglass, Fisher Scientific, Pittsburgh, PA) and Aclar coverslips (12 mm, Ted Pella, Redding, CA) were used as underlying surfaces for the PLL functionalization. Glass or Aclar coverslips were placed in a 24-well tissue culture plate (one coverslip/well) and covered with 1 mL of PLL solution (50 μg PLL/mL in dH_2O) overnight. The coverslips used for the cultures were then rinsed with dH_2O and sterilized with 254 nm UV light using a Spectronics Spectrolinker XL-1500 (Spectroline Corporation, Westbury, NY). The polyamide nanofibrillar scaffolds electrospun on Aclar substrates were obtained from Donaldson Co., Inc. (Minneapolis, MN) and Corning Life Sciences (Lowell, MA). The fiber diameter for the nanofibrillar scaffolds has a range from 100 to 300 nm.

Primary astrocyte cultures were prepared as previously reported [5,6]. The astrocytes cultured on coverslips were fixed in 4% paraformaldehyde for 10 minutes, paraformaldehyde for 10 minutes, rinsed with distilled water and air dried.

Analytical techniques

The AFM investigations were performed using a Nanoscope IIIa (Bruker AXS Inc, Madison WI, formerly Veeco Metrology) operated in contact mode and in ambient air. A J scanner with 125 μm x 125 μm x 5.548 μm x-y-z scan range, and Bruker DNP silicon nitride

probes with a 35º ± 2º cone angle and a nominal 20 nm tip radius of curvature were used for the AFM investigations. For each culture surface, a minimum of ten images were captured from different regions. Cell segmentation, the conventional and new CSI calculations, were implemented with MATLAB version 7.7.0 (R2008b) (The MathWorks, Natick, MA).

EXPERIMENTAL RESULTS

Initial Segmentation Step

CSI analysis requires mathematical segmentation of cell from the cell substrate. The difficulty of segmentation varies depending on the cell and the substrate. There is a serious segmentation issue with AFM investigation of astrocytes on nanofibrillar scaffolds. Cells on nanofibrillar scaffolds interact with these surfaces via nanoscale edges and processes that are not distinguishable from the nanofibrillar background by AFM height, deflection, or phase imaging. This is because the cellular edges and processes are approximately the same order in height as the background nanofibers, ~100 to 200 nm.

In previous work [8], a diagnostic approach based on analysis of discrete Fourier transform of standard AFM section measurements was developed and used to identify quantitative differences in the frequency components of nanoscale cellular edges and processes of neural cells cultured on nanofibrillar scaffolds. Accurate identification of differences formed the basis for the subsequent successful Gaussian high-pass filter (GHPF) design that enabled the edges and processes to be clearly distinguished in AFM images. In the present work, the GHPF was applied to AFM height images and followed by histogram equalization. The final cell boundaries were determined by manual segmentation. The results of the segmentation procedure steps for a representative astrocyte AFM height image were shown in figure 1 (a)-(c).
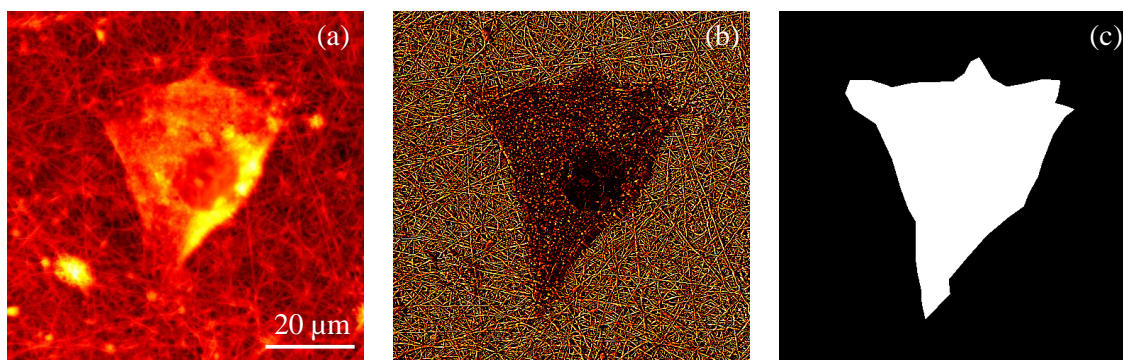

Figure 1. The segmentation procedure results of AFM height images of an astrocyte cultured on nanofibrillar scaffolds. AFM height image of the astrocyte (a), the GHPF and histogram equalization (b), and manual segmentation result (c).

Astrocyte Morphology Investigation by Conventional CSI

After segmentation, the conventional CSI definition shown in equation 1 was used to investigate the quantitative morphometry of the cerebral cortical astrocytes cultured on the four different culture surfaces that presented different nanophysical cues [4]. At 24 h, astrocytes on all

substrates exhibited sufficient variation in their morphologies that histograms (not shown) were used to distinguish majority CSIs from outliers, which were excluded. Standard errors instead of standard deviations were evaluated for the same reason. The conventional CSI results, shown in figure 2, indicated that astrocytes cultured on Aclar surfaces are more stellate than the ones on other surfaces. The mean value of CSI of astrocytes cultured on PLL Aclar and nanofibrillar scaffolds are close, and the standard error of the CSI of astrocytes on nanofibrillar scaffolds is least. The conventional CSI measurements indicated that astrocytes cultured on nanofibrillar scaffolds were the least stellate.
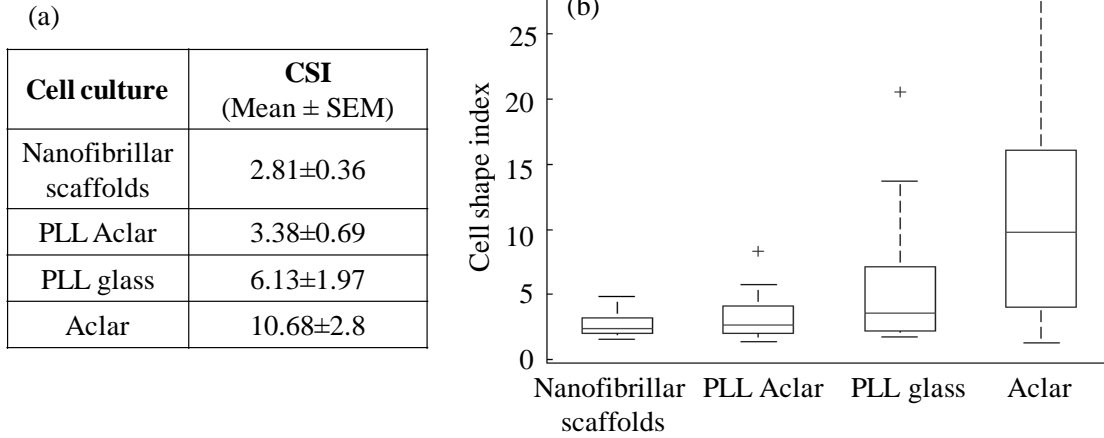
(a)

Cell culture	CSI (Mean ± SEM)
Nanofibrillar scaffolds	2.81±0.36
PLL Aclar	3.38±0.69
PLL glass	6.13±1.97
Aclar	10.68±2.8

Figure 2. The conventional CSI results for astrocytes cultured on cell substrates (a), and CSI results as a box plot (b). SEM denotes standard error of the mean. Solid lines in the boxes show the median.

Astrocyte Morphology Investigation by New AFM-based Cell Shape Index

The new CSI that includes volumetric information extracted from AFM images of cells on surfaces is defined as:

$$\text{New CSI} = (1/18\ \pi) \times (SA_{cell})^3 / (V_{cell})^2 \tag{3}$$

where SA_{cell} is the cell surface area, and V_{cell} is the cell volume. This assumes that the cell is resting on, not penetrating, a surface. For a hemispherical "cell" on a surface the new CSI is:

$$(1/18\ \pi) \times (0.5[4\pi r^2])^3 / (0.5[4/3\ \pi r^3])^2 = 1 \tag{4}$$

Therefore, the new CSI increases as the cell morphology become more stellate and/or the cell spreading increases.

After cell segmentation from the cell substrate, the surface area of each cell was calculated by splitting the AFM topography faces into triangles. The area of a triangle in three-dimensional space was computed using the cross product given by:

$$SA_{triangle} = 0.5\ |(v_2 - v_1) \times (v_3 - v_1)| \tag{5}$$

where $SA_{triangle}$ is the area of a triangle on the cell surface, and the coordinates of the vertices are given by $v_i=(x_i, y_i, z_i)$. The surface area of each triangle was then computed over the segmented cell area, and cell surface area was obtained. The volume of each astrocyte was calculated by assuming each pixel and its z coordinate as a square prism [9]. The volume under each pixel was computed by multiplying the unit pixel area by the height of that pixel. This was repeated for all the pixels of the segmented cell region. The new CSI was then computed as given in equation 3, and results were shown in figure 3. Departure from unity reflects the average departure from a three-dimensional hemispherical volume by both stellation and cell spreading.

The new CSI results indicated that astrocytes cultured on the Aclar surfaces had the *lowest* CSI values (mean and median). This implies that, with the same stellation counted in the new CSI, there is also dominant low spreading behavior. AFM and phalloidin staining results [4] confirmed that astrocytes cultured on Aclar surfaces were significantly more hemisphere-like than the ones on other substrates. The new CSI results further indicated that the astrocytes cultured on the two PLL functionalized surfaces were similar in terms of combined cell spreading and stellation behavior. AFM and phalloidin staining results [4] showed astrocytes had less spreading but more stellation on PLL glass and more spreading but less stellation on PLL Aclar. These two combinations would produce comparable new CSI values.
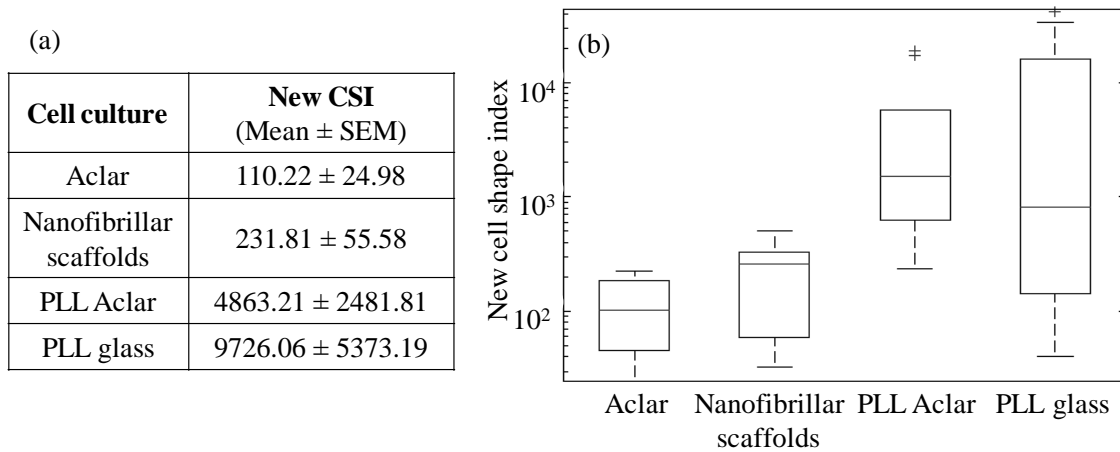
(a)

Cell culture	New CSI (Mean ± SEM)
Aclar	110.22 ± 24.98
Nanofibrillar scaffolds	231.81 ± 55.58
PLL Aclar	4863.21 ± 2481.81
PLL glass	9726.06 ± 5373.19

Figure 3. New CSI of astrocytes cultured on cell substrates (a), and new CSI results as a box plot (b). Solid lines in the boxes show the median.

DISCUSSION

The new CSI definition in this work allows a quantitative evaluation of cell spreading and stellation behavior using AFM height images. Cell-matrix adhesion is mediated by biomolecular interactions between adhesion receptors on the plasma membrane and cell substrate. Cell spreading is an F-actin and myosin dependent process, and it is important for cell growth, and cell motility. So far, cell spreading has been investigated by using confocal microscopy [10], and recently with AFM [4,11]. One of the advantages of AFM over confocal laser scanning microscopy (CLSM) is that AFM resolution with the J scanner used in this work, in z direction is less than 1 nm, whereas it is around 500 nm for CLSM. Thus, cell volume and cell surface area calculations with AFM are more accurate than CLSM. The new cell shape index results in a combined measure of stellation and cell spreading information. The conventional cell shape

index, however, ignores the three-dimensional shape of the cell, and uses only cell perimeter and cell projection area data from two-dimensional images to represent the two-dimensional shape of the cell. The new cell shape index is therefore more accurate than the conventional cell shape index.

The new CSI has been applied to cerebral cortical astrocytes cultured on four different cell substrates. The PLL functionalized surfaces are commonly used as a cell substrate for neural cells to increase cell adhesion. This study showed the cell spreading behavior on Aclar versus PLL Aclar substrates is quite different (Figure 3). The astrocytes cultured on Aclar adopted a hemisphere-like shape, but the ones on PLL Aclar were well spread. Although the conventional CSI of astrocytes on Aclar was highest, the new CSI value of same cells were least indicating the less cell spreading on these surfaces. It is not possible to present the new CSI difference with that accuracy using fluorescence microscopy or CLSM. This important information was also missing from results obtained from the conventional CSI analysis. The new CSI is therefore a new quantitative cell morphology evaluation method that makes use of the volumetric information provided by high-resolution AFM investigation.

CONCLUSIONS

New CSI that reveals quantitative cell spreading and stellation behavior is defined. The new CSI was calculated for the cerebral cortical astrocytes cultured on four kinds of cell substrates that present different nanophysical cues. Significant shape index differences were observed between the astrocytes cultured on the cell substrates by using the conventional and the new CSI. The new CSI is a promising new quantitative cell morphology evaluation method which is expected to be useful for biomaterials research.

REFERENCES

[1] S. Matsutani and N. Yamamoto, *Glia*, **20**, 1, (1997).
[2] K. B. Vartanian, S.J. Kirkpatrick, S.R. Hanson, M.T. Hinds, *Biochemical and Biophysical Research Communications* **371**, 787, (2008).
[3] P. Moshayedi, L.d.F. Costa, A. Christ, S.P. Lacour, J. Fawcett, J. Guck, K. Franze, J. *Phys.: Condens. Matter*, **22**, 194114, (2010).
[4] V.M. Tiryaki, V.M. Ayres, A.A. Khan, I. Ahmed, D.I. Shreiber, and S. Meiners, *Int. J. Nanomedicine*, **7**, 3891, (2012).
[5] S. Meiners, I. Ahmed, A.S. Ponery, N. Amor, V.M. Ayres, Y. Fan, Q. Chen and A.N. Babu, *Polymer Int.,* **56**, 1340, (2007).
[6] R. Delgado-Rivera, S.L. Harris, I. Ahmed, A.N. Babu, R. Patel, V.M. Ayres, D.A. Flowers and S. Meiners, *Matrix Bio*. **28**, 137, (2009).
[7] A. Chvátal, M. Andrová, and F. Kirchhoff, *J. Anat.* **210**, 671, (2007).
[8] V.M. Tiryaki, A.A. Khan, V.M. Ayres., *Scanning*, **34**, 316 (2012).
[9] Y.E. Korchev, J. Gorelik, M.J. Lab, E.V. Sviderskaya, C.L. Johnston, C.R. Coombes, I. Vodyanoy, and C.R.W. Edwards. *Biophysical Journal* **78**, 451, (2000).
[10] M.A. Fardin, O.M. Rossier, P. Rangamani, P.D. Avigan, N.C. Gauthier, W. Vonnegut, A. Mathur, J. Hone, R. Iyengarband and M.P. Sheetz. *Soft Matter*, **6**, 4788, (2010).
[11] W. Shao, H. Jin, J. Huang, B. Qiu, R. Xia, Z. Deng, J. Cai, Y. Chen, *Scanning*, 2012. doi: 10.1002/sca.21040. [Epub ahead of print]