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Probing Mechanical Adaptation of Neurite Outgrowth on a Hydrogel Material Using Atomic Force Microscopy

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Abstract—In this study, we describe the design and initial results of probing mechanical adaptation of neurite growth of lightly fixed neurons on a hydrogel substrate by using atomic force microscopy (AFM). It has been shown previously that cells are responsive to the physical conditions of their micro-environment, and that certain cells can adjust their own stiffness as part of the adaptation to the substrate. AFM, a powerful tool to probe micro- and nano-scale structures, has been utilized in assessing topography, morphology, and structural change of neuronal cells. We used AFM with a robust force analysis approach in this study to probe the mechanical properties of both neurites and the substrate at close proximity. We first confirmed the robustness and consistency of the approach specific to soft materials by comparing measurements made on the same reference material using different methods. Subsequently, it was found that the primary spinal cord neurons that were lightly fixed exhibited different stiffnesses between the cell body and neurites. Furthermore, in comparison to the rigidity of the substrate, the stiffness of the neurites was lower, whereas that of the neuronal cell body was higher.

Keywords—Spinal cord neuron, Stiffness, Topography, Mechanical stiffness, Elasticity map.

INTRODUCTION

Mechanical Response of Neurons to Substrate Stiffness

Interactions between cells and extracellular matrix (ECM) have been the subject of intensive research efforts, both experimentally and numerically in recent years.[2,3] Among critical extracellular cues, physical stimuli, particularly substrate stiffness, have been found to affect cellular behavior in a cell-type-specific manner. The link between cellular response and the mechanical properties of the ECM has been empirically established using soft hydrogel materials that mimic the cellular environment found in natural tissues.[4,7,8,13,21,22] Neurons, whose mechanical responses to physical cues have begun to be appreciated, were proven responsive to the substrate rigidity similar to many other cell types. Using a N,N′-methylene bisacrylamide crosslinked polyacrylamide hydrogel system, Flanagan *et al.*[7] and Georges *et al.*[8] found that primary neuronal behavior is significantly different on soft and stiff substrates. Our group examined the primary spinal cord neuronal response to both static and dynamic rigidities and found that neuron–astroglia interactions affect their responses[9] and that neurons can sense both static and dynamic stiffnesses and adjust their primary neurite structure accordingly.[10–12] Despite these efforts, questions remain about the mechanism of neuronal mechano-response. An understanding of the nature of the process is critical to the rational design of biomaterials for neural tissue engineering applications, especially since different neuronal properties (e.g., axonal length and dendrite number) behaved differently to compliance.[10,12] A recent study has revealed that fibroblasts can alter their own stiffness in adaptation to the surrounding matrix[23]; it would be rational to ask whether neurons exhibit similar behavior.

Probing the Micro-Environment of Neurons by Atomic Force Microscopy

Invented by Binnig *et al.*[1] in 1986, the atomic force microscope (AFM) is among the versatile family of

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scanned-proximity probe microscopes. Continuing development has expanded the capabilities of AFM to include measurements of topography, optical absorption, and magnetism. In AFM, the probe consists of a cantilever to which a sharp stylus is attached. Properties of the scanned object are inferred from the deflection of the cantilever as the tip approaches, and in some cases, contacts sample surface. The inherent advantages of AFM include, but are not limited to: high spatial resolution (up to sub-molecular level), the ability to maintain the probe under physiological conditions (dry or wet), and elimination of the need for surface preparation that is required in other techniques (e.g., scanning tunneling microscopy).

Recently, Lin *et al.*[14–16] proposed a robust approach of automatically extracting elastic properties from AFM force–deflection curves. This method considers the existence of certain adhesive interactions and has proven to be well suited for probing soft tissues with heterogeneity and non-linear elasticity. The implementation of the algorithm in MATLAB (Mathworks) permits elasticity maps corresponding to the scanned region to be generated with minimal user intervention. AFM has been used by a number of investigators in the field of neuroscience[18,26] to study neuronal morphology[6,20] and neuronal structural changes.[24] Although Xiong *et al.*[25] have measured the elastic moduli of neuronal growth cones and sub-cellular structures of live neurons on glass, to our knowledge, there has been no examination and quantification of the stiffness of the ECM and its effect on the elasticity of neurites residing thereon.

Purpose of This Study

We hypothesized that like other types of cells, neurons are able to adjust their own stiffness in response to the rigidity of the surrounding ECM, and that neurites may respond differently from soma. In this investigation, we cultured primary spinal cord neurons on an ECM molecule-coated bis-crosslinked polyacrylamide hydrogel, and after light fixation probed both topography and elasticity distribution of the cell body, neurites, and the substrate in close proximity. It is noted that light fixation was applied to ensure stable conditions of the cells while AFM was being performed. The novelty of this study lies in the combination and analysis of tandem topographic and high-resolution elasticity measurements. This approach may provide further evidence for the existence of perineuronal nets (PNNs) and contribute to a better understanding of the mechanism of neuronal mechano-responses.

MATERIALS AND METHODS

Hydrogel Substrate Preparation

Bis-crosslinked polyacrylamide gels (referred to as 'gels' hereafter) were prepared according to the protocol described elsewhere.[9] In brief, round glass coverslips (12 mm) treated by 3-aminopropyltrimethxysilane and 0.5% glutaraldehyde (Sigma) were used for gel preparation according to the protocol of Pelham and Wang.[22] Two different gels were prepared by pipetting 12 μL of gel solution, containing 5 or 8% acrylamide monomer (acrylamide:*bis*-acrylamide of 29:1, EMD Chemicals Inc., Gibbstown, NJ, USA), 0.1% ammonium persulfate, and 0.1% TEMED on the glass coverslip and covering the coverslip with another siliconized glass coverslip. After polymerization was completed, the top coverslip was removed, and the gel bound to the bottom coverslip was transferred into a 24-well culture plate. Gels were washed and functionalized with Sulfo-SANPAH (Pierce, Rockford, IL, USA). The gels were then coated with poly-D-lysine (Sigma, St. Louis, MO, USA) at 1 mg/mL in water. Care was taken to maintain flatness of the gel surface and hence, minimize the effect of surface topography (roughness). The average thickness of the prepared gels was approximately 100 μm.

Spinal Cord Cell Culture

All animal work was carried out with an approved protocol from the Rutgers University Animal Care and Facilities Committee. Spinal cords were dissected from embryonic, day-16 Sprague–Dawley rats (Taconic, Hudson, NY, USA), and cells were dissociated and plated on gels in 24-well plates at a cell density of 3×10^4 cells/cm^2. Cells were cultured in serum-containing medium [minimum essential medium, 10% horse serum and 0.6% glucose supplemented by antibiotics (penicillin/streptomycin)]. Plates were kept in a humidified CO_2 incubator at 37 °C. One day after cell plating, the medium was changed to Neurobasal medium (Gibco) supplemented with B-27, antibiotics, and β-mercaptoethanol (25 μM). To address concerns about sustained exposure of live cells to the atmosphere during AFM probing, cells were fixed with 3% formaldehyde at day 6 or 7 immediately before probing.

Atomic Force Microscopy

Micro- and nano-indentation of samples was performed using a Bioscope SZ AFM with the latest NanoScope V controller and integrated optical microscopy capabilities. Due to the narrow width of

neurites (a few microns), pyramidal tips (model: DNP; nominal tip diameter: 20 nm; Veeco Probes, Veeco Instruments, Camarillo, CA, USA) were used for the measurements. The approach reported previously[14–16] was adopted in the analysis, particularly for assessing elasticity distribution. The AFM ramp rate was kept between 1.5 and 2 Hz. We have found this rate to be adequately fast such that stress relaxation and fluid exudation are minimized; the mechanical properties thus measured can therefore be considered instantaneous quantities. More than three gel samples for each condition were prepared and more than two were scanned for AFM probing.

RESULTS

AFM Setup for Probing the Neuronal Culture

Our results showed a scan resolution of 32 × 32 to be advantageous over a resolution of 64 × 64 when factoring in scanning speed and the time needed to produce an elasticity map. Due to the presence of the cells cultured on the substrates, surface roughness was high and necessitated imposing restrictions on the size of the areas scanned in order to minimize noise in the AFM signal and to prevent exceeding the vertical range limits of the instrument. Furthermore, to prevent damage to the cells, maximum indentation depths were kept below 100 nm, since previous measurements showed that cell height is on the order of a few microns, consistent with previously reported values (1–5 μm).[26] This precautionary measure also prevented penetration of the cell membrane, often manifested as a sudden change in the curvature of the force curve. Under the imposed conditions, it can be expected that linear elasticity theory can be applied with minimal loss of accuracy in the data analysis. The geometry of the AFM probe and cantilever probing neuronal cell culture is shown schematically in Fig. 1.

Validation of the Elasticity Measurements

We first compared substrate stiffness measured using AFM with that measured using a single-magnet-based method for both the 5 and 8% gels used in this study.[9] The results of both sets of measurements exhibited good agreement, as shown in Table 1. The consistency of AFM in mapping the topography and elasticity of the neural cells on the substrates was demonstrated from multiple scans of randomly selected regions. Representative topographical and elasticity data are shown in Fig. 2. Topography maps from different areas showed similarities, and enabled differentiation of features such as cell body and thin processes from the substrates. There also appeared to be a relatively shallow area surrounding each cell body. The results indicated good consistency in the elasticity measurements. Neuron-like cells exhibited higher stiffness (approximately over 25 kPa) on 5% gels of ~7 kPa, and the area closely surrounding cell soma displayed lower stiffness (<5 kPa).

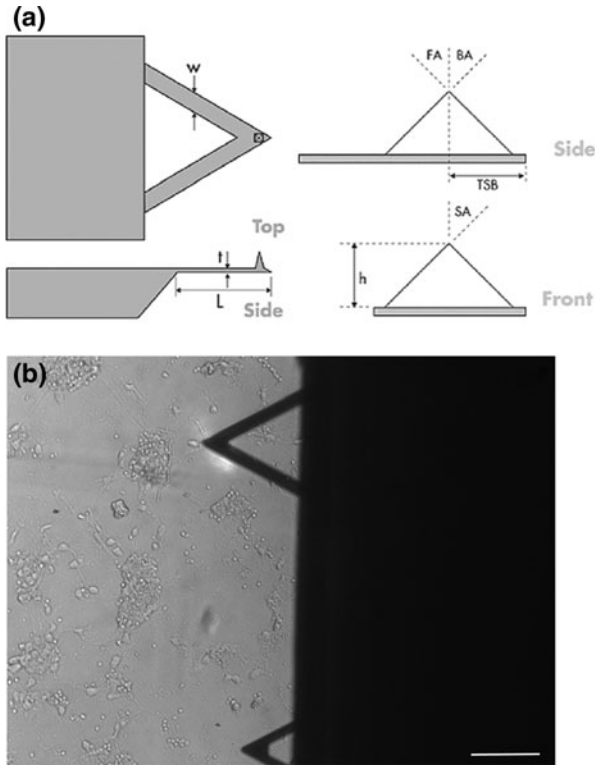

FIGURE 1. AFM probe and cantilever used in this study. (a) Geometric specifications of the Veecoprobe® probe and the cantilever per manufacturer; *L* = 180–212 μm; (b) AFM probe being used to probe the neuronal culture (left portion of the image) on the hydrogel substrate. Neurons were lightly fixed. Two cantilevers attached to the tip of the loading beam were used in the probing of neurons on bis-crosslinked polyacrylamide gels. The larger one was used in this study. Scale bar = 100 μm.

TABLE 1. Mechanical stiffness estimate of the bis-crosslinked polyacrylamide hydrogels by using two different methods: single-magnet-based approach[9] and AFM (stiffness in kPa).

Polyacrylamide gel (%)	Single-magnet	AFM
5	5.8 ± 1.1	6.96 ± 0.7
8	25*	24.6 ± 1.8

* Based on the extrapolation from Jiang *et al.*[9]

Elasticity Distribution of Neurites on Hydrogel Substrates

Probing of neurite outgrowth on gels with AFM made it possible to capture the topographic features

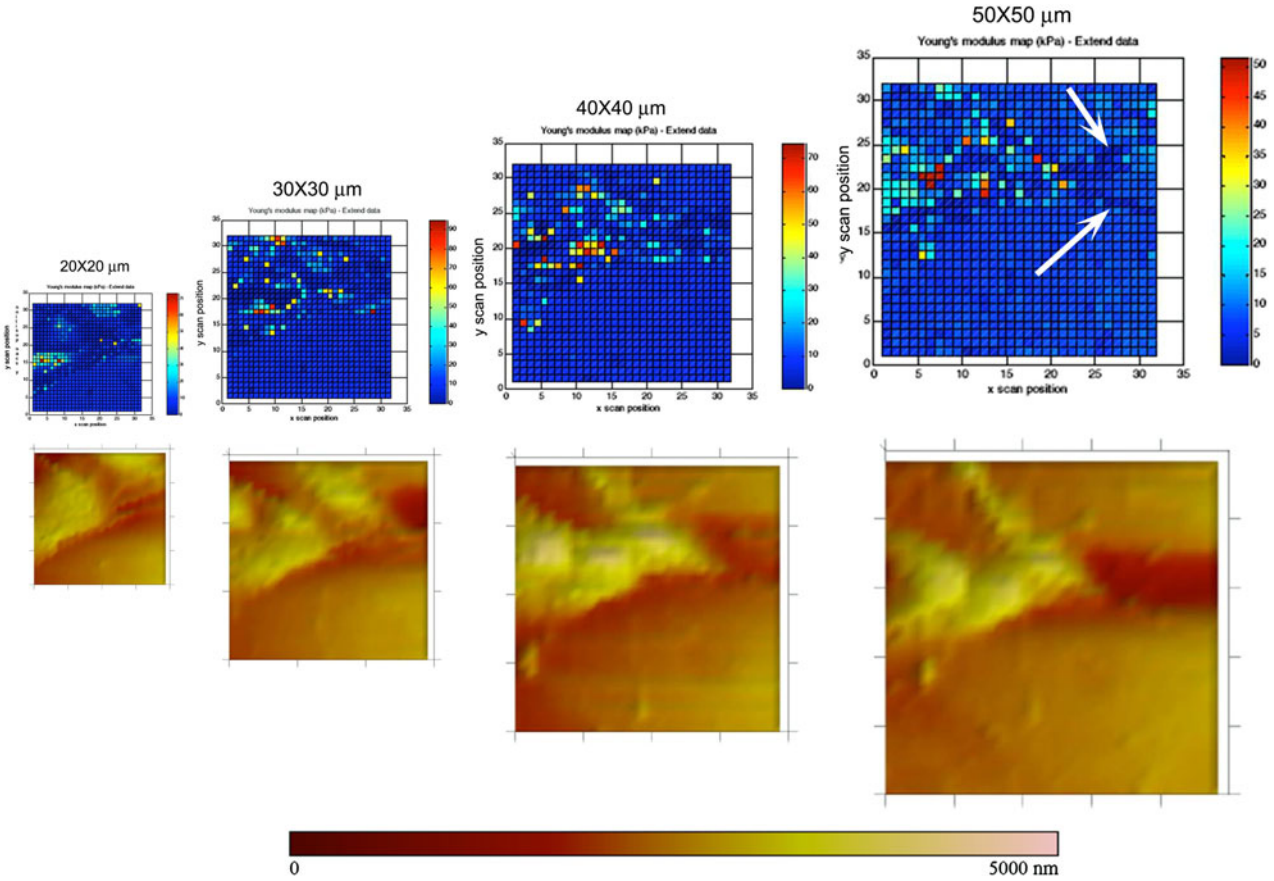

FIGURE 2. Probe of neuronal culture on gels at different scanning area. Scanning areas of 20 × 20, 30 × 30, 40 × 40, and 50 × 50 μm^2 was conducted for the same spot on a 5% gel (~7 kPa). Neurons were lightly fixed. It demonstrates the consistency of AFM measurement for both topography and elasticity with respect to scanning area. Top row for elasticity map and bottom row for topography. Dark-blued elasticity area mostly coincides with area at the edge of cell outline. Arrows point to the neurite-like processes.

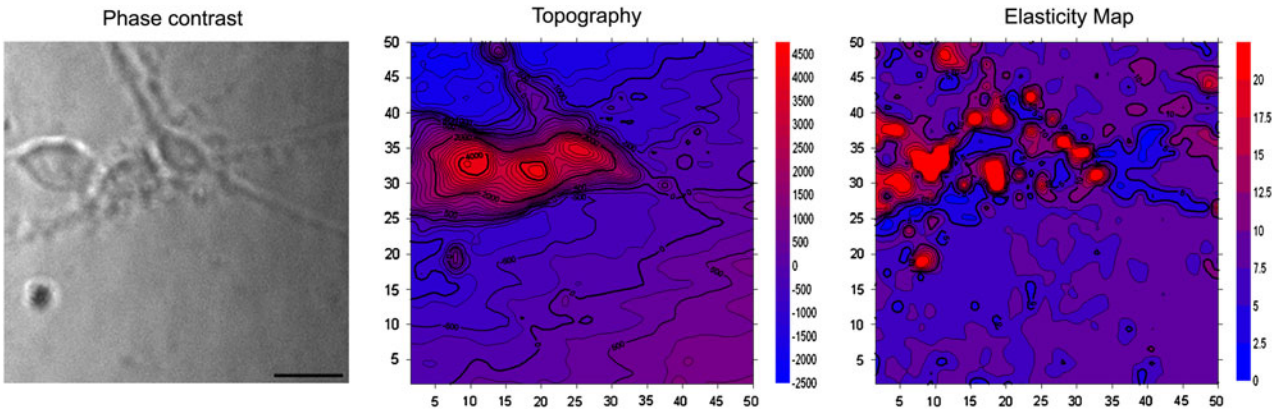

FIGURE 3. Phase contrast, topography, and elasticity maps of the same spot where three neuron-like cells grew on 5% gels. Scale bar = 10 μm. The legend in topography map is at nanometer. The legend for elasticity map is at kPa. The three neuron-like cells reside closely, and the topography image demonstrated that these cells grew on top of the gels with a height of from 4.5 to 6 μm. Neurons were lightly fixed.

and elasticity concurrently. A representative image of the phase contrast image, topography map, and elasticity map of the same region of the cell culture on a 5% gel are shown in Fig. 3. AFM is capable of differentiating between neighboring neural cells based on topography, while discrete areas of high stiffness

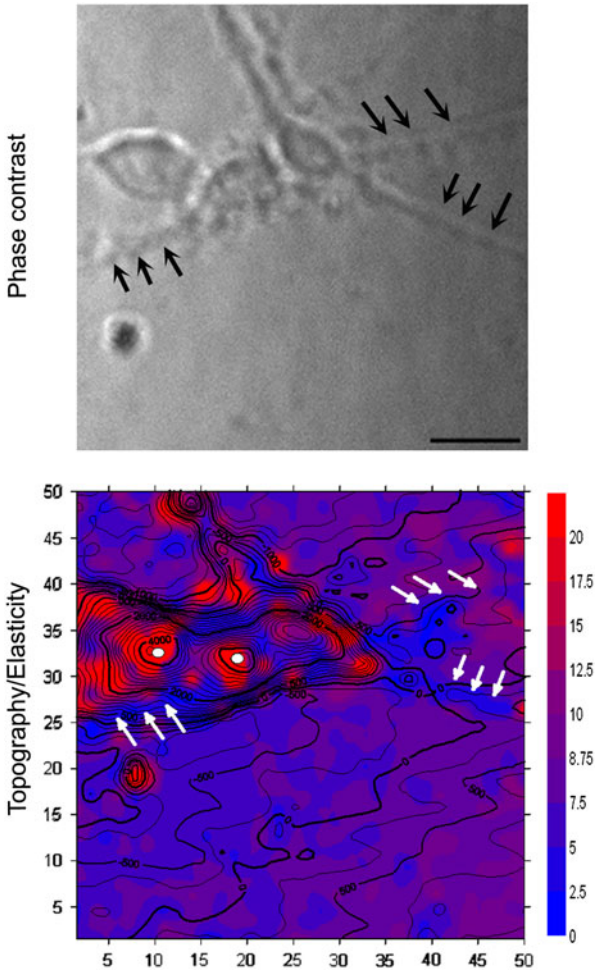

FIGURE 4. Combined topography and elasticity maps, and phase contrast images of the spot shown in Fig. 3. In the combined image, the color contour shows the distribution of the elasticity that AFM probed, and the line contour indicates the topography. Arrows point to the process-like structures of the cells. Scale bar = 10 μm. Neurons were lightly fixed.

(20–30 kPa) corresponding to the soma were also discernible. By overlaying the topography and elasticity maps (Fig. 4), the areas surrounding the cells were found to have the lowest stiffness range on the map. The stiffness of these areas was lower than that of the regions relatively far away from the cell bodies. On 8% gels (Fig. 5), similar observations were made, i.e., the neural cell bodies were generally stiffer than the substrate, or areas relatively far away from the cell bodies. On the stiffer gels (8%, ~25 kPa), cell stiffness (>40 kPa) was also higher than on the softer 5% gels. Near the edges of the cell bodies, areas of lower stiffness (<10 kPa) appeared similar to those of the 5% gels. Neurite-like processes showed low stiffness (<7.5 kPa) (Figs. 2–4). The stiffness values are summarized in Table 2. It is noted that neurons were lightly fixed.

DISCUSSION

AFM proved to be a powerful tool in analyzing the micro-scale features of the cellular environment, with a remarkable capability to probe both topography and elasticity. The instrument used in this experiment was able to identify topographic features down to the submicron scale. Furthermore, resolution of a few kPa in elasticity was also demonstrated, with a range of up to 85 kPa. Finally, we were able to differentiate among neuronal cell soma, neuronal processes or neurites, and areas close to and far away from the cells.

Neural cell soma and processes exhibited different stiffness ranges; neuronal soma was stiffer (~25 kPa on the 5% gels and >40 kPa on the 8% gels) while processes were softer (<7.5 kPa on the 5% gels and ~10 kPa on the 8% gels) than the underlying substrates (~7 kPa for the 5% gels and ~25 kPa for the 8% gels). This observation is consistent with previous reports that neurons prefer softer substrates,[7,8] and may use soft glial cells as substrates *in vivo*.[17] It is pointed out that fixation normally increases stiffness to various degree because it introduces additional crosslinks. However, it is reasonable to assume that the measured trends remain similar for fixed and unfixed cells/tissues in this study. The important novelty of this article is the methodology (mapping the elastic properties by the AFM). In this article, we demonstrate the feasibility of our approach showing examples for neurons grown on different polyacrylamide gels, and in this context fixation is acceptable because stable systems were needed.

It is noted that the area surrounding the neural cell soma possessed stiffness lower than that of hydrogel substrates may suggest the presence of an important structure, the PNN in the culture. One century ago, Camillo Golgi described 'PNNs' enwrapping the cell bodies and proximal dendrites of certain neurons in the adult mammalian central nervous system and suggested that they represent supportive and protective scaffolding. PNN is a specialized form of ECM in the adult nervous system,[5] and its presence in culture has recently been revealed.[19] The thickness of the net can be over 20 μm.[5] Our observations using two different gel stiffnesses seem to indicate the presence of non-cellular structures in the primary spinal cord culture.[19] It is possible that production and secretion of ECM molecules, including proteoglycans, are part of the neuronal response to substrate physical cues. Because PNN has been shown to form and become visible concomitantly with neuronal maturation, even without the presence of the glia[19]; further investigation to verify or refute its presence may shed light on our observation of significant stiffness disparity between the soma and processes and neurons mechano-sensing and mechano-responses. We made observations on the cell

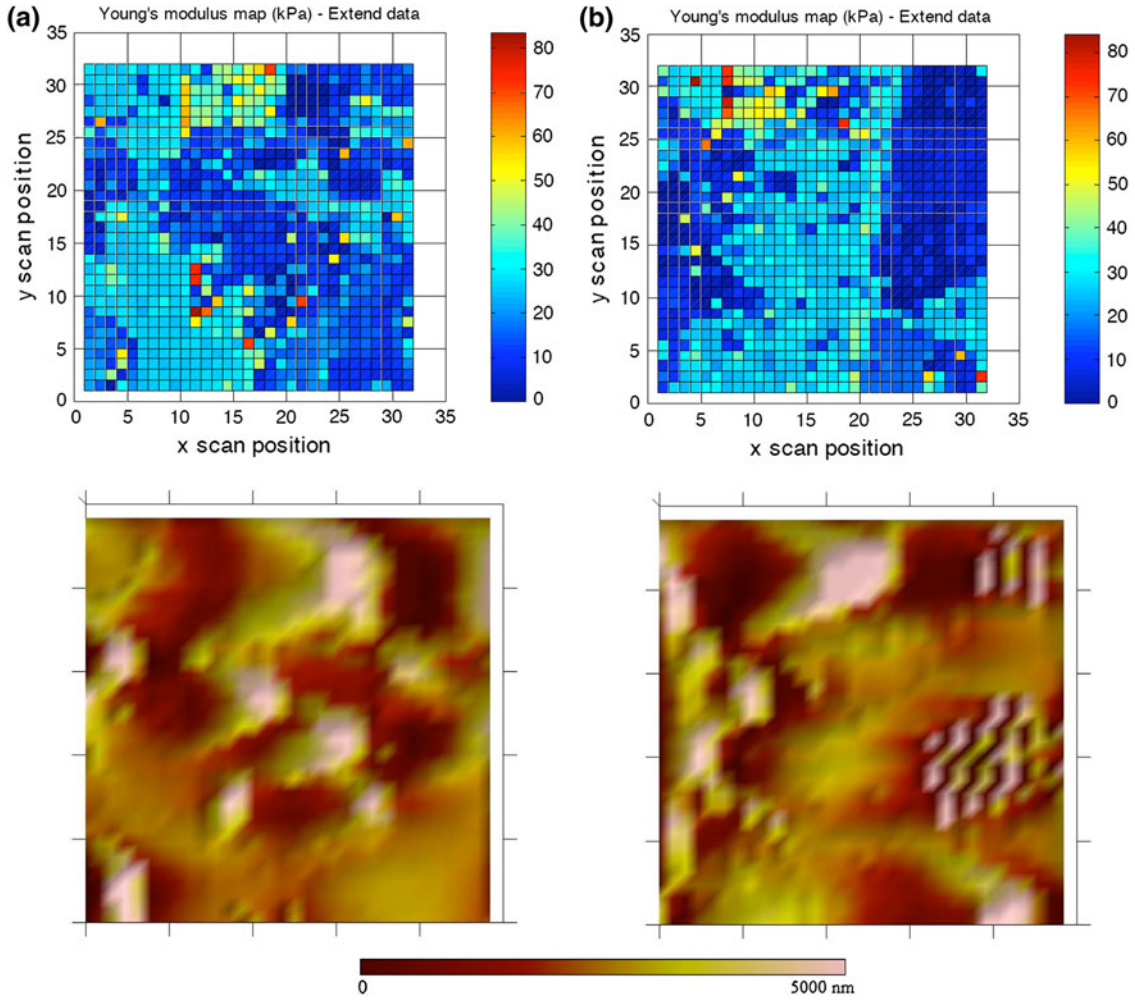

FIGURE 5. AFM images of neuronal culture on 8% gels show cell body of high stiffness, and low stiffness in the close proximity suggesting the possible presence of PNN. Top row for elasticity map and bottom row for topography. Scan area is 50 μm^2 and 32 pix^2. The legend in elastic map is in kPa, and that in topography map in nanometers. Neurons were lightly fixed.

TABLE 2. Mechanical stiffness estimate of lightly fixed primary spinal cord neurons grown on polyacrylamide hydrogels by AFM (stiffness in kPa).

Polyacrylamide gel (%)	Cell soma (ratio to that of substrate)	Area close to soma (ratio to that of substrate)	Neurite (ratio to that of substrate)	Substrate
5	25 (>3)	<5 (<1)	<7.5 (<1)	7
8	>40 (>1)	10 (0.4)	N/A (N/A)	25

Notes: Data are averaged from four cultures on each gel and three 32 × 32 regions from each culture.
N/A, not available.

culture after 6–7 days *in vitro*, which is consistent with the practice described in the literature.[17]

It is noted that identification of neurons with immunocytochemistry is helpful, although based on the previous studies,[9,10,12] the multiple-process-bearing morphology has been proven to be a good indicator of the presence of neuronal cells in the primary spinal cord culture, and has been used in this study. Immunocytochemistry will surely be necessary when investigating the potential differential responses of neurites (e.g., dendrites vs. axons).

AFM excels in characterizing the micro-environment of the substrates (i.e., wet conditions and minute features). Its multi-functionality (e.g., concurrent imaging and mechanical probing, availability of different tips, and the ability to probe samples submerged

in liquid) makes it an ideal tool for probing the mechanical properties of sub-cellular structures under near native conditions. Further studies will exploit more of its features (e.g., using tips with better defined geometry) to fully realize its potential. It would also be meaningful to perform similar analyses using different substrate materials such as collagen to see whether the observations made here were material-specific. Finally, since we lightly fixed the cells immediately before the measurements as a way to preserve the cell state, it may be interesting to see whether doing so affects cell stiffness. Probing cell state without fixation will be desired, and further studies are ongoing.

CONCLUDING REMARKS

This study is among the first attempts to utilize the capability of AFM in probing nano- and micro-topographical and mechanical properties of neuron-like cells and their underlying substrates under physiological wet conditions. Our data show that neural cells display higher stiffness than the soft hydrogel substrates they reside on, and that the areas close to their soma appear to be of lower stiffness than the soma. We speculate that this may indicate modification of the neurons to the substrate or presence of PNN; studies to support or refute this hypothesis are ongoing. The information obtained will assist in understanding the mechanism of neuronal response to mechanical cues, and facilitate biomaterial and bio-scaffold design for neural tissue engineering. Further investigation currently underway will also test the hypotheses of PNN presence and neuronal mechanical response to the substrates.

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