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A. Cricenti
Istituto di Struttura della Materia del Consiglio Nazionale delle Ricerche, cricenti@vaxism.ism.fra.cnr.it

G. De Stasio
Istituto di Struttura della Materia del Consiglio Nazionale delle Ricerche

R. Generosi
Istituto di Struttura della Materia del Consiglio Nazionale delle Ricerche

P. Perfetti
Istituto di Struttura della Materia del Consiglio Nazionale delle Ricerche

M. T. Ciotti
Istituto di Neurobiologia del Consiglio Nazionale delle Ricerche

See next page for additional authors

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ATOMIC FORCE MICROSCOPY OF NEURON NETWORKS

A. Cricenti*, G. De Stasio, R. Generosi, P. Perfetti, M.T. Ciotti[1], D. Mercanti[1]

Istituto di Struttura della Materia del Consiglio Nazionale delle Ricerche, via Enrico Fermi 38, 00044 Frascati, Italy

[1]Istituto di Neurobiologia del Consiglio Nazionale delle Ricerche, viale Marx 15, 00100 Roma, Italy

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Abstract

We imaged uncoated neuron networks by an atomic force microscope in the repulsive regime of contact mode. Images of granule cells and their axons have been clearly revealed with details smaller than 20 nm. The good stability of the sample and the mechanical reproducibility of the microscope allowed the imaging of a neuron culture area of several square microns. By combining tens of images, we were able to reconstruct a highly defined neuronal network. Furthermore, the images were very reproducible over repeated scanning acquisition, demonstrating the mechanical and thermal stability of the instrument-sample system.

Key words: Atomic force microscopy, neuron, neuron granule, neuron axon.

*Address for correspondence:
Antonio Cricenti
Istituto di Struttura della Materia del
Consiglio Nazionale delle Ricerche,
via Enrico Fermi 38,
00044 Frascati,
Italy

Telephone number: +39-6-9426335
FAX number: +39-6-9417003
Email: cricenti@vaxism.ism.fra.cnr.it

Introduction

In recent years, the rapid increase of interest in the neurosciences has stimulated major improvements in the understanding of the biochemistry, physiology, pathology, and pharmacology of the nervous system. A number of studies have improved our knowledge on how neurons communicate and how they make complicated but well-defined networks (Goldman and Selemon, 1990). Neurons in the central and peripheral nervous systems are organized in populations and subpopulations with different chemical and structural properties. Many unexplained aspects of the growth, development, differentiation, and aging of the nervous system must still be clarified. Therefore, it would be extremely useful to analyze the local structural architecture of individual cells.

Before the development of scanning probe microscopies, the only tools for the investigation of the surface of neurons were the optical and electron microscope. However, the resolution achieved with the optical microscope is unfortunately limited to roughly half of the wavelength of light, while in the case of electron microscopy, a nanometer scale resolution can be easily achieved on negative stained samples. In both cases, there is no possibility of making precise structural measurements along the z-axis. Atomic force microscopy (AFM) allows us to overcome the aforementioned problems. In fact, the AFM technique has been strongly developed in recent years (Binnig *et al.*, 1986), and many interesting results have been obtained on non-conductive samples. In particular, biological applications of AFM have clearly shown entire circular filaments of DNA (Bustamante *et al.*, 1992; Thundat *et al.*, 1992), *Escherichia coli* bacteria (Gould *et al.*, 1990), red blood cells (Gould *et al.*, 1990; Haberle *et al.*, 1991), chromosomes (Putman *et al.*, 1992), dried human lymphocytes (van der Werf *et al.*, 1993), and MDCK cells (Le Grimellec *et al.*, 1994; Shoenenberger and Hoh, 1994). Also applications on living cells have greatly increased in the last few years (Henderson *et al.*, 1992; Kasas *et al.*, 1993; Putman *et al.*, 1994).

AFM has been recently applied to study a cultured

neuronal population (Umemura *et al.*, 1994). However, only the rough contour of some cells could be imaged when the microscope was operated in contact mode and the images changed their features on repeated scannings. Umemura *et al.* (1994) tried to improve their images by working in tapping mode, where the lateral forces are lower than in the contact mode, and they could get higher resolution images of the cells showing particles of several tens of nanometers in width.

In this paper, we present images of uncoated primary rat cultures of neurons (granule cells), grown on a gold plated stainless steel substrate, obtained by means of our home-made atomic force microscope operating at constant force. Granule cells and their axons were observed and followed by moving x-y direct current (DC) motors, allowing the observation of the neuron network over a region of several tens of micrometers with a lateral resolution of few tens of a nanometer.

Materials and Methods

Sample preparation

Neuron granule cells were prepared by enzymatic and mechanical dissociation of the cerebellar tissue obtained from eight-day old rats according to the procedure described by Levi *et al.* (1984). Dissociated neurons were plated at a density of 2 x 10^5 cells/cm^2 in Petri dishes containing a gold coated stainless steel substrate previously coated with poly-l-lysine. Cells were cultured in Basal Eagle's medium (BME) containing 25 mM KCl and 10% heat-inactivated fetal calf serum (FCS) in a humidified 5% CO_2 atmosphere at 37°C. Cultures were used at 8 days after plating when full differentiation is attained. Cells were fixed for ten minutes in 4% formaldehyde in phosphate buffered saline (PBS) solution, rinsed with double-distilled water and dehydrated in a desiccator at a reduced pressure for 24 hours. With the material used and this sample preparation, the expected size of neurone granule cell is less than 5 μm.

Atomic force microscopy

Our AFM is described in detail elsewhere (Cricenti and Generosi, 1995). In short, it consists of a unit made of two separable cylindrical supports. The lower one contains the sample holder mounted on top of a piezoelectric scanner (movements 6 μm x 6 μm x 3 μm), contained in a motor controlled x-y-z stage (movements 8 mm x 8 mm x 1 mm). A laser deflection circuit is mounted onto the top cylinder. The AFM is mounted inside a double wall stainless steel chamber through a vibration isolation system. The space between the walls is pumped down to 10^{-2} mbar in order to reduce acoustic and electromagnetic noise. After sample loading, the chamber is closed so that the sample is left in a controlled environment that does not vary from time to time. Constant force images have been obtained with the microscope working in the repulsive mode with a force less than 1 nN from zero cantilever deflection. Gold coated Si_3N_4 microlevers (Park Scientific Instruments, Sunnyvale, CA) having a diamond integrated pyramidal tip, with a spring constant of 0.023 N/m were used. An optical microscope (up to 120x magnification) together with a millimeter x-y stage allowed us to select suitable areas of the sample, thus avoiding lumped aggregates of cells. Friction measurements have also been performed and will be discussed in a forthcoming paper. All the images shown are unfiltered with only rigid plane subtraction. Unlike the results of Umemura *et al.* (1994), the features in our images did not change on repeated scanning acquisition, i.e., images taken on the same area after one week did not show any appreciable modification.

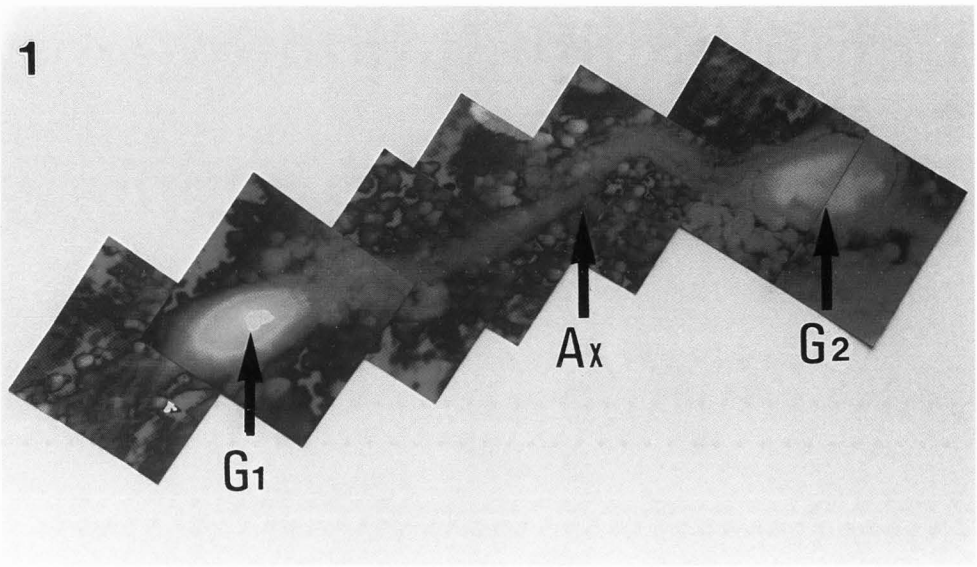
Figure 1. Composite of seven AFM images (6 μm x 6 μm each) taken in constant force mode. The images are in a top-view representation and have been normalized in order to get the same color for similar heights: yellow color for structures with a height of 1.5-2.0 μm, blue color for structures with a height of 250-350 nm, red color for structures with a height of 40-50 nm. The movements of the sample have been performed by moving the x-y DC motors with submicrometer precision.

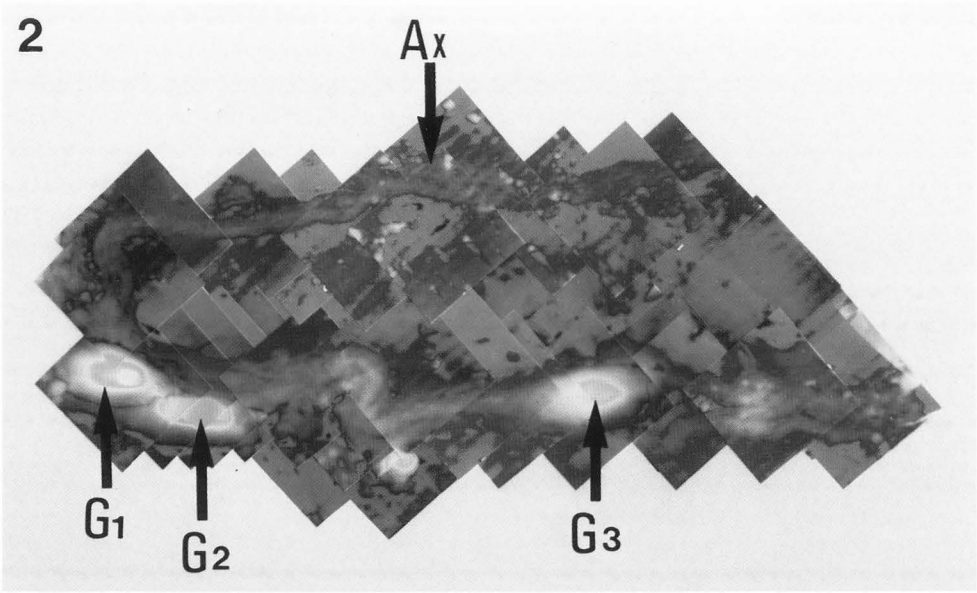
Figure 2. Composite of twenty-two AFM images (6 μm x 6 μm each) with the same normalization and the same colors set in Figure 1. The image shows three granule cells (G1, G2 and G3) interconnected by axons (Ax).

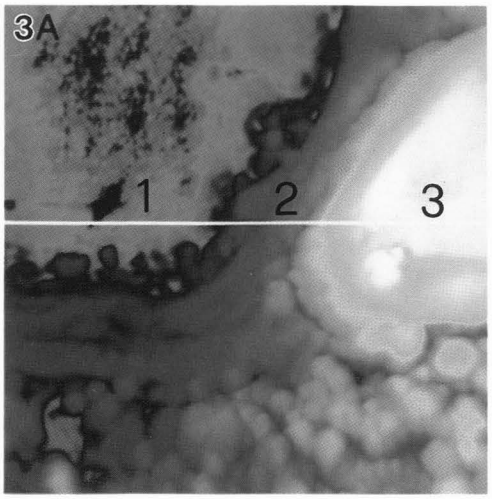
Figure 3. (**A**) AFM image (6 μm x 6 μm) of a part of a granule cell (region 3), an axon (region 2) and the gold substrate (region 1). (**B**) The corrugation of the white line trace in Figure 3A. The gold substrate (red region) has variations of a few tens of nanometers, the axon (blue feature) has a height of a few hundred nanometers, while the neuron cell (yellow feature) has a height of 1.1 μm and variations of several tens of nm (colors are set as in Fig. 1).

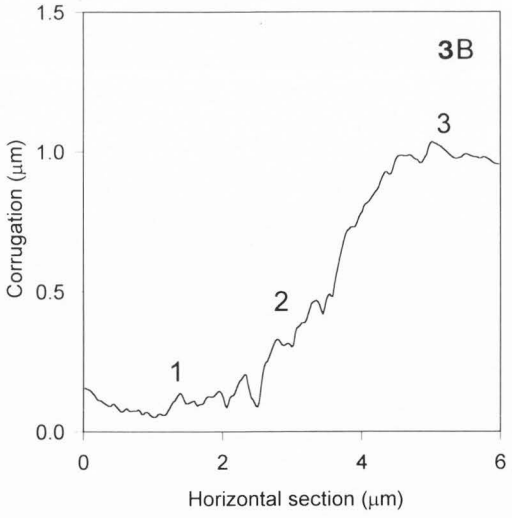
Results and Discussion

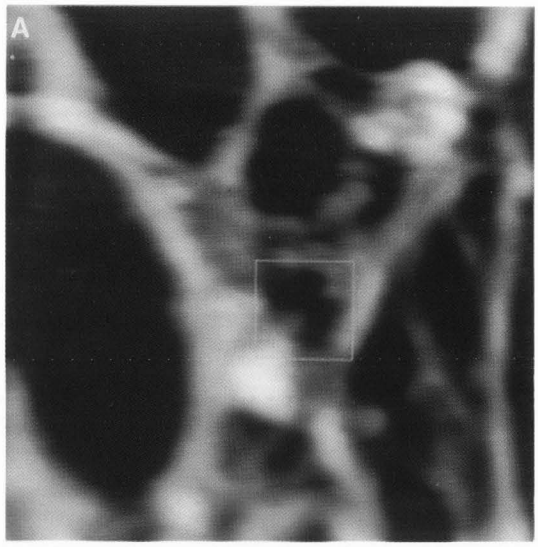
Figure 1 shows a composite of several AFM images (6 μm x 6 μm each). Protruding structures (G1 and G2 on the figure) of xyz size comparable to that expected for granule cells (yellow part of the images, 3.0-5.0 μm diameter, 1.5-2.0 μm height) and axons (blue part of the images, width and height of a few hundred nanometers) are visualized on the bare gold substrate (red part of the

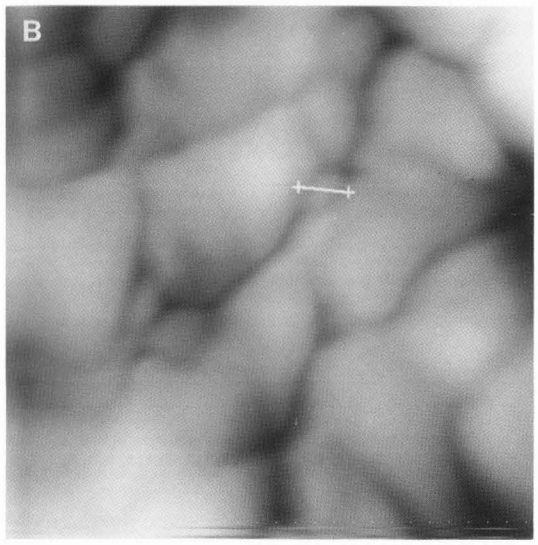

1
Ax
G2
G1


2
Ax
G1
G2
G3


3A
1
2
3


3B
1.5
1.0
0.5
0.0
Corrugation (μm)
0
2
4
6
Horizontal section (μm)
1
2
3





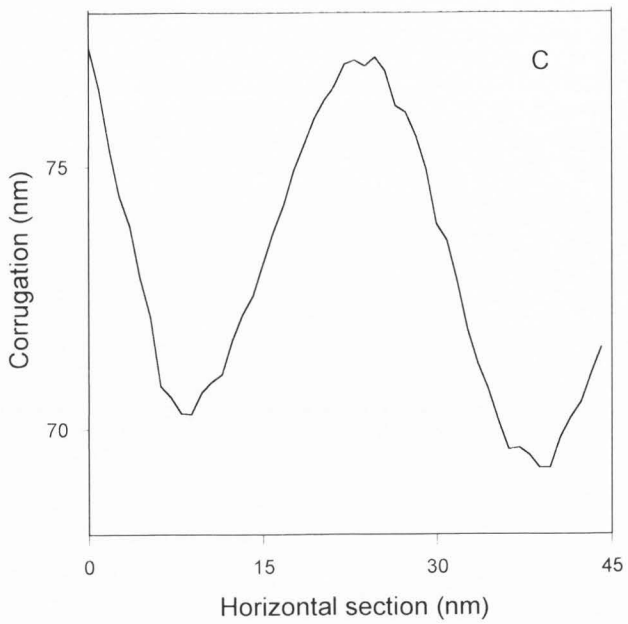


Figure 4. (**A**) AFM image (6 μm x 6 μm) of a network made of several axons (white part of the image) on the gold substrate (dark regions). (**B**) AFM image (500 nm x 500 nm) of the square in the center of Figure 4A. (**C**) Line trace between the two crosses at the center of Figure 4B.

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images, variation of few tens of nanometers). After taking one image and recognizing a neuron cell (G2), the sample was moved a few micrometers with the x-y motors in order to follow an axon (Ax on Fig. 1) structure departing from the cell and reaching another cell (G1) approximately 15 micrometers apart. The height in the images has been normalized so that the yellow features are the neuron cell bodies, the blue ones are the axons and the red region is related to the gold substrate.

Figure 2 shows a few granule cells (G1, G2 and G3) interconnected by axons (Ax) on an area of several tens of square micrometers. It is interesting to observe the contact inhibition between two granule cells (G1 and G2 on the left of Fig. 2) that are proximal to one another but still not in contact: several images of different clusters of granule cells always showed this peculiarity.

Figure 3A is a single AFM image (6 μm x 6 μm) taken from the upper part of Figure 1 and shows a single granule cell (white-yellow part of the image). The line trace drawn on Figure 3A is represented in Figure 3B and shows the different heights of the axon (region 2) and of the cell body (region 3) with respect to the gold substrate (region 1). In this case, the variations of the gold substrate (first part of the line), are of the order of 40-50 nm; the axon is around 400 nm and the granule's height is 1.1 μm. Unlike previous AFM results from Umemura *et al.* (1994), the neuron cell surface appeared highly corrugated, with a corrugation amplitude of several tens of nanometers. Presumably, this higher resolution is due to better stability of the sample-tip system.

Figure 4A shows a AFM image (6 μm x 6 μm) of a network made of several axons (white part of the image) on the gold substrate (dark regions). Note the great capability of AFM to reveal fine details (of the order of a few tens of nanometer) on the specimen, such as, neural fibers that connect individual granule cells. The square in the center of Figure 4A indicates a 500 nm x 500 nm zone observed with the microscope and shown in Figure 4B. It is interesting to observe the fine details of this cluster. Figure 4C is a line trace between the two crosses at the center of Figure 4B showing a structure with a mean width half maximum of the order of 15 nm and a height of 7 nm.

It is worth noting that the morphology of the neuronal cellular system presents very characteristic shapes which, therefore, are easily identified in our micrographs. This is an advantage for distinguishing cellular features from substrate artifacts or possible contaminants. The images also show that the property of the neurons to grow as a monolayer over a flat substrate is almost ideal for AFM experiments. The specimens were found to be very stable over long periods of time: studies performed over approximately one month did not detect any alterations. Such results are an important step towards the possibility of: (1) studying living neuron cells in their culture medium in order to investigate, for example, the growth process and, possibly, the propagation of the nerve pulse; and (2) observing native and functional neurons under physiological and pathological conditions. With this perspective in mind, we note the importance of the high reproducibility and the high quality that we obtained on neuron networks with AFM in air, as preliminary results. Microchemical modifications of neuron cells under treatment are currently being studied.

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Discussion with Reviewers

N.F. van Hulst: The images are similar to scanning electron micrographs and do not prove the real potential of AFM, i.e., to operate on living biological samples in their natural environment. Please comment.

Authors: We do not agree that the real potential of AFM is to operate on living biological samples because much important information can only be obtained on dried samples where scanning electron microscopy (SEM) images cannot be similar to AFM ones since, in SEM, a metal coating is required to render the sample conductive. In particular, on dried neurons, there are many aspects that have been studied with ultra-high-vacuum techniques, such as, spectromicroscopy with synchrotron radiation [see, e.g., Mercanti *et al.* (1991)].

G.M. Roomans: It has been known for a long time that air-drying or drying at reduced pressure is a poor way to prepare biological tissue for high-resolution microscopy. This procedure results both in collapse of the tissue and in damage of the specimen surface due to the expanding water vapor. This is why in SEM, specimens are prepared by critical point drying or freeze-drying. Why then did you use drying at reduced pressure from an aqueous solution in your case?

Authors: This way of preparing neuron samples is the best one for obtaining good and reproducible results when working with ultra-high-vacuum techniques [see, e.g., Mercanti *et al.* (1991)]. We can say the same for the AFM results since the samples are not damaged when dried and are stable for a long period of time: one sample was observed one year after preparation, and in the AFM observation, both the granule cells and the axons maintained the expected size.

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