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Nano-probing of the membrane dynamics of rat pheochromocytoma by near-field optics

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Abstract

High-resolution analysis of activities of live cells is limited by the use of non-invasive methods. Apparatuses such as SEM, STM or AFM are not practicable because the necessary treatment or the harsh contact with system probe will disturb or destroy the cell. Optical methods are purely non-invasive, but they are usually diffraction limited and then their resolution is limited to approximately 1 μm. To overcome these restrictions, we introduce here the study of membrane activity of a live cell sample using a Scanning Near-field Optical Microscope (SNOM). A near field optical microscope is able to detect tiny vertical movement on the cell membrane in the range of only 1 nm or less, about 3 orders of magnitude better than conventional optical microscopes. It is a purely non-invasive, non-contact method, so the natural life activity of the sample is unperturbed. In this report, we demonstrated the nanometer-level resolving ability of our SNOM system analyzing cardiomyocytes samples of which membrane movement is known, and then we present new intriguing data of sharp 40 nm cell membrane sudden events on rat pheochromocytoma cell line PC12. All the measurements are carried out in culture medium with alive and unperturbed samples. We believe that this methodology will open a new approach to investigate live samples. The extreme sensitivity of SNOM allows measurements that are not possible with any other method on live biomaterial paving the way for a broad range of novel studies and applications.

Keywords: PC12; SNOM; Cardiac myocites; Membrane; Cell; Dynamics

1. Introduction

PC12 cells originate from rat pheochromocytoma cell line from adrenal medulla; when grown in a serum-containing medium, PC12 cells divide and resemble precursors of adrenal chromaffin cells and sympathetic neurons. Upon addition of Nerve Growth Factor (NGF), they extend long, branching neuronal-like processes, gradually attaining the phenotypic properties of sympathetic neurons; thus the PC12 cell line is widely used as a model system of neuronal cells [1] and has been successfully used over the years to study neuronal functions [1–3].

The effects of NGF on PC12 cells are reversible; about 3/4 of the cells lose their processes and cell multiplication returns to control rates, respectively, 24 h and 3 days after their return to NGF-free medium [1]. Degeneration, rather than withdrawal, seems to account for the first phenomenon; removal of NGF does not, however, appear to affect the integrity of the cell bodies [1]. PC12 cells grow in suspension in medium containing foetal bovine serum (FBS) and horse serum and their doubling time is long, about 92 h; cell growth is unsatisfactory when horse serum is omitted and they undergo to apoptosis in approximately 48 h in a serum-free medium [1,4].

PC12 cells adhere poorly to plastic tissue culture dishes and well to collagen-coated substrates; in growth medium they have a round or polygonal shape and tend to grow in small clumps [1]. The line we used here has a homogeneous and near-diploid chromosome number of 40 that consist of 38 autosomes and an XY pair [1]. Since PC12 that have

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undergone approximately 70 generations from their isolation from a solid tumour have shown no major changes in their properties or NGF sensitivity, the homogeneity and near-diploidy of the cell chromosome number suggest that the line will tend to remain stable (genotypically and phenotypically) in vitro for many generations [1].

The PC12 cells also contain considerable amounts of norepinephrine and dopamine. In contrast to adrenals, however, dopamine is predominant; epinephrine is not detectable [1,5]. PC12 also possess mechanisms for both the Ca^{2+}-dependent exocytotic release and the cocaine-sensitive high-affinity uptake of catecholamines [5–10].

Submicrometer studies of cell ultrastructure have been performed with scanning- and transmission-electron microscopes (SEM and STM), and atomic-force microscopes (AFM). However, living cells cannot be examined with the first two instruments because those systems require cell fixation and observation in vacuum, and images of living cells obtained with the AFM may be compromised by direct contact between the cantilever and the sample-deforming soft tissue.

SNOM systems [11] include a sharp optical fiber used as a probing element to collect the optical field created in proximity of a sample. There are a number of optical configuration models in which a SNOM can be operated. The most common ones are collection, reflection, and illumination modes [12].

We have previously reported the observation of live-cell dynamics by noncontact scanning near-field optical microscopy (SNOM) modified to work with living biological samples that are fully immersed in liquid using cardiac myocytes in culture. We aimed at detecting morphological activity of a rhythmically contracting syncytium of these cells and we could examine the dynamics of rhythmically beating cardiac myocytes with extremely high vertical sensitivity below the nanometer range [13,14].

In this study we aimed at detecting the morphological activity by using rat pheochromocytoma cell line PC12 in which membrane movement is not well known and/or detectable, and we detected for the first time tiny sharp membrane movements. These unexpected results indicate that the investigation method is able to expose new membrane phenomena of extreme interest.

2. Materials and methods

Undifferentiated rat pheochromocytoma PC12 cell line were routinely cultured in 100 mm-diameter plates in D-MEM/F-12 (Dulbecco's modified eagle medium: nutrient mixture F-12 Ham 1:1, GIBCO, New Zeland) with L-glutamine, sodium bicarbonate and pyridoxine hydrochloride, supplemented with 10% heat-inactivated fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA) and 5% horse serum (GIBCO, New Zealand) in a humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C. For the experiments, PC12 cells were plated onto 35 mm-diameter collagen-coated dishes in fresh growth medium and maintained in the humidified incubator until they reached ~90% confluence. Medium was changed every 3 days. Then the culture was transferred to the optical setup for examination. The area surrounding the sample was maintained at ~37 °C in a humidified atmosphere by circulation of warm humid air under the plastic curtain that surrounded the optical system to guarantee the cell survival in optimum conditions during the experiments.

2.1. Cardiac myocites

The hearts were removed from 4 neonatal Sprague–Dawley rats (age 1–2 days) and minced. The chopped hearts were then washed three times in ice-cold Ca/Mg-free HBSS (Hanks balanced salt solution, Kanto Kagagu – GIBCO BRL – Inagi Soukasi, Saitamaken, Japan). After washing, the HBSS was replaced with 8 ml of 0.05% crude trypsin (Sigma-Aldrich Japan K.K., JL Nihonbashi Building, Tokyo) in versene buffer (GIBCO) and after 8 min of incubation at 37 °C the supernatant was discarded. The chopped hearts were then enzymatically dissociated by the addition of 100 μl DNAase Type II solution (10 000/ml, Sigma) followed 1–2 min later by the addition of 2.5 ml of 0.05% crude trypsin/versene and stirred for 8 min at 37 C. The supernatant was collected, leaving the undissociated tissue in the centrifuge tube, and added to 4 ml of HEPES buffered Hams F10 (GIBCO) containing 0.5% ITS (insulin, transferrin, selenite solution, GIBCO) and 36% FCS (fetal calf serum, GIBCO) to block trypsinisation, centrifuged for 5 min at 1500 U/min, re-suspended in 0.5–1.5 ml of ice-cold HEPES buffered Hams F10 containing 0.5% ITS and 10% FCS and stored at 0–4 C. Meanwhile, the undissociated chopped heart tissue remaining in the 20 ml conical centrifuge tube was once more enzymatically dissociated. This cyclical enzymatic dissociation procedure was repeated 4 times. The collected cell suspensions were pooled and differentially adhered to a 25 cm^2 tissue culture flask for 1 h. The heart cells were then plated onto glass slides coated with 2 μg/cm^2 fibronectin (Sigma) at densities of 106 cells/ml and incubated at 37 C for 24 h in HEPES buffered Hams F10 containing 0.5% ITS (insulin, transferrin, and sodium selenite, GIBCO), 2.5 ml/100 ml of an antibiotic solution (consisting of 200 mM glutamine, Sigma; 5000 U/ml Penicillin/streptomycin, GIBCO; 250 μg/ml of Fungizone, GIBCO) and 10% FCS. The cells were fed daily with pre-warmed HEPES buffered Hams F10, containing 0.5% ITS, antibiotics, and 3% FCS (feeding media). For SNOM imaging, cells were maintained alive by keeping them bathed in feeding media and the examination was done under the plastic curtain as explained above.

Cell viability was assessed by trypan blue dye exclusion prior to all experiments. Cells with compromised cell membrane appeared blue due to accumulation of dye, and

were considered as dead. Cells with ~96.0% viability were employed in all the experiments here reported.

3. Experiments and results

One of the most important developments in the SNOM measurements of a biological sample is the ability to study it in a liquid medium; this potentially allows the examination of living biological samples. Briefly, the SNOM system (Fig. 1) consists of a piezoelectric three-dimensional actuator mounted on a standard fluorescent microscope (Olympus 1X70). In this configuration, light can be applied to the underside of the sample in transmission mode or in total internal reflection mode [12]. The resulting near-field signal is then collected from the upper surface of the sample with a SNOM tip and passed through a suitable filter before being fed into the optical detector. In all of our experiments the sample was mounted on the piezoelectric actuator, and the SNOM probe was mounted on a holder bolted to the light microscope stage. It consists of a glass body (optical fiber) tailored to a conical shape. We constructed our own optical probes with simple techniques involving the chemical etching of optical fibers [15–17]. The optical configuration that we chose for our purpose is shown schematically in Fig. 2. Light illuminated the sample from the bottom, creating a spot of light that was transmitted throughout the whole sample. Above the sample, a fiber tip placed in its proximity was used as a probing element to collect the intensity of the optical field in a submicrometer-sized localized area. If there were variations of the morphological status of the cells, the coupled field would change accordingly, allowing us to record a signal associated to the cell activity. The sample holder was mounted on a piezoelectric three-dimensional actuator that scanned an area of the sample, yielding an image of the specimen under investigation. The setup and the control software that we developed allowed us both to scan and to stop at any point of the image that was forming on the screen of our monitor. When the scan was halted we were able to start recording the localized light field in that point.

Sample-probe separation was controlled by shear force only during approach over a clean area of the substrate. In other words, the shear force approach was used as a $z=0$ reference. Then we moved z back of about 2–3 μm and translated the probe over the cell membrane with the aid of optical monitoring through the microscope (our system allowed us to see the sample and the nano-probe simultaneously). Once over the sample, we started scanning in free running mode, sometimes adjusting the z manually to improve SNOM image. In accordance with near-field theory [11,12] we assumed in optimal condition the probe to be between 100 and 200 nm far from the cell membrane. We had no means to measure directly this value; nevertheless we made an approximate estimation by observing the response of the signal to minor probe shifts in horizontal direction (for higher sample-probe distances we had lower or null sensitivities to probe displacement). Once an interesting area was found, we would stop scanning and begin an in situ recording of the optical signal dynamics. After the recording was completed (approximately 30 sec),

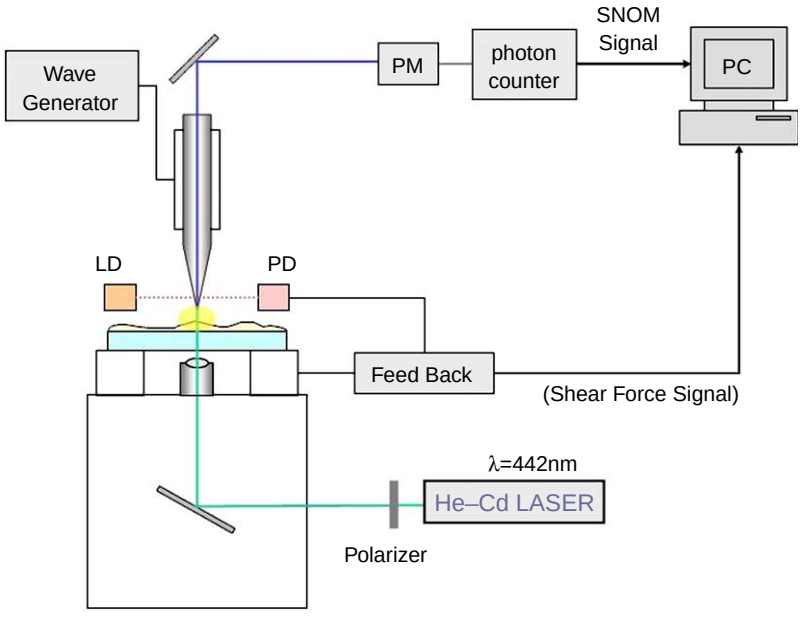

Fig. 1. The SNOM apparatus. It consists of a piezoelectric three-dimensional actuator mounted on a standard fluorescent microscope. In this configuration, the light could be applied to the underside of the sample in transmission mode. The resulting near-field signal was then collected from the upper surface of the sample with a SNOM tip and then fed into the photomultiplier (PM). Sample-probe distance was controlled through an optical Shear Force feedback consisting of a low power laser diode (LD, 680 nm) and a photodiode (PD). In the experiments in liquid we initially approached the glass substrate to have a reference and moved a few microns away from it. Subsequently we placed the probe over the cell culture and recorded in free running mode.

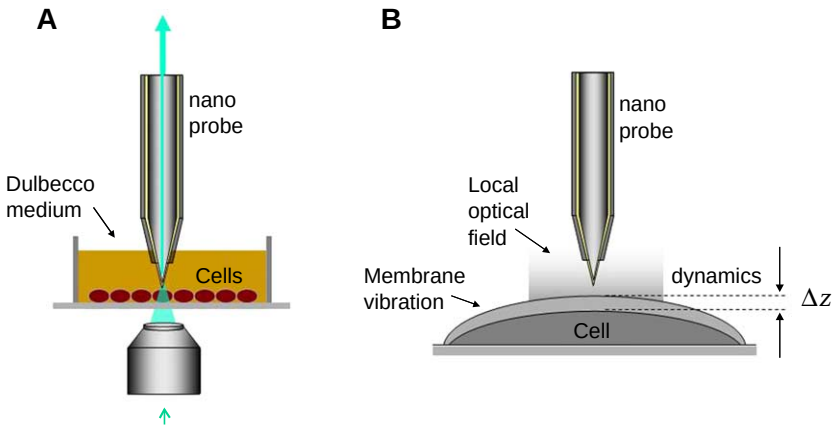

Fig. 2. (A) Schematic view of the liquid cell, core of the apparatus. (B) A close-up representation of the method for signal detection. Nanometer-sized vibrations of the cell membrane perturb the local optical field in the proximity of the cell membrane; a nano-probe locally detects and records these variations. The probe is a metal-coated tapered optical fiber. The apex aperture is approximately 200 nm; the cone angle is approximately 15°.

we would move the tip to another region of interest and stop there for following recordings.

In Fig. 3 we show two scans of the cardiac myocytes used as a positive control, and the relative recordings at two points (P_1 and P_2). These profiles were the result of the beating cell's modifying the optical coupling between tip and sample. The beating is composed of a main peak followed by a much smaller one slightly delayed in time. All the peaks in this recording had similar profiles, indicating that we were observing a complex dynamic feature of the cell's contraction. The recording P_2 revealed that the amplitudes of contractions could change dramatically between adjacent recording areas. The recording sites P_1 and P_2 were adjacent, separated by 0.9 μm; the two profiles showed distinct behavior, revealing that the dynamic properties of the membrane cell were discriminated at different points.

The recording shown in Fig. 4 is referred to the undifferentiated rat pheochromocytoma cell line PC12, called PC12-like neuronal cells because of their properties and behavior. These cells do not have any known movement or variation of their morphological status associable and/or comparable to the beating of cardiomyocytes because of their different nature. The measurements were made in the same conditions used for cardiomyocytes; PC12 cells were plated onto 35 mm-diameter collagen-coated dishes in fresh growth medium and the culture was transferred to the optical setup for examination as described in Materials and methods. The optical fashion in which the data were collected allowed a completely noninvasive recording of the signal. The sample was not disturbed or influenced by the process of measurement.

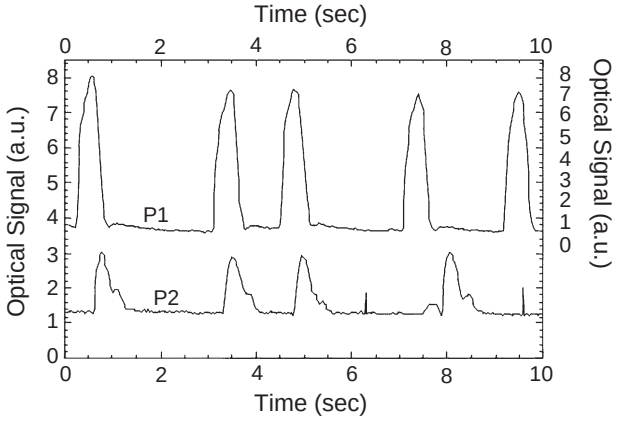

Fig. 3. Recording of the membrane dynamic time profiles of P19 cardiomyocytes. Two symbols P_1 and P_2 represent recordings over the points separated by less than 1 μm on the cell membrane. Each single profile was reproducible and self-similar, however different points on the membrane showed dissimilar time profiles. This fact pointed out the ability of the apparatus to discriminate diverse dynamical feature of the cell membrane vibrations. Right and left scales refer to P_1 and P_2, respectively. Recordings were taken about 30 sec apart. The signal was acquired directly from the photomultiplier and converted by the photoncounter. Optical signal in arbitrary units (a.u.) corresponds to the photoncounter voltage level.

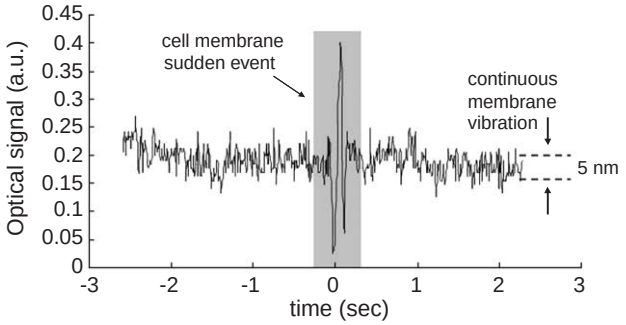

Fig. 4. Recording of undifferentiated PC12-like neuronal cells. Even if these cells do not have known movement during physiological conditions, we could detect for the first time an evident spike of signal corresponding to approximately 40 nm membrane displacement. We also observed a continuous membrane vibration in the 5 nm range. Our system had a noise level of approximately 1–2 mV in the absence of signal. Signal was measured in V, 100 mV corresponded to a displacement of approximately 10 nm (see text for details). Optical signal in arbitrary units (a.u.) corresponds to the photoncounter voltage level, so values are actually in V. See text for discussion about calibration.

The data showed a continuous noisy signal, as indicated in Fig. 4. This signal was observed along the culture, independently of the cell observed and for the majority of the time. When probing over specific cells we observed unexpected phenomena such as the spike indicated in the plot. These sudden vibrations where rare, but occurred regularly; the temporal profile of the spike was also reproducible as shown by other recordings in Fig. 5.

To estimate the extension of the membrane spikes observed we used as a reference the voltage signal detected on the cardiomyocytes in Fig. 3. From previous calibration studies [13,14] we recognized that 100 mV corresponded to approximately 10 nm of actual vertical membrane displacement on the cardiomyocytes. Assuming this calibration as correct and applying it to the observed peaks on PC12, we could conclude that they had an actual vertical extension of approximately 40 nm, in contrast to the cardiomyocytes cell "beating" of approximately 0.5 μm. We understood that the optical voltage signal from a phototube could rescale significantly if – for example – the optical condition of the probe was changed, or for other not controllable phenomena. If this was the case, the calibration proposed would be altered. A factor that pointed to the correctness of our estimation was that the continuous "noisy" signals we observed when there were no membrane spikes or beating, were, with the current calibration, matching relatively well. This was compatible with the fact that differentiated P19 cardiomyocytes and PC12 cells are similar and presumably

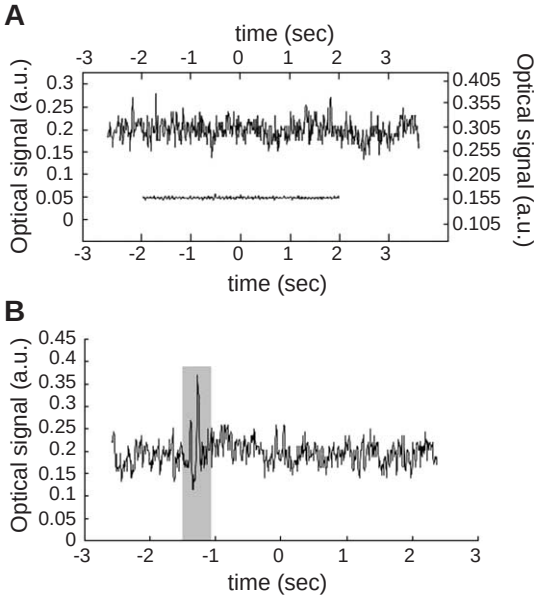

Fig. 5. (A) In situ recordings on the membrane of PC12 cells showing a continuous membrane vibration. This represents a typical signal characterized by a noise-like pattern of approximately 50 mV amplitude, corresponding to about 5 nm actual membrane displacement. Noise without sample is approximately 6–7 mV; corresponding scale is on the right. Rarely but regularly we observed sudden spikes of signal as in the plot shown in panel (B). The results here shown corresponded to a membrane displacement of approximately 30 nm.

showed a continuous membrane vibration pattern of the same extension. However, our calculations should be interpreted as our best estimation of the extent of the cell membrane movement, not of its exact value.

These minuscule movements have never been observed previously and we did not understand fully their actual nature. However, they were apparently associated with the physiological activity of the cell membrane, such as structural cytoskeletal rearrangements due to cell movements or to processes related to cell duplication.

Our study demonstrates that it is possible to detect and investigate these tiny and sudden vertical vibrations as small as 40 nm on the membrane of undifferentiated PC12-like neuronal cells that are not known to have evident movement during physiological conditions.

4. Discussion

The cytoskeleton of eukaryotic cells is fundamental to the spatial organization; the usefulness of the cytoskeleton filaments depends on accessory proteins that link the filaments to other cell components as well as to each other. Cytoskeletal systems are dynamic and adaptable, they can change or persist, according to need; thus the individual macromolecular components that make up these structures are in a constant state of flux. The dynamic assembly and disassembly of cytoskeleton polymers, the regulation and modification of their structure by polymer-associated proteins, and the actions of motor proteins moving along the polymers are all activities coordinated to define a cell's shape, to able/enable it to crawl, to divide or to achieve the ideal structural rearrangement when conditions change. Many cells move by crawling over surfaces; long-distance crawling is fundamental to the construction of the entire nervous system. Cell crawling is a prime example of such complex coordinated cytoskeletal action. It is a highly complex integrated process, dependent on the actin-rich cortex beneath the plasma membrane. In some crawling cells, such as keratocytes, these activities are closely coordinated, and the cells seem to glide forward smoothly without changing shape. In other cells, these activities are more independent, and the locomotion is jerky and irregular and involves variation on the cell's shape. Furthermore, cells undergo cyclic duplication and division: a phenomenon that is called cell cycle, in which a cell duplicates its content and then divides in two. The details of the cell cycle vary from cell to cell and at different times depending on the organism and/or tissue the cell belongs to, however, certain characteristics are universal. DNA in each chromosome must first be faithfully replicated to produce two complete copies and the replicates chromosomes must then be accurately distributed to the two daughter cells; in addition to duplicating their genome, most cells also duplicated their other organelles and macromolecules, otherwise, they would get smaller with each division. To maintain their size,

dividing cells must coordinate their growth (i.e. their increase in cell mass) with their division.

The undifferentiated rat pheochromocytoma cell line PC12 that we used in our experiments were in good and healthy conditions as shown by trypan blue exclusion. We used them in their physiological conditions, without any kind of compound that could cause variation in their physiological status and/or cell death; our purpose was to detect their membrane activity, if there was one detectable, using the same system used for cardiomyocytes, that is the high-sensitive SNOM system that allowed us to work in a liquid medium, allowing the examination of living biological samples. The results shown in Fig. 5 gave us an exciting proof of the high sensitivity of our system, allowing us to detect a very small morphological movement, a vertical sudden spike as small as 40 nm that was not comparable to the evident beating of cardiomyocytes that we used as a positive control in the same physiological conditions shown (Fig. 3). Since we were using PC12 cells in their physiological conditions, this small movement could be associated to the physiological activity of the cell, which is dynamic of cytoskeletal system to achieve the ideal structural rearrangement and/or to be able to crawl. Or it could be associated to cyclic duplication and division that are associated to the duplication of the cell's organelles and macromolecules and cause an increase in cell mass. All these activities could be associated to a movement of the cell, even if small. We have here demonstrated the high sensibility of our system without processing the cells to any treatment such as those needed for using SEM, STM, and AFM but directly using living cells and maintaining them in good conditions during our experiments. Further experiments will be carried out to assign the movement to a specific activity of the cell; since the PC12 line appears to be a useful model system for the study of numerous problems in neurobiology and neurochemistry, it could be interesting to supervise the mechanism of action of NGF and explore its role in the development and differentiation of neuronal stem cells and initiation and regulation of neurite outgrowth. In addition, further investigation will be carried out to evaluate the metabolism, storage, uptake and release of catecholamines under stress insult and changes on morphology after apoptotic stimulus.

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