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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