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Citation	Japanese Journal of Applied Physics. Pt. 1, Regular papers, short notes & review papers, 41(7B), 4952-4955 https://doi.org/10.1143/JJAP.41.4952
Issue Date	2002-07
Doc URL	http://hdl.handle.net/2115/33072
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Type	article (author version)
File Information	JJAP_HUSCAP.pdf

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Improvement of Force Modulation Mode with Scanning Probe Microscopy
for Imaging Viscoelasticity of Living Cells

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We improved the force modulation mode with scanning probe microscopy (SPM) in order to make a quantitative evaluation of the viscoelasticity of living cells. Taking account of the viscosity of liquid medium, the vibration frequency of the cantilever was selected to be 500 Hz, and analysis of cantilever vibration was adopted for evaluation of the viscoelasticity of the samples. Consequently, we have succeeded in determining viscoelasticity distribution on living cells. The values of Young's modulus and the coefficient of viscosity vary from 10 to 50 kPa and from 20 to 40 Pa·s on a cell, depending on its internal cellular structure.

KEYWORDS: atomic force microscopy, cell dynamics, mechanical property, elasticity, Young's modulus, viscosity, fibroblast

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1. Introduction

Cell migration is a very common biological phenomenon at the tissue level. For example, it plays a central role in healing of wounded tissue, self-organizing of the early embryo, and metastasis of tumor cells. Many researchers have intensively studied molecular processes involved in the mechanisms of cell migration. However, cell migration is considered to be a difficult phenomenon to explain at the microscopic level, because it results from complex interactions among numerous proteins. Recently, it was suggested that macroscopic mechanical properties like elasticity relate to cell migration .[1–6]

We have studied the temporal change of local stiffness of a cell to clarify the mechanism of cell migration. Two methods of measuring the stiffness of biological samples were proposed with scanning probe microscopy (SPM); force modulation mode and force mapping mode.[7,8] The force mapping mode has the advantage of obtaining quantitative distribution of static elasticity (Young's modulus).[9,10] However, this mode takes longer than 30 min to capture an elasticity image with 64×64 pixels. Such temporal resolution is too low for a detailed discussion on cell migration. On the other hand, the force modulation mode can capture an elasticity image with higher spatial resolution in less than 10 min which is the same time scale as that for elongating leading edges and contracting posterior parts producing cell migration. Furthermore, it should be noted that this mode can evaluate not only elasticity but also viscosity of biological samples.[11,12] However, the obtained data in liquid medium were often incredible, even qualitatively. For example, cells sometimes looked stiffer than a hard glass substrate in the elasticity images. Therefore, this mode requires some improvements for measuring the viscoelasticity of living cells in liquid cultured medium.

In previous papers, we reported an evaluation method for qualitative viscoelasticity under the assumption that the viscosity of liquid medium is negligible.[13,14] In the present study, we selected the measurement conditions and adopted the analysis of cantilever vibration, taking the viscosity of liquid medium into account. We have succeeded in quantitative evaluation of the elasticity (Young's modulus) and viscosity (coefficient of viscosity), using the improved force modulation mode.

2. Materials and Methods

2.1 SPM imaging of living cells

For the SPM measurements, we used a commercial instrument composed of a SPA400 and SPI3800 equipped with a piezo scanner with a maximal $X - Y$ scan range of 100 μm in each direction and a Z range of 10 μm (Seiko Instruments Inc., Chiba, Japan). It is important to select a suitable spring constant for the cantilever, since the spring constant equivalent to that of the cells is more sensitive for measuring the viscoelasticity. Thus, we selected commercially available silicon-nitride cantilevers with a length of 200 μm and width of 20 μm, and a pyramidal tip with a typical radius of curvature of about 50 nm (ThermoMicroscopes, Sunnyvale, CA). The typical spring constant of this cantilever is 0.02 N/m. This cantilever satisfies the requirements.

Samples were prepared similarly to those reported in the previous papers.[13, 14] The cultured fibroblasts (NIH3T3) were purchased from RIKEN Cell Bank (Tsukuba, Japan). The cells grown in low glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (GIBCO BRL, Basel, Switzerland) were maintained at 37°C and 5% CO_2 in a humidified incubator. The confluent cells were trypsinized and released from plastic flasks for culture. The cell suspension was plated on a glass petri dish precoated with fibronectin (Boehringer Mannheim, Mannheim, Germany) for the SPM imaging. This petri dish was then placed in the incubator for at least one night. To maintain physiological conditions, preheated HEPES buffer (pH 7.2~7.3) was substituted for the culture medium in the dish before the measurement and the temperature of the buffer was controlled at 33±0.5°C during the measurement.

2.2 Force modulation mode

When sinusoidal stress is applied externally to samples, the viscoelasticity can be estimated from an induced sinusoidal sample strain. On measuring the viscoelasticity in the force modulation mode, either a cantilever or a sample stage is vibrated sinusoidally during scanning in the contact mode. Figure 1 shows a schematic of the instrument. To vibrate the sample stage, we applied sinusoidal voltage to a scanner with a function generator (AFG310; SONY, Tokyo, Japan). In this mode, deflection signals of the cantilever

consist of two components which reflect the morphology (DC component) and viscoelasticity (AC component) of samples. These signals are detected by the position of the laser on a sensitive photodetector. To evaluate viscoelasticity, the AC component of the deflection signals is extracted through a two-phase lock-in amplifier (Model 5210; PAR, Oak Ridge, TN), and is then visualized as amplitude and phase shift images.

It is difficult for elasticity and viscosity of samples to be estimated from the amplitude and phase shift, because the detected oscillation at the detector is not that of the sample strain but that of cantilever deflection. Furthermore, the influence of the viscous drag of the liquid medium should be taken into account for quantitative evaluation. Thus, we adopted the analysis of SPM cantilever vibration including effects of both resistance force due to sample viscoelasticity and liquid drag against cantilever vibration.[15, 16] A major modification on this analysis is to analyze the vibration of an elastic cantilever touching a viscoelastic sample in viscous medium by means of a one-dimensional equation (see Appendix). This analysis allows the evaluation of one-dimensional elasticity and viscosity from the amplitude and phase shift of cantilever deflection signals. Young's modulus E_s and coefficient of viscosity η_s can be obtained from these values, on the assumption that the depth of indentation of the samples by the cantilever is constant (about 200 nm).

3. Results and Discussion

We measured the frequency dependence of the amplitude of cantilever deflection when an external vibration with an amplitude of 10 nm was applied to the cantilever (Fig. 2). The cantilever deflection is affected strongly by the viscous drag of the liquid medium in contrast to the sample viscoelasticity when the frequency exceeds 1 kHz. In this case, it is quite difficult to evaluate the viscoelasticity of samples. On the other hand, it takes more time to capture a viscoelasticity image when the frequency selected is lower. Thus, we selected 500 Hz as the measuring frequency. A 128×64 pixel image can be obtained in only 6 min under this condition. The spatial and temporal resolution of the SPM measurements is 10 times the higher than that of the force mapping mode. However, the influence of the viscous drag of the liquid medium is still comparable to that of the viscoelasticity of samples even at this frequency. This result means that taking account

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of the viscous drag of the liquid medium is necessary for an accurate analysis of SPM cantilever vibration.

Figure 3 shows SPM results for a living fibroblast measured in the improved force modulation mode. These are topography (a), deflection (b), amplitude (c), phase shift (d), elasticity (e) and viscosity images (f). The height of this cell is about 4 μm. The highest region in the topography represents a cell nucleus (an *oval* in (a)). Many filaments running beneath the cell surface can be seen clearly (*arrows* in (a) and (b)). We confirmed that these filaments reflect stress fibers composed of actin filaments and myosin II beneath a cell membrane by immunofluorescence observation (data not shown). In the amplitude (c) and the phase shift images (d), the values on the cell indicate smaller amplitude and larger phase shift than those on the glass substrate, respectively. In the elasticity image (e), brighter areas represent greater hardness. The values of elasticity are not uniform over the surface but vary from 10 kPa to 50 kPa depending on the position of the cell. Stress fibers are stiffer than their surroundings (*arrows* in (e)). This is in good agreement with results obtained previously in the force mapping mode. In the viscosity image (f), brighter areas represent higher viscosity. The values of viscosity are not uniform over the cell. The coefficient of viscosity of the cell is about 25 Pa·s. The nuclear region appears less viscous than the periphery.

In the force modulation mode, the viscoelasticity data are taken during lateral scanning of the cantilever on sample surfaces. We compared the line profiles of elasticity and viscosity with those of height and deflection to evaluate the influence of topographic roughness of samples. The line profiles between the *white arrowheads* on Fig. 3 (a) are shown in Fig. 4. Many stiffer peaks appear on the line profile of the elasticity (*filled circles*). Peaks of the topographic (*filled triangles*) and deflection profiles (*filled squares*) are observed at the same positions as the elasticity peaks. However, the heights of the elasticity peaks have no correlation with those of the topographic or deflection peaks. There are no large sharp peaks in the line profile of the viscosity (*open circles*). These results clearly verify that the surface roughness does not affected the elasticity and viscosity data under the present condition (scan speed = 0.5 line/s).

4. Conclusions

We improved the force modulation mode with SPM by considering the viscous drag of liquid medium. We have succeeded in measuring both quantitative elasticity and viscosity of living cells in this mode. Imaging viscoelasticity in high temporal and spatial resolution will reveal the cell dynamics involved in migration in the near future.

Acknowledgements

Appendix

The analysis of SPM cantilever vibration

The one-dimensional differential equation on the cantilever vibration is expressed as follows.

$$m^* \frac{\partial^2 y(x,t)}{\partial t^2} + c^* \frac{\partial y(x,t)}{\partial t} + E_c I \frac{\partial^4 y(x,t)}{\partial x^4} = p(x,t), \tag{1}$$

where $y(x,t)$ and $p(x,t)$ are deflection and loading force of the cantilever at position x $(0 \leq x \leq L)$ and time t, respectively. L is the length of the cantilever. m^*, c^*, E_c, and I are the following mechanical properties of the cantilever, respectively; effective mass, effective damping coefficient, Young's modulus, and geometrical moment of inertia. On the left-hand side, the first term expresses inertia. The second and third terms are viscous damping and spring elasticity of the cantilever, respectively. On the other hand, the cantilever moves under the following external force at the end of the cantilever.

$$p(L,t) = \left[K_s \{\phi(t) - y(L,t)\} + C_s \frac{\partial \{\phi(t) - y(L,t)\}}{\partial t} \right], \tag{2}$$

where K_s and C_s are one-dimensional elasticity and viscosity of the sample, respectively. $\phi(t)$ is a vibration applied to the sample externally. This equation allows the evaluation of one-dimensional elasticity and viscosity from the detected cantilever deflection.

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Figure captions

Fig. 1. Schematic of SPM for the force modulation mode. The raw deflection signal is transmitted to both a lock-in amplifier and a band elimination filter (*black arrow*). This filter excludes only AC component with frequency of the loading vibration and transmits only its DC component to the Z-controller (*dashed black arrow*). The Z-controller transmits feedback signals to a scanner by the DC component (*dashed white arrow*) and visualizes topography and deflection images. External oscillation from a function generator is sent to the scanner and the lock-in amplifier for vibration of sample stage and reference signal, respectively (*gray arrow*).

Fig. 2. Frequency dependence of the amplitude of cantilever deflection in liquid medium. Amplitude ratios to input vibration are plotted under two different conditions where the cantilever is set on the glass substrate and apart from it, as shown in the inset.

Fig. 3. Topography (a), deflection (b), amplitude (c), phase shift (d), elasticity (e) and viscosity images (f) of a living fibroblast measured in the improved force modulation mode. The scale bar for elasticity in (e) is shown on a log scale. The bar in (a) is 10 μm.

Fig. 4. Line profiles (indicated by *arrowheads* in Fig. 3 (a)) of elasticity, viscosity, topography, and deflection images.
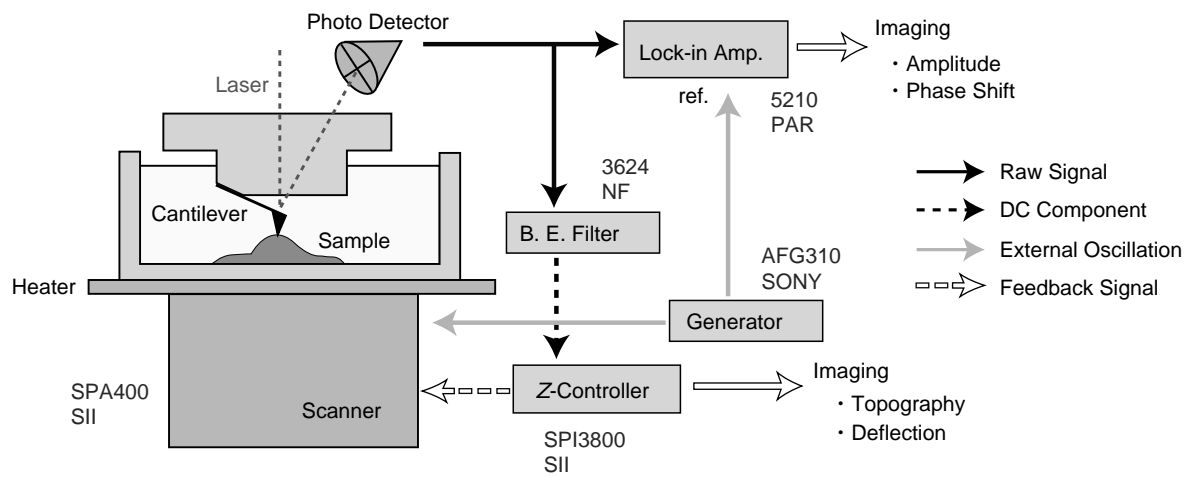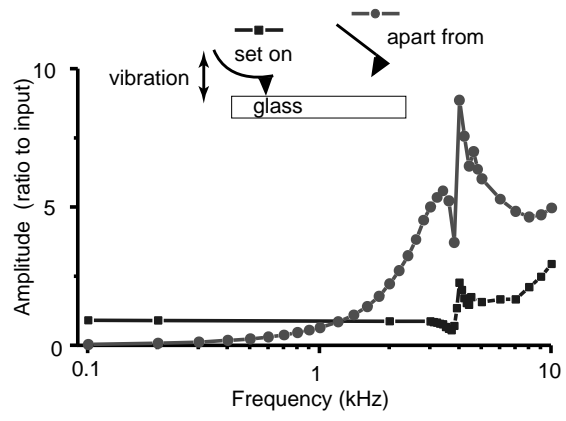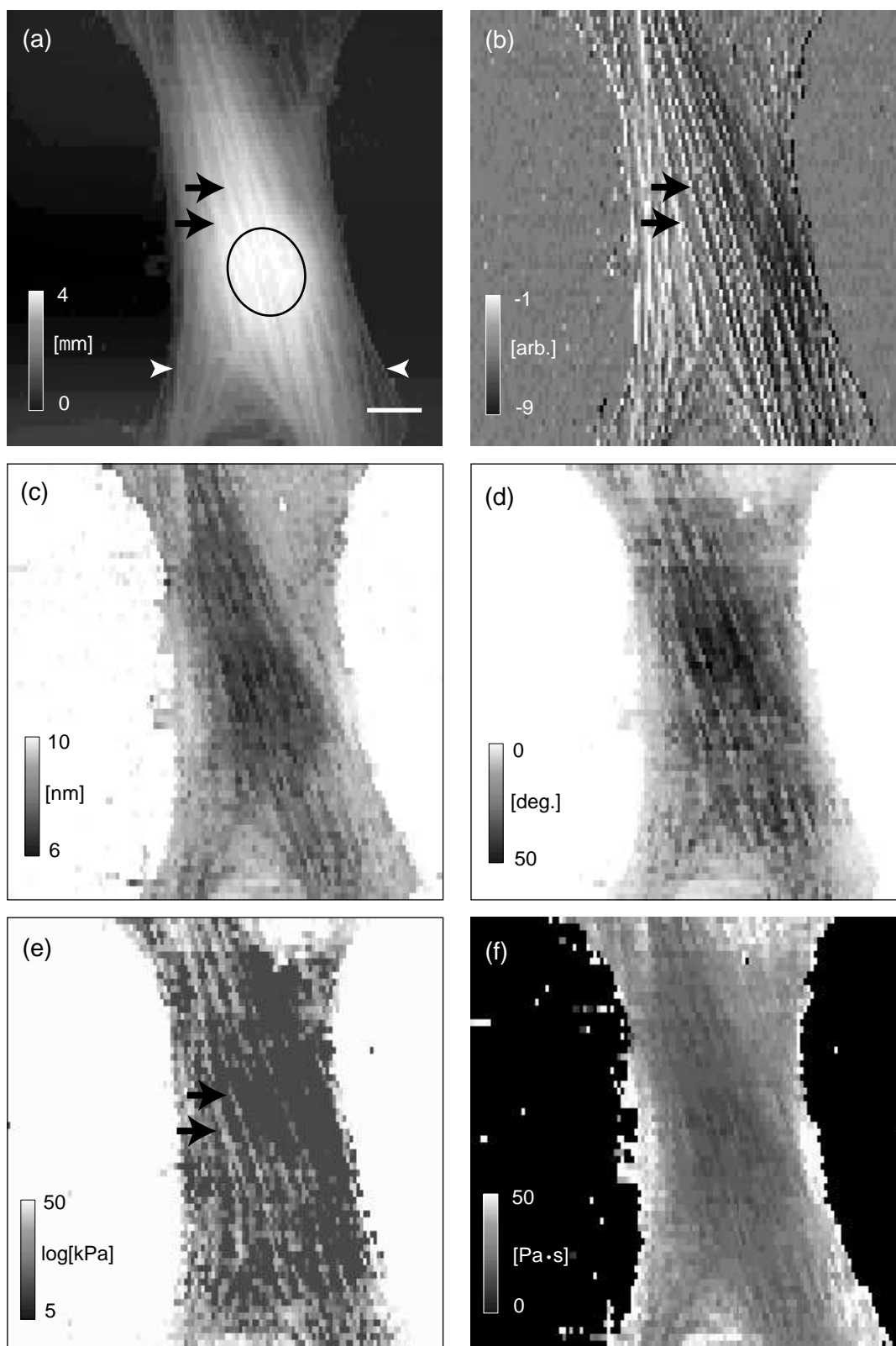
Figure 1

Figure 2

Figure 3

Figure 4