A $\lambda/30$ Resolution Laser Speckle Pattern Biosensor for Dynamic Studies on Live Samples

Maria Fernanda Avila[1], Shigeki Yamaguchi[2], Hideho Uchiyama[3], Ruggero Micheletto[4]

Graduate School of Nanobiosciences, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, JAPAN

[1]maria-o_o@i.softbank.jp
[2]i080704b@yokohama-cu.ac.jp
[3]hideho@yokohama-cu.ac.jp
[4]ruggero@yokohama-cu.ac.jp

***Abstract*—Nowadays there is a big interest in the research of cell behaviour in different sciences, like biology, physics, and medicine. For this reason, many interdisciplinary research projects have been developed in many countries. The main goal of the realization of the proposed biosensor is to obtain a super high resolution optical detection of nano-scaled movements of live cells. We used a very straightforward principle, the interference of laser light with the membrane of the cells under investigation. The laser light is focused on the target cell, while observing the picture through an optical microscope. The laser light creates an interference image (speckle pattern) that is projected on a screen and monitored by a CCD camera. This interference pattern is perturbed by any movement or displacement of the cells, and this interaction is recorded in real time by the CCD. While the contrast in standard optical microscopy is very low, the advantage of this approach is that the coherence of laser light produce constructive or destructive patterns that can be detected with very high signal-to-noise ratio. The displacement resolution we can achieve is better than $\lambda/30$, that is in the order of 20nm.**

I. Introduction

Sub micron studies of cell ultra structure have been performed using scanning and transmission electron microscope (SEM, TEM) or Atomic Force Microscope (AFM). However, live cells cannot be examined using SEM and TEM methods because these systems require cell fixation and observation under vacuum, and images of live cells obtained using AFM systems may be compromised by the direct contact between the cantilever and sample deforming soft tissues altering the tests [1].

We want to investigate the dynamics of cytoskeleton activities in order to evaluate and estimate the performance of a speckle pattern analysis. The cytoskeleton of eukaryotic cells is fundamental to the spatial organization of the cell components. Cytoskeletal systems are dynamic and adaptable, they can change or persist, according to need. Cellular structures must be assembled, disassembled and reorganized during the life of the cell; thus the individual macromolecular components that make up these structures are in a constant activity. Since cells are small and complex, it is hard to detect their structure and movements, and what we can learn about cells dynamics depends on the tools available, and on the adoption of different techniques[2].

We used in previous studies a SNOM system [3], [4] to detect nano-scaled movements of a cell. SNOM is a purely non-invasive, non-contact method and therefore the natural life activity of the sample is unperturbed. However this instrument is very complex, expensive, and difficult to use. This was the main reason we decided to develop this research by designing a new kind of microscope, with similar resolving ability, but simpler to use and much more economical to realize.

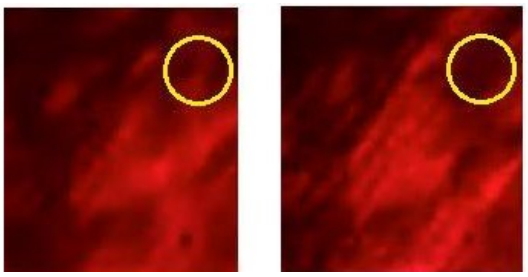

Fig. 1. The speckle pattern variation in function of time: two pictures of the screen (device number 7 in figure 3) taken at about one second interval. The circled area shows a variation of intensity easily detectable, corresponding to about 500nm spatial displacement, a value hardly observable with conventional microscopy.

The system we have constructed uses a low power laser beam to interfere with the sample and produce an interference image (speckle pattern, fig. 1) that is recorded and analysed for dynamical activity. A speckle pattern can be analysed by means of several mathematical and statistical tools and provide numeric or visual information on its magnitude. Because the number of scattering centres is very high, the collective phenomenon is hard to interpret and the individual contributions to the final result may be difficult to be inferred. The measurements that are obtained by means of the analysis tools display the activity level as a sum of the contributions of phenomena of the scattered light as well as any other existing phenomena (time variations of the refractive index of the sample, etc). A biological sample, for example, that is a material that contains a huge number of mobile scattering centres, displays refractive index variations in the materials that compose it with power changes as well as many other effects, thus increasing the complexity in the identification and

isolation of these phenomena. Then, the complete interpretation of the activity of a sample, by means of dynamic speckle, presents huge challenges[5]

A. Cell preparation method and characteristics

To test the characteristics of our Speckle Pattern Biosensor (SPB) we used P19CL6 cells, an embryonal carcinoma (EC cell) derived from C3H mouse [6]. This cell line (Resource No. RBRC-RCB2318) supplied by Riken Bioresource Center (Tsukuba, Japan).

Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in 0.1% gelatin-coated 60mm dish (TPP Inc., Switzerland) with 10 mL of culture medium made up of MEM-alpha supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 microgram/mL streptomycin (all from GIBCO, Invitrogen Inc.). In passages, cells were washed with phosphate-buffered saline (PBS), dissociated with 0.25% Trypsin-EDTA (GIBCO, Invitrogen Inc.), suspended in fresh medium, split 1:20, and inoculated to a fresh dish with 10 mL of fresh medium at 2 days intervals. In differentiation experiments, 1% (v/v) dimethyl sulfoxide (DMSO, Infinitely Pure Grade, No.045-24511, Wako Pure Chemical Industries, Osaka, Japan) was added to the culture medium and the cells were continuously cultured and passaged in this DMSO-added medium. Usually, 7 to 10 days later several spots of beating cell clumps of various sizes appeared, and the beating could last for one to two weeks.

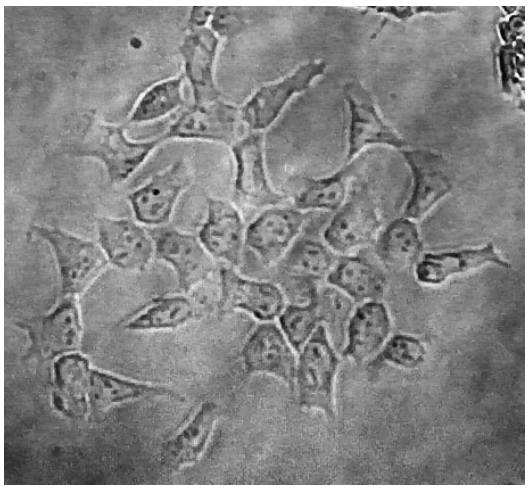

Fig. 2. An optical microscope picture of the P19CL6 cells that will differentiate to cardiomyocytes

P19 cell and its derivatives are embryonal carcinoma cells (EC cells) and they maintain pluripotency, which means that they can differentiate into various cell types according to the stimulus they receive. This time the treatment was DMSO, known to stimulate them to differentiate into cardiomyocytes. P19CL6 cells have higher differentiation tendencies into cardiomyocytes than the original P19 cells. Adding DMSO at 1% (v/v) to the medium and the cells are continuously cultured in this medium, the cells will differentiate into cardiomyocytes in about one week and start beating (see fig. 2). The beating corresponds to a maximum displacement of about 500nm (0.5 μm) and this is what we are going to measure to validate the resolving power of our biosensor instrument.

II. The instrumental apparatus and calibration tests

Figure 3 shows a scheme of the experimental apparatus. A standard optical microscope (Olympus BX51-W1) is fitted with a solid state Laser (670nm) that focus on the sample. Cells are placed on a transparent Petri dish, so that the interference pattern is transmitted and reflected on a screen. Any perturbation of the interference pattern due to displacements of the cytoskeleton of the cell is recorded in real time by the CCD camera on a solid state memory. The data are then analysed off line and a time profile of the variable domains of interference is generated (see figure 4 for an example).

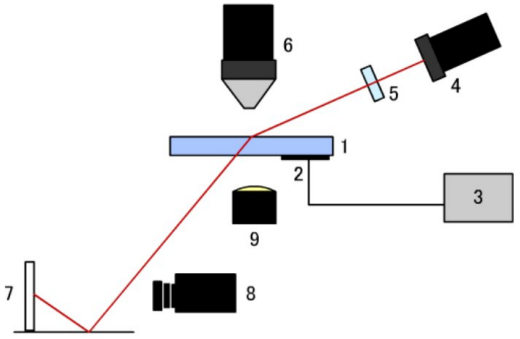

Fig. 3. Layout of the apparatus used. 1. Biosample, 2. Piezoactuator, 3. Function Generator, 4. Red Laser, 5. Focus Lens, 6. Microscope objective, 7. White screen, 8. Video camera, 9. Lamp

The instrument was firstly calibrated for sensitivity using a piezo actuator to give a known displacement to a blank sample and observing the instrument response as speckle pattern changes. The driving stimulus was a 400Hz signal. The intensity was changed and the instrument response was used to determine the average sensitivity (see table I). We could demonstrate a sensitivity of 0.7 nm/mV, a value of the same order of magnitude as our previous work with a SNOM probe[7], [8], but obtained in this case with a much simpler and low cost instrument.

TABLE I
CALIBRATION DATA SHOW A SENSITIVITY OF ABOUT 0.7 mV/nm

Signal Voltage [V]	**Response** Vpp [mV]	**Signal** Voltage [V]	**Response** Vpp [mV]
8	24	1	17.8
10	28.8	2	17.4
12	29.8	4	19.6
14	33.6	7	23.6
16	34.0	8	22.8
18	32	9	30.2
20	32.4	10	24.2
		13	28.4
		15	24.6
		17	28.6
		20	33.8

III. Results on Cell's behaviour

The tests were carried out on samples in condition of strong and live beatings. Sample was placed on the transparent Petri dish and with the aid of the optical microscope an aggregate of cardiomyocytes is chosen as the target of the investigation (as in fig. 2). The laser is focussed on a single cell of the group. The corresponding speckle patter is monitored for changes initially optically. As shown in figure 1 there are domains in the pattern that show a highly recognizable variation. These variations are barely visible or not at all noticeable in the standard optical microscope. This fact motivated us to construct a device able to study the speckle pattern and record all its variations with high sensitivity and in real time.

Initially, with the aid of a single photodetector (a high sensitive photodiode) we recorded the intensity on a confined region of the interference pattern. If the cell is beating the spatial displacement associated alters locally the speckle structure and the beating profile is revealed as a variation of potential in the detector circuit.

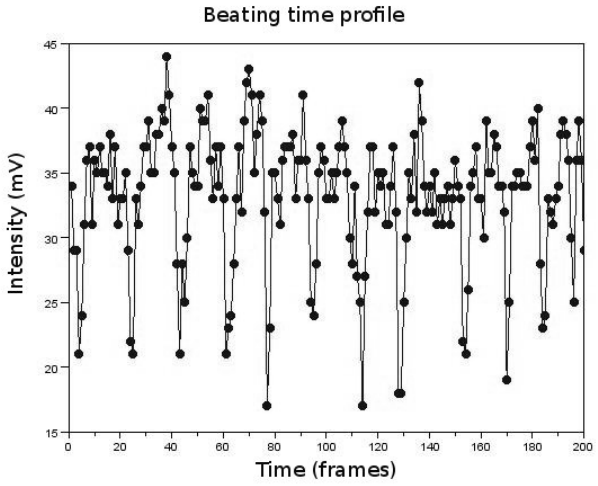

Fig. 4. The luminosity time profile of confined area of the speckle pattern detected by a single photodiode detector. The beating characteristics of cardiomyocytes cells are recorded in high contrast and are clearly recognizable. Time scale is in frames, 30 frames correspond to about 1 second.

In figure fig. 4 we plot the raw data detected by the photodiode. The plot clearly shows the oscillation of the luminosity of the interference, revealing the rhythmic movements of the cardiomyocytes cells. This *beating* of the signal, could be due to many causes, as rearrangement of sub-cellular components. Our method does not reveal directly the original cause of the optical variation. However, we can validate this signal as associated to the cell wall vibration, because of its similarity in frequency and temporal profile, to well known previous experiment with cardiomyocytes [3], [4].

To produce an universal system able to investigate a wider area of the biosample, we replaced the single photodiode device with a CCD camera sensor. This device collects simultaneously a much wider area of interference, allowing the researcher to localize interference pattern of higher contrast. The device records in this case complete moving pictures in AVI file format, that can be stored on solid state memories for later analysis.

The files detected by the CCD camera, are complex data structures difficult to handle; for this reason we developed a bespoke software able to isolate the desired pixel in the file and plot that pixel intensity against time (frame number).

The data obtained in this way can be investigated pixel by pixel for a full mathematical analysis. The cardiomyocytes beating characteristics can be evaluated not only from the point of view of time profile, but also Fourier analysis, spectrum of beating, chaos and any other standard investigation typical in the study of dynamical systems can be performed off line by the instrument.

In the lower inset of figure 5 is shown the Fourier spectrum of the cardiomycytes corresponding to the dynamical profile plotted above. A clearly recognizable peak at about 0.8 seconds is observed. General studies of drug dependence, culture media composition, temperature, acoustic perturbation, fatigue and other investigations are possible with this approach.

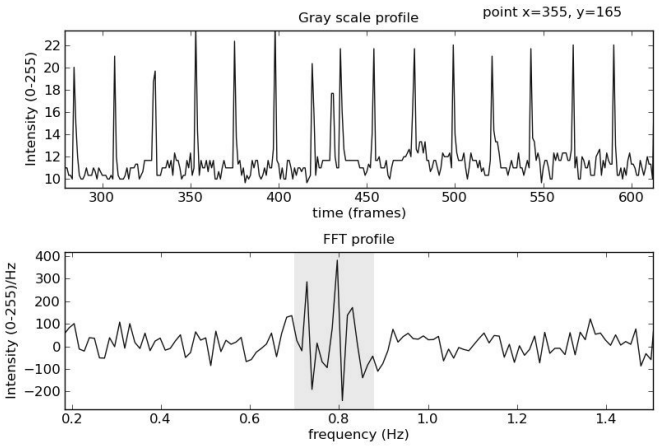

Fig. 5. In the upper panel the beating of cardiomyocyties is detected by a CCD device with high contrast and high resolution. The single pixel plot demonstrates a vertical minimum discrimination of about 20nm, in the order of $\lambda/30$. This value is lower than that obtained with the blank calibration tests, however well below the diffraction limit of optical microscopy. In the lower panel is plotted the Fourier spectrum of the same beating profile showing in detail the frequency distribution of the cell beating with a noticeable peak of about 0.8 Hz (greyed).

IV. Conclusion

In this study we succeeded in detecting tiny mechanical displacements of the cytoskeleton of P19 line stem cells differentiated as cardiomyocytes. The beating of single cells was isolated and observed with extremely high resolution estimated in the order of 20 nm.

We are aware that the optical signal that we recorded, in common with other optical microscopes, does not contain pure morphological information. Differences in optical coupling could arise also from other phenomena in the cell body, such as local modifications in the index of refraction and movement

of inner cellular structures. In the hypothesis that the morphological displacement plays the major role in our measurements, we can estimate the vertical sensitivity of our recordings. As we stated above, contractions of cardiac myocytes are known to extend vertically approximately ΔZ=500 nm. We could observe the beating by using a noncalibrated optical signal ranging between 0 and a maximum of 255 units (8 bit scaled images). The strongest peaks were observed to be about ΔS=20 units from the base to the top of the peak. Considering a minimum discrimination of δ_m=1 unit, the minimal cell displacement detectable δ_o was calculated with the following relation that resulted to be in the range of 20 nm, about $\lambda/30$.

$$\delta_o = \frac{\Delta Z}{\Delta S}\delta_m = 20nm$$

These values are intended to be only estimates, since they assume linearity between signal and contraction displacement. This linearity is not guaranteed, because of the complexity of the phenomena involved. However, it is a reasonable approximation of the order of magnitude of the vertical sensitivity of our instrument, lower than that we obtained in the calibration tests, but well below the diffraction limit of standard optical microscopy.

The system we constructed uses a CCD camera that records a wide area of the interference pattern, and a software that isolates single pixel profiles for data treatment and analysis. Fourier spectroscopy could demonstrate the beating characteristics of the cell line and observe variations in function of time, fatigue, culture media composition and other environmental parameters that condition the cell life activities. This device will be optimized and we believe it will give a contribution as a non-invasive biosensor for the dynamical study of nanoscaled movements of cells and in all live biosamples that have even tiny dynamical activity in general.

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