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In situ observation of cell-detachment process initiated by femtosecond laser-induced stress wave

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Abstract When a stress wave generated by focusing a femtosecond laser is loaded on an animal cell adhered on a substrate, the cell is detached from the substrate. There are two possible mechanisms for the cell detachment: (a) The cell is detached from a scaffold coated on a glass plate, and (b) the cell is detached from the glass plate with the scaffold. In this work, we have studied the cell-detachment mechanism by visualizing the scaffold with a fluorescence probe of quantum dots. When the cell was detached from the substrate, fluorescence from the scaffold simultaneously disappeared from the glass plate, although the scaffold was not irradiated by the laser. This indicates that detachment due to the stress wave is attributed to mechanism (a). On the other hand, when the cell was detached from the substrate by a trypsin treatment, the fluorescence from the scaffold remained, suggesting mechanism (b). By comparing both results, it is considered that physiological damage of the cell membrane during the detachment process by femtosecond laser-induced stress wave is less than that due to the trypsin treatment.

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1 Introduction

When an intense 800 nm-femtosecond laser is focused into water through an objective lens, shockwave and cavitation bubble are generated at the laser focal point due to multi-photon absorption, resulting in the propagation of a stress wave from the laser focal point [1]. Compared with that of other lasers with longer pulse duration, these phenomena are induced with lower pulse energy by the femtosecond laser [2]. When the laser pulse energy is adjusted near the threshold energy, the photothermal and photochemical effects expect for the laser focal point can be neglected, so that the stress wave is used purely as a mechanical force to manipulate micro-objects near the laser focal point [3].

We have already demonstrated that the stress wave provides a promising tool for single-cell manipulation [4–7]. The cell culture medium, biological cells and cell scaffolds are generally transparent at 800 nm. This allows a stress wave to be generated selectively at the laser focal point in an aqueous culture medium even when the laser is focused nearby the biological cells and the cell scaffold. In fact, when the stress wave is generated near an animal cell adhered to a substrate, we can detach it individually from the substrate [4]. Furthermore, we have applied this cell-detachment technique to cell isolation and cell patterning [5–7]. This cell manipulation technique is performed in a culture medium without exposing the cells to air, so physiological damage to the cell is minimized. Alternatively, optical tweezers are known to be a useful tool for precise, single-cell manipulation [8, 9]. However, their photon pressure was demonstrated through experimentation to be too weak to detach cells from the substrate. In comparison with the laser tweezers, the stress wave is capable of more active and versatile cell manipulation, including single-cell detachment from a substrate.

According to typical procedures used to culture animal cells on a substrate, a glass or plastic plate is coated with a cell scaffold such as collagen, on which the cells are then seeded. As a result, the cells connect to the scaffold through cell adhesion molecules, such as integrins, and grow on the substrate. In order to detach the cells from the substrate for re-seeding and analysis, trypsin is used as an enzyme to chemically digest the cell adhesion molecules. Because both the cell adhesion molecules and the membrane proteins likely interact with the enzyme, it is presumed that the cell surface receives slight chemical damage during the treatment [10, 11].

On the other hand, when using the femtosecond laser-induced stress wave method, cells are mechanically detached from the substrate. Therefore, cell detachment using the stress wave is indicated to cause less damage to cell membrane than the trypsin treatment method. Furthermore it is expected that the cell-detachment is not fatal for the cell activities such as cell multiplication, cell migration, cell death, and cell differentiation. Actually this possibility has been suggested in our previous investigations, as follows. When NIH/3T3 fibroblast cells were detached by the stress wave, over 80% of the cells were adhered again and migrated on the substrate. This rate is comparable with the trypsin treatment [6]. Furthermore, PC12 cells were employed to confirm how the stress wave influences cell differentiation [12]. PC12 cells, which are derived from a rat pheochromocytoma, are induced to differentiate into the cell with neurites by nerve growth factor (NGF) [13]. We evaluated the differentiation activity after cell detachment due to the stress wave and compared with that due to trypsin treatment. This result also suggests that the stress wave had little effect on cell differentiation.

Now our attention is directed to mechanistic understanding of cell detachment from a scaffold on a glass or plastic plate by a stress wave. In this work, the cell-detachment mechanism was confirmed by visualizing the collagen scaffold. Fluorescence quantum dots were dispersed in the collagen scaffold covering the glass plate before PC12 cell culture. The behavior of the collagen when the cell was detached by the stress wave was investigated by monitoring its fluorescence under the cell by fluorescence microscopy.

2 Experiments

To prepare the cell culture substrate, a glass plate was coated with a collagen matrix as the cell scaffold as follows. The glass plate (9.5 × 2 × 0.5 mm) was dipped into 0.3 mg/ml collagen (Cell Matrix collagen type-I, Nitta Gelatin Inc)/1 mM hydrogen chloride for thirty minutes. After the plate was dried in order to fix a collagen scaffold on the plate, it was placed in 80 nM aqueous solution of

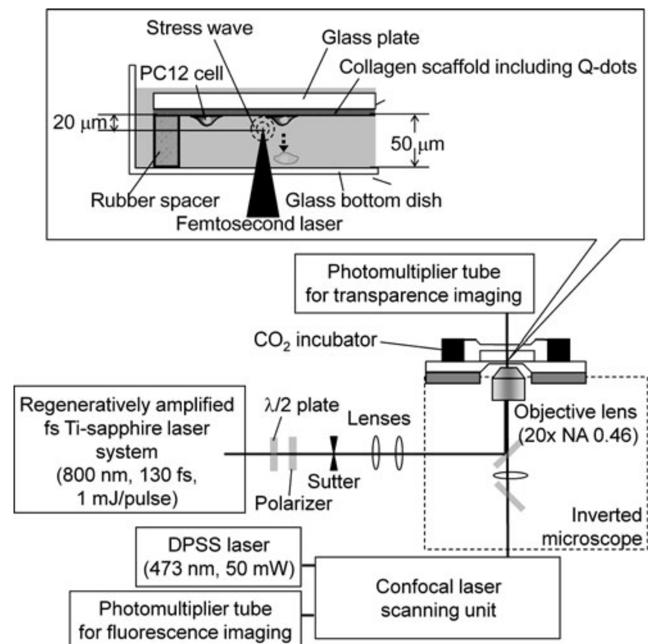

Fig. 1 Experimental setup for observation of cell detachment by femtosecond laser-induced stress wave

quantum dots (Qdot ITK Carboxyl Quantum Dots, Invitrojen, Life Technologies Corporation) for seven days at 4°C, during which the quantum dots penetrated the collagen and bound to the collagen electrostatically.

PC12 cells (RCB0009, Riken Bio Resource Center) were seeded on the substrate at an initial concentration of about 2000 cells/cm^2 and cultured in a CO_2 incubator, in which temperature, moisture, and CO_2 concentration were kept at 37°C, 90%, and 5%, respectively. The cell culture medium is Dulbecco's Modified Eagle Medium (Wako) supplemented with 10% fetal bovine serum (GIBCO) and 10% horse serum (GIBCO), 100 μg/ml streptomycin (GIBCO), and 100 unit/ml penicillin (GIBCO). After the 1-day incubation, the substrate was placed upside-down on a glass bottom culture dish (35 mmϕ IWAKI) as shown in Fig. 1. The space between the substrate and the culture dish was maintained using silicone rubber spacers with a thickness of 50 μm and filled with the culture medium. The sample was set on a sample stage of a confocal fluorescence microscope system (FLUOVIEW FV 300 system, Olympus). The cell culture was carried out under the above-mentioned conditions.

Femtosecond laser pulses generated by an amplified Ti: Sapphire laser system (Hurricane, Spectra-Physics, 800 nm, 130 fs, 1 kHz) were led to the sample stage through a 20× objective lens (N.A. 0.46). The single shot pulse was extracted from the pulse train and focused on the sample stage. The laser power under the microscope was tuned by a half-wave plate and polarizers. Before the laser irradiation, the laser pulse energy through the objective lens was measured by attaching a laser power meter (ORIEL, Model AN/2) to

the objective lens. The diameter of the focal spot was estimated from etching behavior of a carbon-doped polymer film, whose laser ablation should be initiated by one-photon absorption. The diameter of the laser focal point was about 3 μm.

The cell adhered on the substrate was detached by focusing the laser into the culture medium in the vicinity of a target PC12 cell below the collagen scaffold at a distance of 20 μm. The fluorescence from the quantum dots in the collagen matrix on the glass plate was detected by the confocal fluorescence microscope system before and after the laser irradiation. A 473 nm DPSS laser (Shanghai Dream Lasers Technology Co. Ltd. 50 mW) was used as the fluorescence excitation light source. Simultaneously, light scattering images of the sample were obtained using the DPSS laser as the transmitted light source.

Cell detachment due to the trypsin treatment was also investigated. The sample mentioned above was set on the sample stage and observed using the confocal fluorescence microscope system. The culture medium of the dish was replaced with phosphate-buffered saline solution (D-PBS(-), Wako) to avoid the inactivation of trypsin. Adding 0.05 g/l trypsin, 0.02 g/l EDTA4Na/1 ml D-PBS(-) into the dish, the substrate was put on the stage for fifteen minutes. After the cells were detached completely by pipetting, the collagen matrix was observed by the system.

3 Results and discussion

When a single shot pulse with energy of 850 nJ/pulse, which is about 10 times larger than the threshold energy of the stress wave generation, was focused into the culture medium, a stress wave was generated at the laser focal point and propagated to a nearby cell as an impulsive force. This was the almost minimum pulse energy required to detach PC12 cells effectively, but it should be noted that cell damage, such as bursting, was not observed even when the pulse energy was 10 times larger than the present case. The cell was detached by loading the impulsive force at a few positions near the cell. A representative result is shown in Fig. 2, where the laser pulses were focused in the culture medium at a position of 20 μm away from the edge of the cell. The cells in the light scattering image A1 were detached when the laser was sequentially focused at the two positions indicated in the light scattering image B1. Because the distance between the laser focal point and the edge of the cell is longer than the radius of the laser focal point, the cell was not irradiated directly with the laser. The detached cell sank down in the culture medium and reached on the glass bottom dish a few minutes after the detachment, as shown in the light scattering image C1. The white patterns of fluorescence images A2, B2, and C2 indicate the collagen scaffold upon the cell-detachment process, corresponding to light scattering images A1, B1, and C1, respectively. The fluorescence image is blocky because the collagen matrix is not uniform. Fluorescence at the position of the target cells (white circles in A2) disappeared at the time of cell detachment (B2). Additionally, fluorescence was observed from the cell transferred to the glass bottom dish (white circles in C2). Photobleaching cannot be the cause of the fluorescence, because the laser is not directly focused on the cell. These results indicate that PC12 cells and collagen scaffold detachment

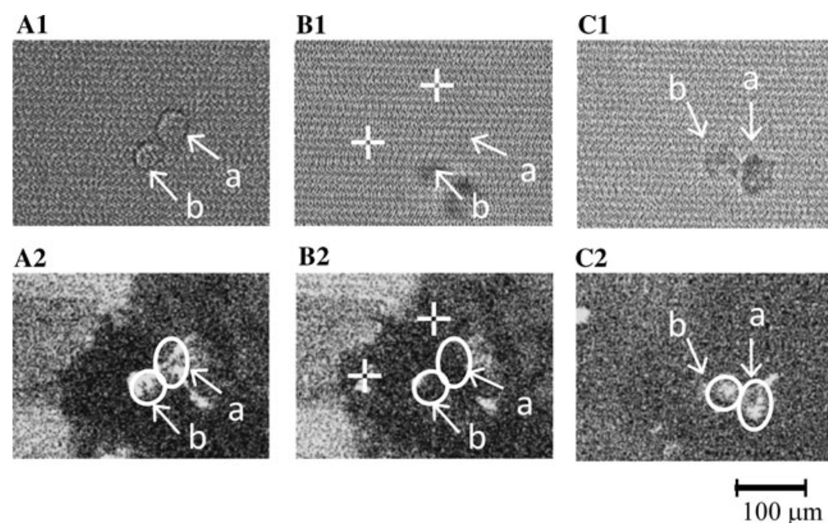

Fig. 2 Light scattering (*top*) and confocal fluorescence (*bottom*) images of PC12 cells as they are detached from a glass plate coated with a scaffold by femtosecond laser-induced stress wave. The cells on the scaffold before and immediately after cell detachment are shown in **A** and **B**, respectively. Cells transferred to the glass bottom dish after detachment are shown in **C**. Laser pulses were sequentially focused at the center of the *crosses* in these figures. *Arrows* (a, b) in images indicate detached cells and *circles* trace the cell figure. The white dots in the fluorescence images are fluorescence from quantum dots included in the collagen

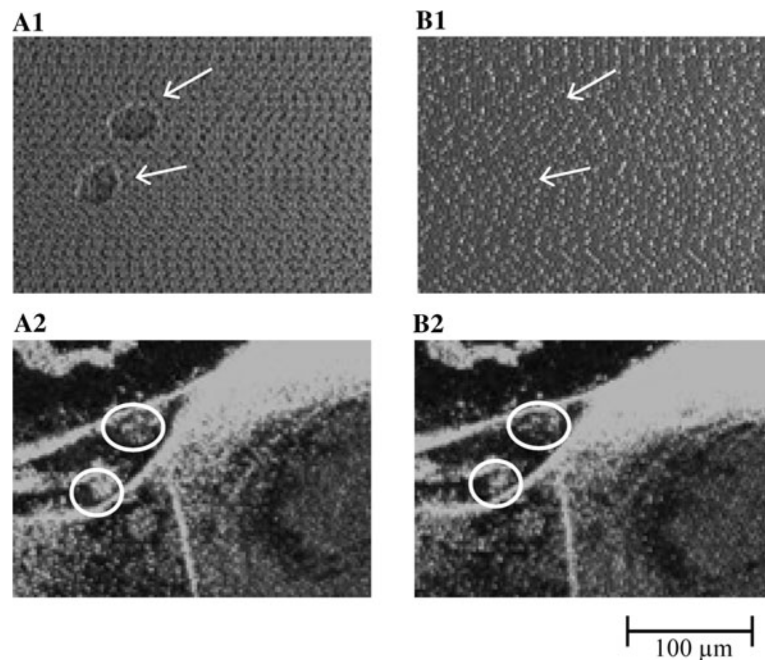

Fig. 3 Light scattering (*top*) and confocal fluorescence (*bottom*) images of PC12 cells before (**A**) and after (**B**) adding trypsin to the culture medium. *Arrows* in light scattering images indicate the target PC12 cells and *circles* trace the cell figure. The white dots in the fluorescence images are fluorescence from quantum dots included in the collagen

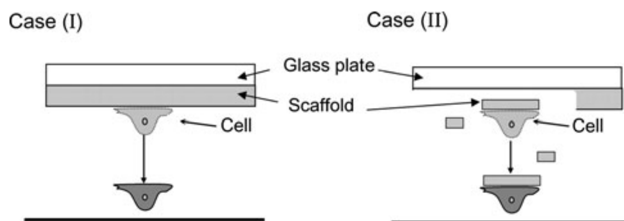

Fig. 4 Possible detachment mechanisms of a cell and collagen scaffold

from the glass plate occurred simultaneously, due to the femtosecond laser-induced stress wave.

On the other hand, when the cell was detached by trypsin treatment, the collagen scaffold under the cell remained even after the cell was detached (white circles of A2 and B2 in Fig. 3). This indicates that PC12 cells were detached solely from the collagen scaffold and the collagen was not digested with trypsin. The cell-detachment mechanism was different from that of the femtosecond laser-induced stress wave.

These results are summarized in the schematics shown in Fig. 4. The mechanisms of cell detachment due to trypsin treatment and femtosecond laser-induced stress wave are classified into Case I and Case II, respectively. In Case I, protein binding between the collagen scaffold and collagen binding proteins (i.e., integrins on the surface of the cell) are broken by detachment. Since trypsin hydrolyzes the binding proteins [11], the detachment due to trypsin should be attributed to this case. On the other hand, when the connection between the cell and scaffold is undisrupted during the detachment process, the cell detaches together with the collagen scaffold from the glass plate as shown in the Case II. This fact suggests that the physiological damage to the cell membrane during the cell-detachment process is less than that due to the trypsin treatment.

We also observed the cell shape change due to the stress wave. Furthermore, in µs time region after the laser irradiation, the cell shape is probably disturbed by the impact of the shockwave and cavitation bubble. Such shape changes would modify the chemical/biological state of the cell and can lead to cell deactivation [14–16]. Separately, we are studying cell differentiation after cell detachment by femtosecond laser-induced stress wave and by trypsin treatment. The PC12 cells were transferred to a glass bottom dish and cultured for 1 day in a culture medium including NGF. In both cases, about 45% cells were differentiated to cells with neurites on the glass bottom dish. In view of cell differentiation, we can say that the cell damage due to the stress wave is comparable with that due to trypsin treatment. This result suggests the possibility that cell damage is induced by the cell shape changes caused by the impact of the stress wave. Meanwhile, in this experiment, the damage to the cell membrane was found to be less than chemical damage to it due to trypsin treatment.

4 Conclusion

The cell detachment brought about by femtosecond laser-induced stress wave was elucidated by observing the collagen scaffold with a fluorescence probe of quantum dots. From these results, it was demonstrated that the cell is individually detached from the glass plate with the collagen scaffold by our method, although the cell is detached from the scaffold by trypsin treatment. Further studies are being

extended systematically, with the aim to clarify the advantages and disadvantages of the stress wave method and to confirm that cell detachment by femtosecond laser can be applied as a single-cell manipulation technique as a standard method in biotechnology. Details of the inhibition and activation of cells due to the stress wave will be investigated by combining biological analyses for single cells and reported in the near future.

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