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Morphological evaluation of cell differentiation after the isolation of single cells by a femtosecond laser-induced impulsive force

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Abstract When nerve growth factor (NGF) is interacted with PC12 cells derived from rat pheochromocytoma, they are partially differentiated into neuron-like cells with neurites. In this work, PC12 cells differentiated by NGF were selectively isolated using a localized impulsive force in a μm-scale area, which was generated by focusing an infrared femtosecond laser into a cell culture medium. In order to evaluate the ability of the isolation method, differentiated and undifferentiated cells were isolated and their morphological changes after the isolation were compared. In both cases, their neurites were once contracted and some of them gradually regenerated day by day. When differentiated cells were isolated, the percentage of differentiated cells with regenerated neurites, 6 h after the isolation, was about 3.3 times higher than that when undifferentiated ones were isolated. This result was compared with a control trypsin experiment. In the comparison, it was indicated that the same degree of cell function was maintained when the present isolation method was used.

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1 Introduction

Recent progress in single cell biotechnology has led to a demand for a single cell isolation technique to purify cells with specific phenotypes. For example, although stem cells and precursor cells can be differentiated by growth factors or chemical compounds (Xian and Zhou 2004; 7: Huang et al. 2009), perfect control of differentiation is currently impossible because of the wide variety of cell fates. This inability to achieve precise cell differentiation is a bottleneck to applications of embryonic stem (ES) and induced pluripotent stem (iPS) cells (Evans and Kaufman 1981; Martin 1981; Lei et al. 2009; Takahashi and Yamanaka 2006; Okita et al. 2007). Although the phenotype can be categorized according to cell shape or behavior on a scaffold substrate, it is generally difficult to isolate cells with the same phenotype from a cell group on the scaffold. For example, when flow cytometry (FCM) is applied to the isolation (Roda et al. 2008; Zborowski et al. 1999; Crosland-Taylor 1953; Fluwer 1965; Hulett et al. 1969), it is impossible to select specific cells by the shape or behavior on the substrate, because cells must be once dispersed into solution. Optical tweezers have been demonstrated to be a promising tool for such cell manipulation (Ashkin et al. 1987); however, since the driving force of the tweezers due to optical pressure is too weak to detach individual cells from a substrate, it cannot be applied to such isolation. Hence, for isolating cells with a specific shape from a cell group on a substrate, establishment of a technique to detach a specific cell on a scaffold one by one is desired.

Previously, we have applied femtosecond laser-induced impulsive force as an external force to manipulate single cells precisely. When an intense femtosecond laser is focused into a cell culture medium through an objective lens, a stress wave due to a shockwave and a cavitation bubble is generated at the laser focal point, propagating a very short wave packet like a Tsunami (Hosokawa et al. 2004). When the laser pulse energy approaches the threshold for generating a stress wave, an impulsive force due to the stress wave is localized in the vicinity of the laser focal point (a few tens of μm). We have successfully demonstrated that a single animal cell can be detached from a cell culture substrate made of several kinds of materials, such as glass, plastic, or collagen matrix (Kaji et al. 2007). As far as we know, except for this method, it is too difficult to detach a specific cell adhered on a scaffold at the single cellular level.

In this work, we are investigating the application of the above cell detachment process to achieve single cell isolation. One of the requirements of single cell isolation using a femtosecond laser is that cell function must not be disturbed by the impulsive force. Namely, it is important that cell functions, such as cell division, cell differentiation, and cell death, are not affected by the impulsive force manipulation. Previously we demonstrated that when NIH3T3 cells were individually detached from a collagen coated substrate, 80% of the cells were adhered again on another substrate (Kaji et al. 2007). This percentage is comparable or higher than that of cells after reseeding by trypsin treatment. Furthermore, in order to elucidate the influence of the impulsive force on animal cell differentiation, we have investigated the behavior of PC12 cells (Takizawa et al. 2008) derived from rat pheochromocytoma (Greene and Tischler 1976). Since these cells are differentiated to neuronal phenotype by nerve growth factor (NGF), they have been widely used in the study of animal cell differentiation processes. When cells were randomly detached by the impulsive force, the percentage of differentiated cells after detachment was almost the same as that after reseeding by trypsin treatment. In order to establish the application of the impulsive force as a single cell isolation method, the behavior of individual cells during differentiation should be clarified. Here, individual PC12 cells were monitored one by one after single cell isolation by the impulsive force using a multi-point time lapse imaging system and the differentiation was evaluated on the single-cell level. On the basis of these results, the performance of this single cell isolation method will be summarized.

2 Experiments

PC12 cells (from RIKEN BioResource Center) were maintained in 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) at 37°C, 90% moisture, and 5% CO_2 concentration. About 300 cells were seeded on a source substrate of a glass plate (9.5×2×0.5 mm) coated with collagen-I (Nitta Gelatin Inc.). After incubation for 1 day, nerve growth factor (2.5 S NGF Mouse, Wako) with a concentration of 50 ng/ml was added to the culture medium on the source substrate to induce the cell differentiation as the microphotographs show in Fig. 1a.

After the 3 days of incubation, the source substrate was turned upside-down and placed on a glass bottom cell culture dish (35 mmφ, MatTek co.) coated with collagen-I as shown in Fig. 1b. The space between the source substrate and the culture dish was maintained using 50 μm silicone rubber spacers and filled with the culture medium including NGF. The sample was set on a motor-driven microscope stage (BIOS-102 T and BIOS-115 T, Sigma Koki) which was mounted on an inverted microscope (IX-71, Olympus). The temperature, moisture, and CO_2 concentration around the sample were regulated to the above-mentioned conditions using a microscope-equipped CO_2 incubator (MI-IBC, Olympus).

Femtosecond laser pulses were produced by a regeneratively amplified Ti: Sapphire laser system (Hurricane, Spectra-Physics, 800 nm, 130 fs, 10 Hz), then led to the microscope and focused into the culture medium, 20 μm below the source substrate, with a 10× objective lens (PLN 10X, Olympus, N.A. 0.25). A mechanical shutter with a gate time of 100 ms was used to extract the single shot laser pulse from the pulse train with 100 ms intervals. The laser pulse energy under the microscope was tuned by a half-wave plate and a pair of polarizers. The laser focal point in the image plane was adjusted to the vicinity of the target cell on the source substrate using a joystick to position the microscope stage, after which a single shot laser pulse was fired by opening the mechanical shutter. After the laser pulses were shot at a few positions surrounding the target cell, the cell was detached from the source substrate and transferred to the culture dish. The cells on the dish were observed by a home-made time-lapse imaging system, in which the specific positions on the dish were captured sequentially by synchronizing a CCD camera (ICD-878, Ikegami) with the microscope stage as shown in Fig. 1c. The observation was executed over a 4-day period using this system.

Before and after the transfer, since the culture medium contains NGF, some of the PC12 cell neurites were elongated as shown in the microphotographs of Fig. 1a. In observations using the time-lapse system, we classified the monitored cells into the following three types: differentiated cell with one or more neurites longer than its body length,

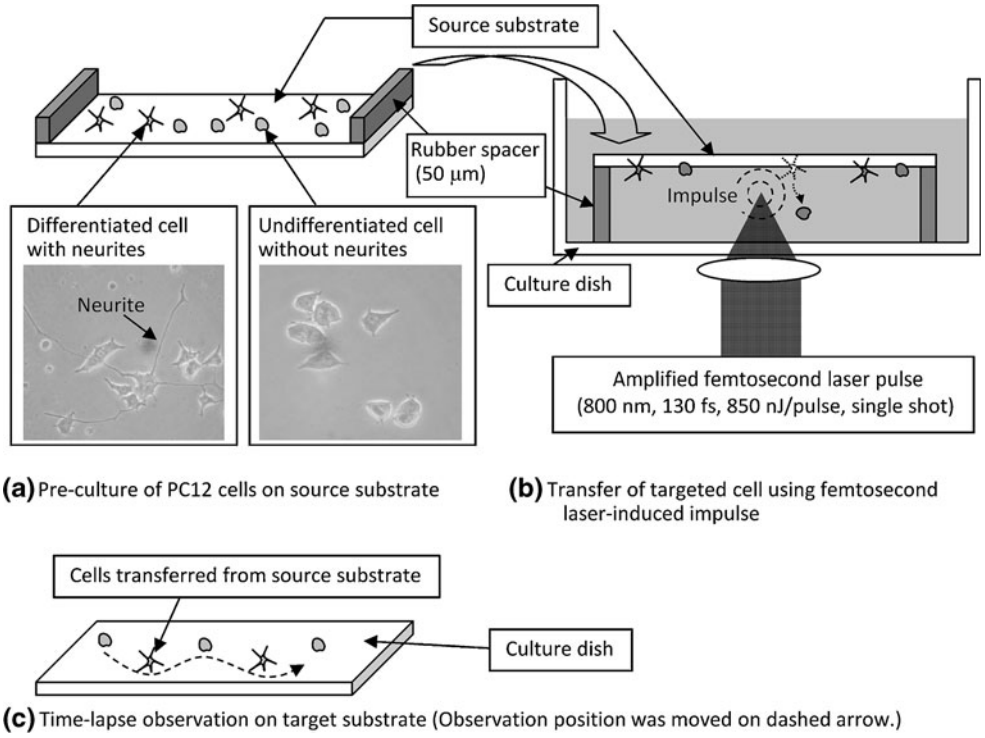

Fig. 1 Experimental procedures for isolating (**a**, **b**) and observing (**c**) single PC12 cells differentiated to neuron-like shape. The PC12 cells were cultured in a medium containing NGF

undifferentiated cell without such neurites, and cells which disappeared from the monitored area. The percentage of each type is indicated according to time evolution in Figs. 2 and 3.

Before the isolation, we must select the laser irradiation condition that minimizes cell damage due to the impulsive force. Hence this laser irradiation condition for the isolation was determined according to the following procedure. The source substrate with cultured cells was put on the culture dish, where the substrate was not turned upside-down and the silicone rubber spacer was not used. This experimental setting is considered to be better to evaluate

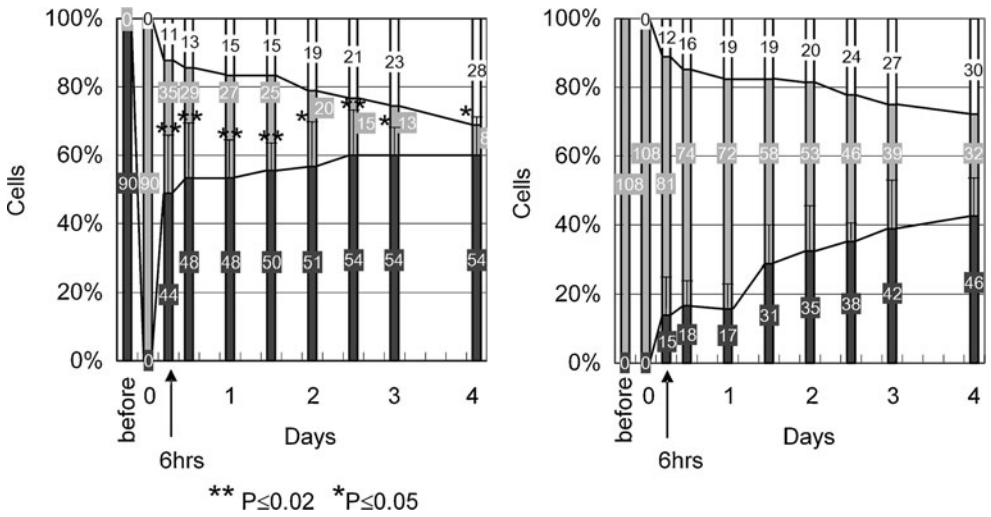

Fig. 2 Time evolutions of cell differentiation percentages when differentiated (**a**) and undifferentiated (**b**) cells were isolated from the source substrate and cultured on the dish filled with a medium containing NGF. The black, gray, and white bars indicate percentages of differentiated, undifferentiated, and lost cells, respectively, whose definitions are specified in the experimental section. The first and second time-lapse observations on the culture dish were preformed immediately after (0 days) and at 6 h after the isolation, respectively. The amount of classified cells is indicated in the bar. Error bar on the top of the black bar is standard deviation of percentage of differentiated cells. Significant difference between percentage of differentiated cells of (a) and (b) is evaluated by *t*-test (* $p \leq 0.05$, ** $p \leq 0.02$)

the pure influence of the impulsive force on the cell, because a target cell after the force loading is not detached from the culture dish. The impulsive forces were loaded at 2 or 3 positions surrounding the target cell, and the cell behavior immediately after the laser irradiation was observed in real-time imaging. When the 850 nJ laser pulse was used, the target cell was transiently vibrated by the laser irradiation, with no effect on the neighboring cells. When a greater laser pulse energy (e.g. >1 μJ) was used, the vibration due to the impulsive force was not only observed for the target cell but also for neighboring cells. On the other hand, when a lesser laser pulse energy (e.g. <700 nJ) was used, the clear vibration was not observed even for the target cell. The behavior of the cell differentiation of target cells after the 850 nJ pulses irradiation was estimated by adding NGF to the medium immediately after laser irradiation and compared with that without the impulsive force loading. The percentage of target cells differentiated after the force loading, which was calculated to divide number of target cells differentiated into total number of target cells, increased from 0% to ca. 45% during the 4-day period. Although some target cells were detached from the dish by the impulsive force loading, this rate was nearly the same as that without irradiation. Therefore, we considered a pulse energy of 850 nJ to be optimal for the isolation.

The percentage of cells differentiated after detachment by trypsin treatment was also confirmed and compared with that after isolation due to femtosecond laser induced impulsive force. After the cells were cultured with NGF on the substrate for 3 days, they were washed using a phosphate buffer, detached by adding 2.5 g/l trypsin / 1 ml phosphate-buffered saline solution (D-PBS(-),Wako), reseeded on the culture dish, and cultured in a culture medium containing NGF. The cell behavior after the reseeding was observed using the same procedure as that described above.

In one experiment of the isolation due to the impulsive force and the control trypsin experiment, about 35 cells were simultaneously captured by the time-laps system. The experiment was repeated three or four times for each case and standard deviation of the experimental error was estimated. Comparison between experimental data in the discussion was performed based on two sample *t*-test.

3 Results and discussion

First, PC12 cells with long neurites (differentiated PC12 cells) on the source substrate were targeted and isolated from the source substrate. When each cell was detached, the neurites contracted and the shape of the cell became like a droplet, which was the same as that of undifferentiated cells after detachment. The detached cells sunk into the culture medium and reached the culture dish within 5 min. Since the convection between the source substrate and the culture dish was negligible due to a time span which was less than 30 min, the position of the cell on the culture dish was almost exactly opposite its original position on the source substrate. Migration of the cells due to Brownian motion on the culture dish stopped less than 30 min. after the transfer, suggesting that the cell had again adhered onto the culture dish. The time evolution of the cell differentiation after the cell transfer is summarized in Fig. 2a. Although the neurites had completely disappeared immediately after the cell transfer, ca. 50% of the cells had regenerated their neurites within 6 h after the transfer. An additional ca. 2% of the cells had differentiated after each day, reaching a total of ca. 60% differentiated cells at the end of the 4-day period. It should be noted that ca. 10% of the cells had been lost from the observation area at 6 h after the isolation, reaching a total of ca. 30% lost cells at the end of the 4-day period.

Next the PC12 cells without long neurites (undifferentiated PC12 cells) were isolated from the source substrate as summarized in Fig. 2b. Neurites of ca. 15% cells were elongated at 6 h after the transfer. The percentage of differentiated cells was about 3.3 times smaller than that of the former case (Fig. 2a). Meanwhile the increase in differentiated cells was faster than that in the former case. The percentage of lost cells at 6 h after isolation was ca. 10%, reaching a total of ca. 30% lost cells at the end of the 4-day period.

The percentage of differentiated cells when the PC12 cells were reseeded by trypsin treatment is shown in Fig. 3. Before reseeding, ca. 30% of the cells formed neuron-like shapes with long neurites. Also in this case, the neurites had completely disappeared immediately after reseeding. After 6 h of incubation, ca. 20% of the cell neurites had elongated and the percentage of differentiated cells had reached ca. 50% at the end of the 4-day period. The percentage of lost cells was ca. 5% at 6 h after reseeding and had reached ca. 20% at the end of the 4-day period.

Result of two sample *t*-test also indicates that, when differentiated cells on the source substrate were selectively isolated, the percentage of differentiated cells after isolation was clearly larger than that when undifferentiated cells were isolated. These results mean that phenotype after the isolation depends on that before the isolation. Meanwhile, in the former and latter cases, the differentiated and differentiated cells after the isolation were not 100 and 0%, respectively. This reason may not be due to negative effects caused by the impulsive force, but may be due to the following (Greene and Tischler 1976): NGF induces cell differentiation for almost all PC12 cells, but speed to

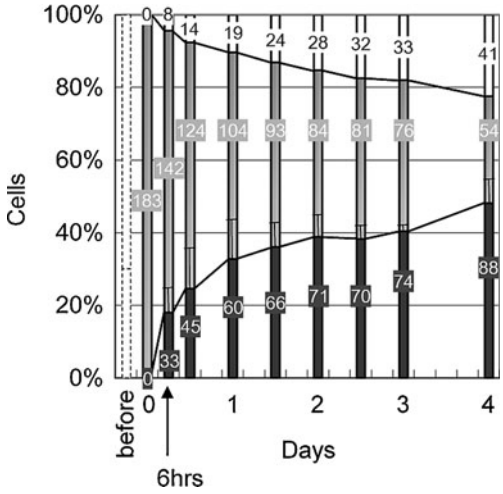

Fig. 3 Time evolutions of cell differentiation percentages when the cells were reseeded by trypsin treatment and cultured on a dish filled with a medium containing NGF. The time-lapse observation was performed with same manner as the isolation using the femtosecond laser. The black, gray, and white bars indicate percentages of differentiated, undifferentiated, and lost cells, respectively. Error bar is standard deviation of percentage of differentiated cells

generate neurites of the differentiated cell depends strongly on individual cells. Actually the same phenomenon was observed in the reference experiment by trypsin treatment. For example, as shown in Fig. 3, when 30% differentiated and 70% undifferentiated cells were reseeded by the trypsin treatment, ca. 20% cells were differentiated at 6 h after the reseeding. If 30% differentiated and 70% undifferentiated cells were isolated by the impulsive force, the percentage of differentiated cells at 6 h after the isolation would be estimated as

$$50\% \times 0.3 + 15\% \times 0.7 = 25.5\%.$$

This percentage is a little larger than that by trypsin treatment. The same tendency was confirmed throughout the 4-day period. This suggests that the cell differentiation by NGF is largely unaffected by the impulsive force.

As for the reasons that some cells disappeared from the observation area immediately after isolation due to the impulsive force (Fig. 2) and reseeding by trypsin treatment (Fig. 3), the 2 following explanations are most likely: (a) the cell was no longer observable because it died during the cell detachment process; (b) the cell left the observation area due to migration. As a result of time-lapse observation with a time interval of about 60 min, it was confirmed that case (a) was predominant and that case (b) was very rare both for the isolation due to the impulsive force and for the reseeding. Nevertheless, the number of cells immediately after the isolation and reseeding (at 0 h) cannot be compared accurately, because only the cells deposited on the culture dish were captured in the case of the trypsin reseeding, though all cells isolated by the impulsive force were captured after the isolation. In other words, we cannot count the cells disappeared in the reseeding process, though it is presumed that the cell membrane received chemical damage as a result of the trypsin treatment (Weiss 1963; Easty et al. 1960). Previously, the cell detachment process from the source substrate due to trypsion treatment has been compared with that due to the impulsive force. This comparison was performed by visualizing a collagen scaffold on the source substrate with a fluorescence probe of quantum dots (Maezawa et al. 2010). When cell was detached from the substrate by the impulsive force loading, fluorescence from the scaffold simultaneously disappeared from the substrate, although the scaffold was not irradiated by the laser. This suggests that the cell was detached along with the collagen scaffold. On the other hand, the scaffold remained on the substrate when it was detached by trypsin treatment. This result clearly indicates the difference between the two cell detachment processes i.e., although cell detachment due to the trypsin treatment is induced by chemical dissociation between the collagen scaffold and cell membrane, such dissociation is negligible in the detachment due to the impulsive force. Therefore, it is considered that the physiological damage to the cell membrane during the detachment process by the impulsive force loading is less than that by the trypsin treatment. Alternatively, the inside of the cell may receive physical damage caused by the impulsive force loading due to femtosecond laser irradiation. Although the experiment was performed under laser irradiation conditions to minimize cell deactivation, physical damage during the cell detachment process seems to result in the loss of cells after isolation. Nonetheless, cell deactivation due to the impulsive force loading was less than that due to reseeding by trypsin treatment.

4 Conclusion

PC12 cells differentiated to neuron-like shapes on a source substrate were selectively and individually isolated by femtosecond laser-induced impulsive force, and the differentiation property after isolation was confirmed. When differentiated cells on the source substrate were selectively isolated, the percentage of cells differentiated after isolation was obviously larger than that when undifferentiated cells were isolated. Cell deactivation due to the impulsive force loading was not larger than that due to reseeding by trypsin treatment. In the present experiment, since the cells of the same phenotype were divided according to the stages of differentiation, even cells that were initially undifferentiated

were eventually differentiated after isolation. If cells containing 2 or more differentiation phenotypes were isolated using this technique, cells of desirable phenotypes could be obtained selectively. Therefore, we conclude that our isolation method will be a promising technique for purifying cells of a specific phenotype from differentiated cells.

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