PARALLEL MANIPULATION OF ADHERING LIVING CELLS BASED ON PHOTO-INDUCED CELL CAPTURING

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Introduction

In recent years, demands for cell processing are becoming diversified in response to the rapid development of cell engineering. However, most existing technologies such as flow cytometry, magnetic cell sorting system and optical trapping can handle the cells only in the suspended state; to apply them to the anchorage dependent cells cultured on the substrate, it is essential to remove the adhering cells from a culture substrate in advance. Since anchorage dependent cells, which consist most of organisms, maintain their biological functions basically in the adhering state, removing them from a culture substrate may damage seriously the function and activity of the cells. Additionally, "these cells I am observing now" will mingle into the many other cells once the cells are detached from a culture substrate and suspended in the medium. After that, one cannot distinguish the cells, which had been characterized by the shape, the degree of extension and the positional relation with others in their adhering state.

In such a situation, we proposed a concept of photo-manipulation of living cells in adhering state as a principle of novel biotool, and have been promoting the development of elemental technologies to implement the concept (1). The implementation of this method is expected to enable the researchers in the field of cell engineering to fix "these cells I am observing now" selectively on a culture substrate though simple operation on a PC monitor. After flushing evenly, the cells other than captured are removed. Since this method can handle the adhering cells on culture substrate, we can choose the cells to capture corresponding to the characteristics of the cells, which can be observed in the microscopic image as mentioned above. Further, this method can be used also as a means to form cell patterning, but in a completely different way from any existing methods. In clear contrast to the conventional cell patterning using a previously patterned substrate, the area to retain the cells can be defined even after cell seeding, and the selectively captured cells can continue to grow freely beyond the defined area in further incubation. Up to now, we have developed several substrates having photoresponsive properties and demonstrated the photo-control of cell adhesion (1, 2). Recently, we reached the finding of the conditions to capture living cells firmly onto the substrate just by irradiating light without any assist of photoresponsive materials. In this study, we demonstrate cell pattern formation by using a newly developed apparatus, which enables us to project arbitrary micropattern onto the surface of culture substrate under microscopic observation. Further, we investigate the characteristics of this photo-induced cell capturing in order to examine the feasibility of manipulation of adhering cells based on this novel technology.

Experimentals

We composed a cell manipulation system based on photo-induce cell capturing by installing a PC-controllable microprojection unit in an optical system of an IX-71 inverted microscope (Olympus). The schematic illustration of the system is shown in Fig.1. We confirmed that the system can project the

light with the wavelength of 365 nm, which induced cell capturing and cannot be irradiated with the existing microprojection system based on commercialized LCD projector (3), in arbitrary pattern with the resolution of 7 micron in the area of 3.7 mm x 2.7 mm at the intensity up to 200 mW/cm^2.

For the experiment of cell pattern formation, we used CHO-K1 cells, which had been seeded uniformly and cultured for 24 h on a fibronectin-coated 35mm-diameter culture dish (No. 354457, BD Biosciences) at the density of 1.1 x 10^5 cells/cm^2. We irradiated the light with the wavelength of 365 nm at the energy density of 18 J/cm^2 focusing a computer-generated micropattern at the bottom surface of the cell culture dish. Then the culture medium was substituted by phosphate buffer saline (PBS) containing 1 mM EDTA, which is known to remove divalent metal ions such as Ca^{2+} and Mg^{2+}. After 10 min, we flushed the bottom surface of the dish with PBS to remove the weakly adhering cells. In order to obtain a constant flushing condition, we set a proprietary attachment on the dish to form a flow path with the upper clearance of 0.1 mm (Fig.2), and the flow velocity was set to be 2 m/sec. The microscopic images of the cells on the culture dish were taken by using a VB-7000/7010 cooled CCD camera system (Keyence) installed on the IX-71 inverted microscope.

We examined the influence of light irradiation to the cell viability in the following ways. At first, after cell pattern formation through photo-induced capturing, we stained the cells with CellTracker Red CMTPX (Invitrogen), which stains solely the living cells maintaining esterase activity adequately, and observed their fluorescence image with a confocal laser scanning microscope FluoView 300 (Olympus). Further, we irradiated the light with the energy density of 24 J/cm^2 to the CHO-K1 cells seeded on tissue culture polystyrene 96-well plates (No. 354409, BD Biosciences) and compared these growth with that of non-irradiated cells. Number of cells cultured in a well was estimated by treating with CyQUANT Cell Proliferation Assay Kit (Invitrogen) and measuring fluorescence intensity with a GENios plate reader (Tecan).

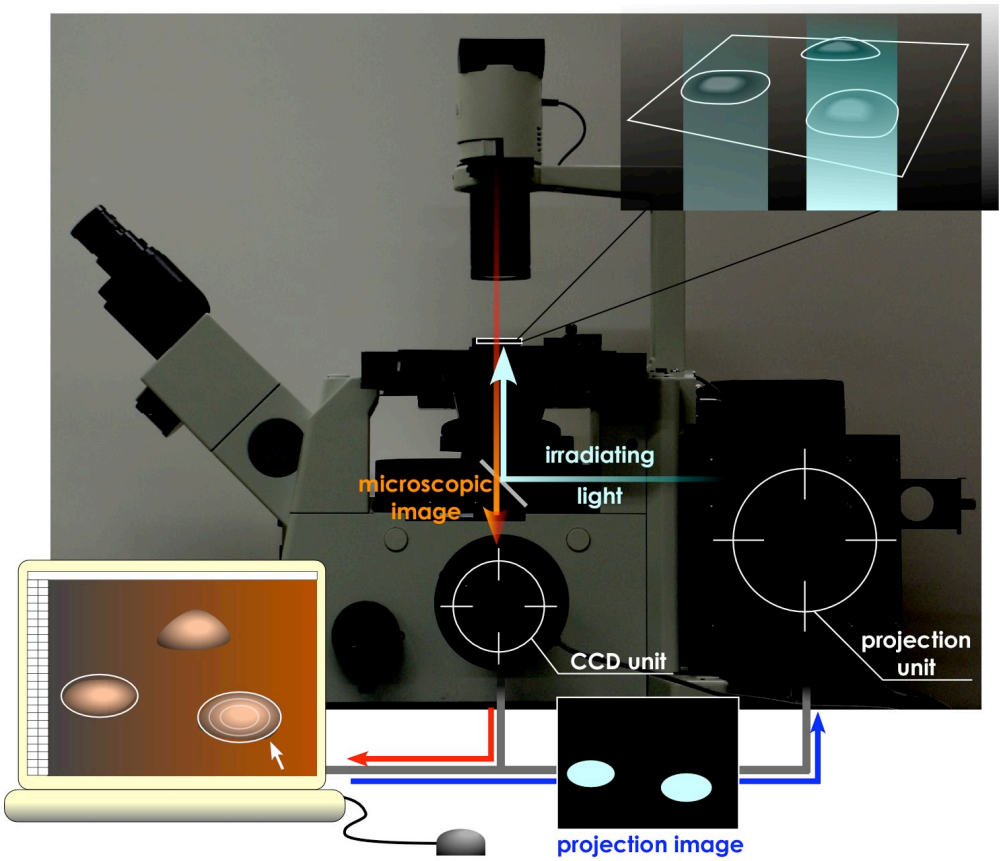

Fig.1. A schematic illustration of the cell manipulation system based on photo-induce cell capturing.

Results and Discussions

Figure 3 shows the microscopic images of the CHO-K1 cells, which were micropatterned by using a newly developed cell manipulation system. We obtained the highly contrasted cell patterns indicating that the cells were captured firmly on the cell culture dish in the irradiated region, while the cells, of which the adhesion was weakened through the EDTA treatment (4), were removed completely from the non-irradiated region. We obtained such a cell pattern with high contrast also when we used PBS containing no divalent metal ions instead of EDTA solution although it took 30 min to weaken the cell adhesion to the required extent. This result suggested that the light irradiation decrease the sensitivity of cell adhesion to the loss of divalent metal ions. Figure 3 indicated also that the cells were captured definitely even by the light irradiation confined within the size of one single cell. It should be noted also that the adhering and extended state of the cells is maintained even after the process of pattern formation including light irradiation, EDTA treatment and flushing with PBS. These features distinguish our technology sharply from the optical trapping, which is also a cell manipulation technique using light (5).

In terms of the influence of the photo-induced cell capturing to the viability of living cells, we confirmed that the cells grew vigorously even after the experience of the photo-induced cell capturing. In addition, we observed sufficient fluorescence from most of the cells, which had been irradiated with the light to induce cell capturing and then stained with CellTracker Red CMTPX, (Fig.4). Further, as a quantitative measure of influence of light irradiation to the cell viability, the comparison of the growth curves is shown in Fig.5. Although some slow-up appeared 1 and 2 days after the light irradiation, the irradiated cells grew similarly to non-irradiated ones from then on. Also we detected no significant difference between their shapes. The UV-A light, into which the light we used to capture cells is categorized, has been reported to have cytotoxicity by many research groups (6. 7). The UV-A light emitted from a usual solar simulator basically contains the light with the wavelength of 320 nm, which is absorbed by DNA to a considerable extent. Although the DNA has no absorbance at the wavelength of 365 nm, and it is highly unlikely that the light used in this study degenerates the DNA directly, the intensity of the light is not low and there is still some possibility that

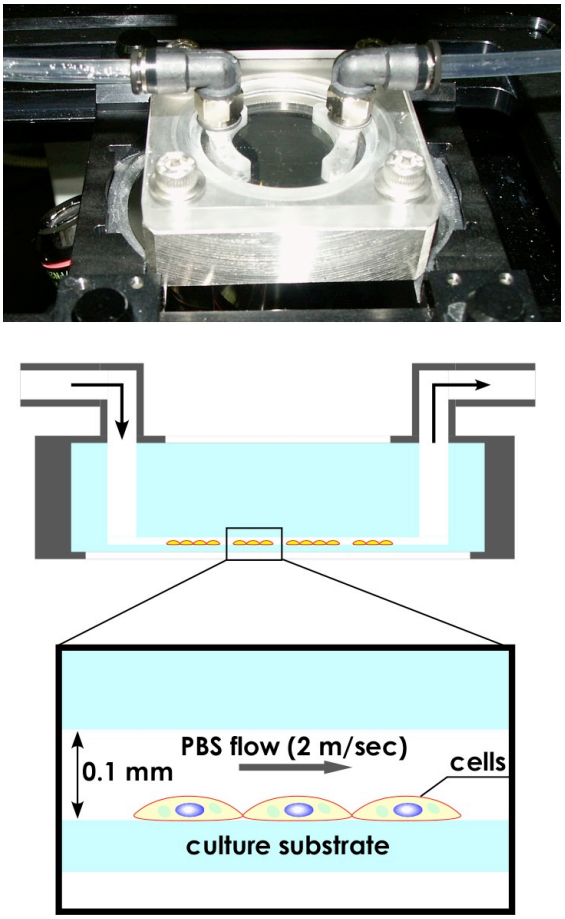

Fig.2. Set up for flushing the surface of culture substrate in a certain flushing condition.

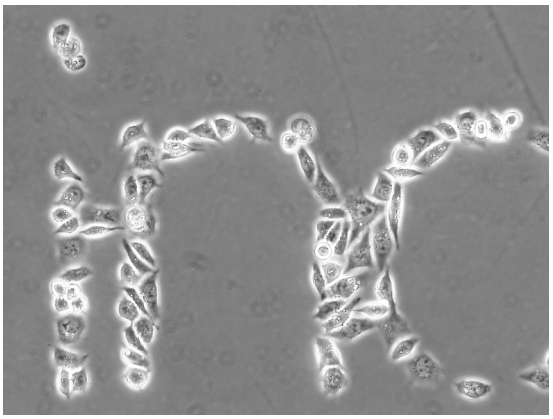

Fig.3. Micropatterned CHO-K1 cells produced by means of photo-induced cell capturing.

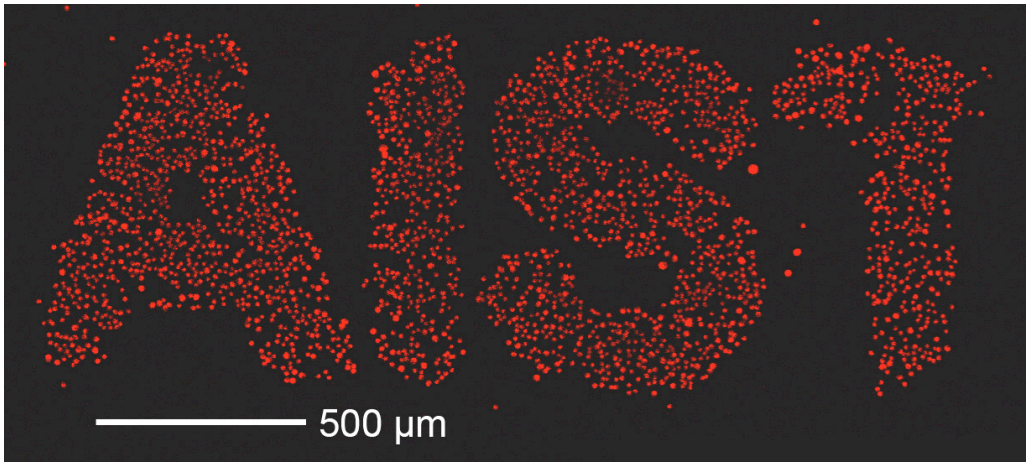

Fig.4. Fluorescence image of CHO-K1 cells, which were patterned by means of photo-induced cell capturing, and then stained with CellTracker Red CMTPX.

the radical oxygen species are generated through some photoreaction and may damage the DNA (8, 9). Further, a part of proteins have a little absorbance at the wavelength and may be altered by the light irradiation. Accordingly, in case that this technology is applied to precise investigation and analysis of the cell properties, more minute examination is considered to be necessary.

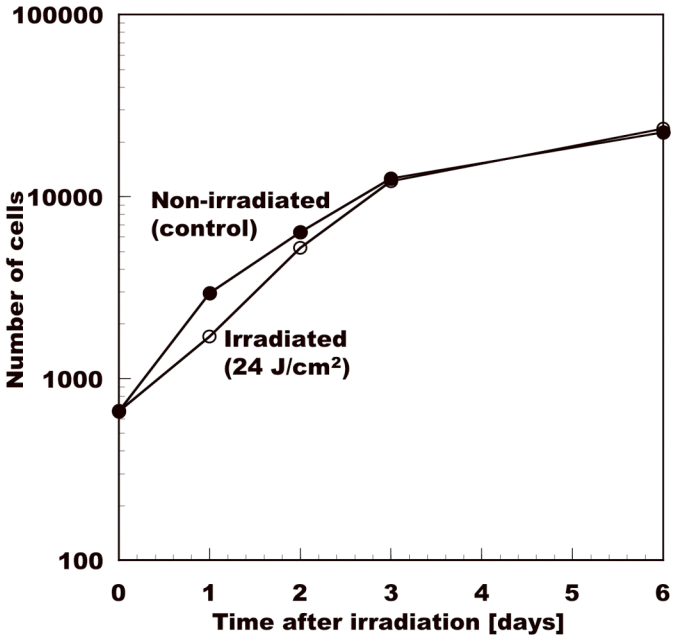

Fig.5. The influence of the light irradiation to the cell growth. Open circles: irradiated condition; closed circles: non-irradiated condition (control).

Conclusions

As a result of the experiments using a newly developed cell manipulation system, we confirmed that the highly contrasted cell patterns were formed with the precision of a single cell size by means of photo-induced cell capturing. We also determined that the area to retain the cells can be defined after cell seeding, and the captured cells can continue to grow freely beyond the defined area in further incubation. Moreover, it was clarified that the process containing light irradiation and flushing has no critical influence to the viability of living cells. On the other hand, the photo-induced cell capturing is basically compatible with electronics and information technology; the practical operation is based on the observation of the cells with CCD and the light irradiation with PC-controllable microprojection unit. Together with this point, the system studied here is expected to provide the research field of cell engineering with the means to manipulate adherent living cells in adhering state, which has never been impremented.

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