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学 位 論 文 題 目	Development and Evaluation of a Method for Micro Object Transfer into Live Cells by Liposome Electrofusion
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「要約」

Living systems consist of hierarchies, such as organs, tissues, cells, intracellular organs (organelles), and molecules. Although cells have various sizes and forms, cells all possess properties such as absorbing extracellular objects, responding to external stimuli, and reproducing their own cellular structures. From a mechanical engineering point of view, the cells (and organelles) are precision machines with absolute autonomy. To investigate the mechanical properties of intracellular structures, conventional techniques such as bend measurements use microneedles, which need to break the membrane and eventually kill the cell. Intracellular components can act quite differently between living and extracted conditions, therefore, non-invasive or low-invasive methods which are able to manipulate intracellular conditions are awaited.

In order to transfer various objects into cells, endocytosis has been widely utilized. In endocytosis, the external solution becomes entrapped in a closed vesicle consisting of a “reversed” plasma membrane. The sizes of transferable objects through this pathway are quite limited, up to several hundred nanometers in diameter. To overcome this, microinjection is generally used. However, such a labor intensive method is not feasible for many applications. Electroporation, a method that make micropores in the cell membrane for transferring DNA/RNA molecules by exposing the target cell to an electric field, is able to treat many cells at once. However, electroporation is limited to the transfer of nucleic acids and small soluble molecules only. Cell-cell fusion is widely used for constructing hybridomas. Particularly, the cell-cell electrofusion by electroporation, causing membrane breakdown, has been well investigated. The breakdown process consists of the following two steps. First, cells form “pearl-like chains” in AC electric fields along the electrical flux by effect of dielectrophoresis. Then a high voltage DC pulse is applied to the electrodes to break the membranes. Intracellular transfer of cellular ingredients takes place at the pore when the DC pulse applied. This fusion has been widely used in bioengineering because it can fuse many cells, and can avoid undesired damage caused by carrier materials such as peptides or viral vectors.

On the other hand, various biocompatible nano-micro particles have been developed, including thermo sensing polymers and DNA origami objects. If the artificial objects are transferred non-invasively to the live cells, they are able to measure various intracellular activities in living conditions. For instance, nuclear transplantation into a foreign cell has been tried, in order to elucidate molecular mechanisms of mitochondria using microinjection. However, this approach is not suitable to analyze the function of the mitochondria-transferred-cell because it is slow and only able to manipulate a single cell. Due to their large size and fragility, there are no other methods to transplant mitochondria at this moment. Shirakashi et al., proposed cell-GUV (Giant Unilamellar Vesicle) electrofusion, where GUVs loaded with low-molecular-weight disaccharides were fused with Jurkat cells. However, neither the efficiency of transfer of the GUV contents (trehalose, raffinose, and KCl in this case) nor cell viability were reported. Their GUVs were prepared by a conventional electroformation, which is not able to enclose large-sized objects.

Here, we describe research aiming at establishing a method to transfer micrometer-sized objects into live cells using cell-GUV electrofusion. In this research we propose to combine the latest reliable method called "w/o emulsion centrifugation" developed for preparing GUVs containing artificial objects larger than 1 μm in diameter and cell-GUV electrofusion. After exposure to the DC pulse, the HeLa cells were cultured on the dishes, and the cells which were stained with cell tracker red and Hoechst 33342 to reveal the cytoplasm (red) and nucleus (blue), respectively (Figure 1(A)), and the z-axial positions were observed. These images show HeLa cells into which no beads, and beads of 0.5, 1, and 2 μm in diameter (green) had been transferred. However, 2 μm microbeads were not observed inside the HeLa cells. The percentage of HeLa cells containing the transferred microbeads was quantified by a flow cytometer. The cells were cultured for 2 days after electrofusion. Figure 1(B) shows histograms of the HeLa cells emitting a green fluorescent signal. The values shown in the inset, for 0.2, 0.5, 1, and 2 μm microbeads, and no microbeads, were 73, 50, 40, 0.4, and 0.3%, respectively. These values were defined as the ratio of cells that demonstrated a fluorescent intensity of more than 10. These data revealed a threshold between 1 and 2 μm for the size of beads transferred into live cells using cell-GUV electrofusion.

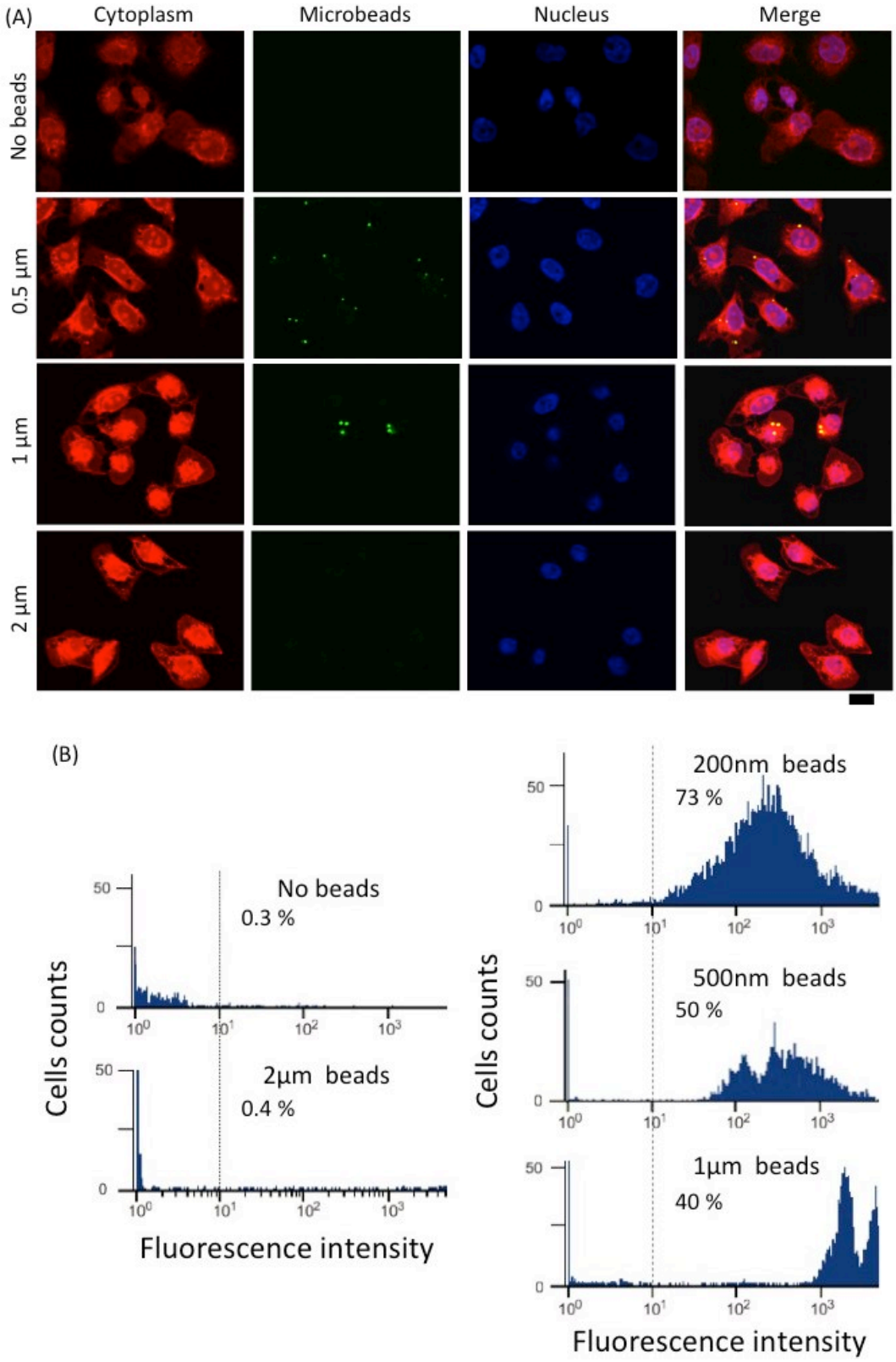

Figure 1 Fluorescent beads with various diameters were transferred into HeLa cells by cell-GUV electrofusion. A: Confocal microscojpic images show the cross-section of the treated HeLa cells. Bar = 50 µm. B: Flow cytometric detection of microbeads transferred into HeLa cells. Created based upon figures from SAITO et al., PLOS ONE 9 (9): e106853.

.By using this proposed method, we evaluate various conditions such as GUV membrane properties (charge and elasticity), electrical conditions, and the numerical ratio of GUVs and cells to improve the transfer efficiency and cell viability. As a result, we succeeded in transferring various objects, such as micro magnetic beads, designed DNA origami, and extracted mitochondria. With fluorescent beads as known size markers, we confirm that beads of up to 1 μm in diameter are efficiently transferred into living cells carrying mammalian plasmid expression vectors. We also found that our method does not severely affect cell viability. Namely, after the electrofusion, cells proliferated normally until confluence was reached, and the transferred fluorescent beads were inherited during cell division. In summary, the best transfer efficiency was achieved using negatively charged GUVs, and by using a large number of GUVs compared with cells. We also tried to transfer a designed nanostructure (DNA origami) into living cells, and succeeded. Interestingly, the fluorescent signal of the transferred DNA origami disappeared after overnight culturing because of nucleic decomposition in the cell. Moreover, the cell-GUV electrofusion method can be applied to transfer extracted organelles into other live cells. We have succeeded in transferring mitochondria into living cells by our proposed method. The mitochondria activity was evaluated by antibody staining and the result showed that the transferred mitochondria in live cells could be recognized as foreign substances but they also maintain their activity.

Overall, the results reported here present a milestone for designing artificial symbiosis of functionally active objects (such as nano- to micro-scale machines) and intracellular organelles in living cells. Moreover, these bioengineered hybrid cells are ready to use in drug delivery system, tissue engineering, and for further elucidation of cellular mechanisms.