A Microfluidic Device for Modulation of Organellar Heterogeneity in Live Single Cells

Ken-Ichi WADA,*† Kazuo HOSOKAWA,* Yoshihiro ITO,** and Mizuo MAEDA*

*Bioengineering Laboratory, RIKEN Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
**Nano Medical Engineering Laboratory, RIKEN Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

The quantitatively controlled organellar transfer between living single cells provides a unique experimental platform to analyze the contribution of organellar heterogeneity on cellular phenotypes. We previously developed a microfluidic device which can perform quantitatively controlled mitochondrial transfer between live single cells by promoting strictured cytoplasmic connections between live single cells, but its application to other organelles is unclear. In this study, we investigated the quantitative properties of peroxisome transfer in our microfluidic device. When cells were fused through a 10 or 4 μm long microtunnel by a Sendai virus envelope-based method, a strictured cytoplasmic connection was achieved with a length corresponding to that of the microtunnel, and a subsequent recovery culture disconnected the fused cells. The peroxisome number being transferred through a 10 μm length of the microtunnel was smaller than that of 4 μm. These data suggest that our microfluidic device can perform a quantitative control of peroxisome transfer.

Keywords Microfluidic device, cell fusion, organellar transfer, peroxisome, mitochondria

(Received October 19, 2020; Accepted November 29, 2020; Advance Publication Released Online by J-STAGE December 4, 2020)

Introduction

It is widely known that cells show different properties even within the same population, namely, cells have individuality. A number of studies have shown an intercellular variety of epigenetic statuses such as DNA methylation, chromatin accessibility and histone modifications, suggesting that nuclear epigenetic factors promote cellular individuality. Added to these, non-nuclear (i.e., cytoplasmic) factors are also likely to contribute to the establishment of cellular individuality. In particular, organellar heterogeneity is a critical factor for cellular individuality. However, it has rarely been investigated, at least partly, due to the absence of a practical method to manipulate the organellar heterogeneity in a live single cell.

A promising method to manipulate the organellar heterogeneity is to perform non-invasive and quantitatively controlled organelle transfer to target cells segregated from the nuclei of donor cells. One direct method for this manipulation is the microinjection of isolated organelles. Although mitochondrial transfer to mammalian cells using a relatively thick microcapillary (∼1 μm inner diameter) has been demonstrated, this method has the risk of imparting serious damage to the target recipient cells by puncturing via the microcapillary. Added to this, the isolation of organelles results in exposure to extracellular environments that may affect the organellar properties. Recently, a novel injection method utilizing pulsed laser- triggered cavitation bubbles has been developed. This method can facilitate mitochondrial transfer and possibly other kinds of organellar transfer without severe trauma to the target cells, although, the isolation of organelles is still required. In contrast to these injection methods, the fusion of cytoplasts (enucleated cells) with target cells can perform organellar transfer without exposure to extracellular environments. A coculture under a certain condition also leads to intercellular organelle transport via an intrinsic cellular structure named a tunneling nanotube. However, the amount of organelles being transferred by these methods is basically uncontrollable. Overall, the methods described above are impractical to manipulate the organellar heterogeneity.

A microfluidic device is now recognized as an advantageous tool for biological analysis. In particular, the manipulation of single cells given by its accurate structures provides a unique analytical platform. Over the last ten years, microfluidic devices that can perform cytoplasmic or organellar transfer between live single cells have been reported by several studies, including ours. In those devices, single cells were fused through a microfabricated aperture. As a result, cytoplasmic components were non-invasively transferred to the target cells, segregating from the nuclei of donor cells. Recently, we found that cell fusion through different lengths of the microtunnel led to quantitative control of mitochondrial transfer to the target cells. Therefore, our method provides a unique experimental platform to modulate organellar heterogeneity in live single cells, at least of mitochondria. However, it is still unclear whether our method is applicable to other organelles. In this study, we focused on peroxisomes because they are involved in the metabolism of xenobiotics and have been shown to negatively affect cell functions.

† To whom correspondence should be addressed.
E-mail: wada.kenichi.833@m.kyushu-u.ac.jp
Present address: R&D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi, Fukuoka 812-8582, Japan

1 DOI:10.2116/analsci.20SCP11
study, we investigated the properties of peroxisome transfer in our microfluidic device to evaluate the validity of our microfluidic device for modulating organellar heterogeneity.

Experimental

Cells
The NIH3T3 fibroblast-derived cell lines Ng3T3 (nucleus is labeled with H2B-EGFP) and CrN3T3 (cytoplasm and nucleus are labeled with mCherry and H2B-mCherry, respectively) were previously established. PXg3T3 (peroxisomes are labeled with EGFP-SKL) was established from NIH3T3 cells by stable transfection with the EGFP-SKL expression vector, which was generated by inserting the TCCAAATTA nucleotide sequence (cording SKL/Serine-Lysine-Leucine, a peroxisome targeting signal) at the C-terminus of the EGFP gene. PXgMTr3T3 (peroxisomes and mitochondria are labeled with EGFP-SKL and Cox8α-mCherry, respectively) was established from PXg3T3 by stable transfection with the Cox8α-mCherry expression vector. For stable transfection, the constructed vectors were transfected into the cells using the Lipofectamine 2000 reagent (Thermo Fisher Scientific, MA); then, the cells were selected with 200 μg/mL Hygromycin B (Wako Pure Chemical Industry Ltd., Japan) or 2 μg/mL Puromycin (Takara Bio Inc., Japan). The resulting colonies were harvested and confirmed the fluorescent signals. Cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) containing 4500 mg/L glucose (Wako Pure Chemical Industry Ltd.) supplemented with 10% fetal bovine serum. To maintain the expression of the fluorescent proteins, Hygromycin B or Puromycin was added to the culture medium, if necessary.

Reagents
Cytochalasin D was purchased from (Wako Pure Chemical Industry Ltd.). Y-27632 was purchased from EMD Millipore (Billerica, MA). Sendai virus envelope (HVJ-E) was purchased from Ishihara Sangyo Kaisha Ltd. (Osaka, Japan). HVJ-E suspension was prepared according to the manufacturer’s protocol, but suspended with DMEM in lieu of the supplied suspension buffer. This was because the suspension buffer inhibited the formation of neurite-like processes penetrating the microtunnel, leading to low efficient cell fusion through a microtunnel (data not shown). The prepared HVJ-E suspension was stored at −80°C.

Microfluidic device
The microfluidic devices were fabricated as previously described. Briefly, the master molds for a poly(dimethylsiloxane) (PDMS) chip were fabricated on Si wafers using SU-8 (MicroChem Corp., MA) by well-aligned double exposure of micro-patterned UV light. The first and second exposure was used for optical modeling of a microtunnel with a 2 μm width, a 2 μm depth and a 4 or 10 μm length; the main channel region had a 500 μm width and a 26 μm depth, respectively. After curing of PDMS on the master mold at 65°C > 1 h, the PDMS chip was harvested and trimmed. A silicone tube with an inner diameter of 5 mm was then connected to create an inlet reservoir. The resulting PDMS chip was reversibly bonded to the bottom of a 35-mm culture dish to make the microfluidic device.

Cell fusion in microfluidic device
Cell fusion between Ng3T3/PXg3T3 and CrN3T3 cells through a microtunnel was induced, as previously described, with some modifications. Briefly, a growth medium containing a 1/40 volume of an HVJ-E suspension and 40 μM Y-27632 was used as the fusion medium. After introducing cells into the microfluidic device, a fusion medium was applied to the inlet reservoir and incubated in a CO2 chamber for 1 h. Cell fusion was detected by the donor cell to the Ng3T3/PXg3T3 cell. After the induction of cell fusion, the medium in the inlet reservoir was replaced with a normal growth medium and further incubated for 24 h (recovery culture).

Cell observations
Fluorescent signals of EGFP and mCherry were observed directly using reverse fluorescent microscopy IX81 (Olympus Corp., Japan) with either a 0.3 NA 10× UPlanFL N objective (Olympus) or a 0.6 NA 40× LUCPlanFL N objective (Olympus) through a blue and green filter, respectively. The images were recorded with a CCD camera (ROLERA-XR, QIMAGING) and LuminaVision software (MITANI Corp., Japan). The acquired images were analyzed using ImageJ ver. 1.45s (National Institutes of Health) software.

The cell behavior during the recovery culture was recorded by 10 min-interval time-lapse observations for up to 24 h using a BioStudio mini (Nikon Engineering Co., Ltd., Japan) placed in a CO2 incubator. We did not perform any fluorescent observation during the recovery culture so as to maintain the cell viability and not to reduce the fluorescent signals. At the endpoint of the time-lapse observation, a fluorescent observation was performed on the fused cell pairs or disconnected recipient cells.

Statistics
Statistical data were analyzed by the Student’s t-test. A value of P < 0.05 was regarded as being significant.

Results and Discussion
In this study, we compared two types of microfluidic devices having 105 CPSs (cell paring structure) with a microtunnel 4 or 10 μm in length (short tunnel, or long tunnel, respectively) (Fig. 1). In our approach, the organellar transfer between live single cells are performed as follows (see Fig. 4A). First, single cells are captured in CPSs to make 1:1 single cells pair across the microtunnel. Then, cell fusion is induced between the paired single cells to promote a strictured cytoplasmic connection, which leads to organellar transfer segregated from the nuclei of the donor cells. Finally, the fused cells are disconnected to terminate the organellar transfer. At first, we optimized the experimental procedures of each step described above using Ng3T3 and CrN3T3 cells. After cell suspension flowing, > 90% of the CPSs were successfully occupied with 1:1 cell pairs, and approximately 30–40 heterogeneous cell pairs were achieved in one microfluidic device (data not shown). Therefore, there was no need to improve the procedure for this step. In previous studies, we used a fusion medium containing 1/10 volume of the HVJ-E suspension. In this condition, supplementation with Y-27632 was essential to induce cell fusion because the reagent was needed to generate neurite-like processes penetrating the microtunnel to achieve cell-cell contact. In this study, we found that a thinner fusion medium containing 1/40 volume of the HVJ-E suspension induced cell fusion through a microtunnel without Y-27632, perhaps due to a decreased inhibitory effect of HVJ-E on neurite-like processes formation. Furthermore, supplementation with 40 μM Y-27632 to this fusion medium increased the fusion rate from 6.2 to 12.8% (Fig. 2C). Given...
the results, we then employed a fusion medium containing a 1/40 volume of HVJ-E suspension supplemented with 40 μM Y-27632 in subsequent experiments. Finally, we investigated how frequent disconnection occurs in the cells fused under the determined condition. The result of time-lapse observations on the fused cells during the recovery culture showed that 9 out of 11 (81.8%) fused cells were disconnected within the first 16 h of the recovery culture, and only 1 out of 11 (9.1%) fused cells maintained the cytoplasmic connection for > 24 h (Fig. 2E). During the recovery culture, no nuclear mixing (i.e., nuclear migration passing through the microtunnel) occurred. These data suggest that the applied protocol reliably promotes a transient cytoplasmic connection between living single cells.

The disconnection of the fused cells is an essential process for organellar transfer between single cells. Although we succeeded to promote a transient cytoplasmic connection through a microtunnel by the recovery culture, the mechanism for the disconnection was unclear. Since many cells quickly moved away from the CPSs after disconnection, we assumed that the force given by the cell migration caused the disconnection. In order to evaluate this, we treated the fused cells with an actin inhibitor Cytochalasin D (CytoD) to reduce the cell migration activity. When cells were treated with 2 μM CytoD after cell fusion, the cells including fused ones became round and their migration activity were severely inhibited, and all fused cells observed (n = 8) kept a cytoplasmic connection through a microtunnel for > 24 h (Fig. 2E). During the recovery culture, no nuclear mixing (i.e., nuclear migration passing through the microtunnel) occurred. These data suggest that the applied protocol reliably promotes a transient cytoplasmic connection between living single cells.

The disconnection of the fused cells is an essential process for organellar transfer between single cells. Although we succeeded to promote a transient cytoplasmic connection through a microtunnel by the recovery culture, the mechanism for the disconnection was unclear. Since many cells quickly moved away from the CPSs after disconnection, we assumed that the force given by the cell migration caused the disconnection. In order to evaluate this, we treated the fused cells with an actin inhibitor Cytochalasin D (CytoD) to reduce the cell migration activity. When cells were treated with 2 μM CytoD after cell fusion, the cells including fused ones became round and their migration activity were severely inhibited, and all fused cells observed (n = 8) kept a cytoplasmic connection through a microtunnel for > 24 h under this condition. In a subsequent recovery culture, all fused cells were successfully disconnected (Fig. 3B). The connecting time of these cells during the recovery culture was similar to that of the cells without CytoD treatment (Fig. 3C). Considering that the inhibitory effect of CytoD is reversible, our data suggests that the cell motility is involved with the disconnection, as we expected. Therefore, the connecting time might be related to the time that cells regain motility.

We have shown that modulation of the length of the
microtunnel promotes the quantitative control of mitochondrial transfer, suggesting that our microfluidic device is applicable to other organelles. Then, in order to evaluate whether our microfluidic device leads to the quantitative control of organellar transfer other than mitochondria, we observed the peroxisome transfer using PXg3T3 and CrNr3T3 cells as a donor and recipient, respectively (Fig. 4). In this observation, the amount of peroxisomes being transferred was evaluated by counting the peroxisome number in the disconnected recipient cells at 24 h of the recovery culture. The dotted EGFP signals brighter than the strongest background signal were regarded as being the transferred peroxisomes. We counted the number of transferred peroxisomes manually.

When a transient cytoplasmic connection was achieved between these cells through a short tunnel, EGFP-labeled peroxisomes (dotted EGFP-SKL signals) were observed in all 7 cases, whereas through a long tunnel, no EGFP-labeled peroxisome was observed in 5 out of 9 cases. The average number of peroxisomes being transferred through a short tunnel was significantly larger than that of a long tunnel (4.14 ± 2.75 and 0.44 ± 0.68, respectively) (Fig. 4D′). However, the observed peroxisome number would be underestimated due to degradation of the EGFP-SKL and/or peroxisomes during the recovery culture. The connecting time was similar between the short and long tunnels (9.9 ± 3.5 and 11.6 ± 5.9 h, respectively) (Fig. 4D′). However, there was no significant correlation between the connecting time and the number of peroxisomes being transferred in the cell pairs fused through a short or a long tunnel (r² = 0.07 and 0.21, respectively) (Fig. 4D). These data suggest that different lengths of the microtunnel provides a quantitative property in peroxisome transfer by a connecting time-independent mechanism. Although the mechanism for the quantitative control of peroxisome transfer is unclear, we think that a vibration-like motion of peroxisomes might be involved because it potentially promotes the diffusion of peroxisomes passing through the microtunnel, thereby resulting in a more severe restriction of peroxisome transfer in the long tunnel because of its longer distance between the donor and recipient cells.

In contrast to peroxisomes, we have shown that the amount of mitochondria being transferred through a microtunnel increases in a time-dependent manner. Therefore, it is likely that the transfer of peroxisome and mitochondria through a microtunnel is driven by a different mechanism. If this is the case, selective peroxisome or mitochondrial transfer might be achieved via discrete interference of their transfer mechanism in our microfluidic device. Then, in order to investigate this possibility, we observed the transfer of these organelles at the same time using the PXgMTr3T3 and CrNr3T3 cells as a donor and recipient, respectively (Fig. 5). The fused cell pairs were observed at 6 h after cell fusion because the Cox8a-EGFP (i.e., mitochondrial) signal in the recipient cells became weaker at 8 h or later perhaps due to degradation and/or diffusion to unlabeled mitochondria of the recipient cells. In this observation, round cells were excluded because they squeezed out their cytoplasm into the fusion partner by cellular shrinkage, obscuring the effect of the microtunnel for regulating organellar transfer. Round cells were found in ∼30% of the fused cells in this observation.

The result showed that the peroxisome transfer segregating from mitochondria, or selective peroxisome transfer, was achieved in 1 of 10 (10%) and 3 of 8 (38%) cell pairs fused through the short and long tunnel, respectively (Fig. 5B). Although the mitochondrial transfer may possibly occur in these cell pairs thereafter, we think that our microfluidic device can perform selective peroxisome transfer because some fused cell pairs were disconnected within 6 h after cell fusion (Fig. 2E). The remaining fused pairs underwent either co-transfer of mitochondria/peroxisomes or no transfer of these organelles.
There was no fused cell pair showing mitochondrial transfer segregating from peroxisomes (Fig. 5B). These data suggest that the selective peroxisome transfer is promoted by the higher restriction of the mitochondrial transfer than that of peroxisomes in our microfluidic device. Cytoskeletons would be involved with this selective peroxisome transfer because they govern peroxisomes and mitochondria positioning and promote different behavior and intracellular distribution. As selective organellar transfer becomes a key technology for the modulation of organelle heterogeneity, the effect of cytoskeletal components on the organellar behavior in our microfluidic system is an important issue to be explored in future studies.

In summary, we succeeded in non-invasive and quantitatively controlled peroxisome transfer using a microfluidic device via promoting a transient cytoplasmic connection between live single cells through a different lengths of microtunnel, and found the possibility that the transient cytoplasmic connection through the microtunnel leads to the selective peroxisome transfer segregating from mitochondria. Importantly, our microfluidic device is completely confined within a 35 mm culture dish without connections to any external machines. With this feature, our method can facilitate organellar transfer using quite simple manipulations, such as pipetting. Furthermore, we have confirmed that our microfluidic device is applicable to a variety of cell types such as 143B, HeLa, MCF7, HEK293, C2C12, MEF (mouse embryonic fibroblast), and ES cells. These features highlight the practical utility of our method for modulating organellar heterogeneity. Considering this practical utility, as well as the non-invasive, quantitative and selective features in organellar transfer, our method will provide a unique experimental platform for analysis on the cellular individuality while focusing on the organellar heterogeneity.

Fig. 4 Quantitative control of peroxisome transfer. (A) Summary of the experiment. (B) An example of time-lapse observation showing disconnection of fused cell. Cell fusion was confirmed in advance of a time-lapse observation (subpanel of 0 min). Solid and dashed arrows represent the tracked PXg3T3 and CrNr3T3 cells, respectively. (C) Two examples of fluorescent microscopic images of disconnected CrNr3T3 cells. (D) Plots of transferred peroxisome number and connecting time. Black and white circles represent the data from experiments using short and long tunnels, respectively. Data of the transferred peroxisome number and connecting time of short (S) and long (L) tunnel in D are separately presented in D' and D". The values are means ± SD.
Acknowledgements

The authors thank the Laboratory for Animal Resources and Genetic Engineering, Center for Developmental Biology, RIKEN Kobe for providing EGFP/mCherry vectors. This work was supported by the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (KAKENHI) (Grant Number 16K07207), Center of Innovation Program from the Japan Science and Technology Agency, and Single Cell Project and Engineering Network Project from RIKEN.

References

1. T. Masujima, Anal. Sci., 2009, 25, 953.
2. G. Landan, N. M. Cohen, Z. Mukamel, A. Bar, A. Molchadsky, R. Brosh, S. Horn-Saban, D. A. Zalcenstein, N. Goldfinger, A. Zundelevich, E. N. Gal-Yam, V. Rotter, and A. Tanayal, Nat. Genet., 2012, 44, 1207.
3. H. Guo, P. Zhu, X. Wu, X. Li, L. Wen, and F. Tang, Genome Res., 2013, 23, 2126.
4. J. D. Buenrostro, B. Wu, U. M. Litzenburger, D. Ruff, M. L. Gonzales, M. P. Snyder, H. Y. Chang, and W. J. Greenleaf, Nature, 2015, 523, 486.
5. T. Nagano, Y. Lubling, T. J. Stevens, S. Schoenfelder, E. Yaffe, W. Dean, E. D. Laue, A. Tanay, and P. Fraser, Nature, 2013, 502, 59.
6. D. A. Cusanovich, R. Daza, A. Adey, H. A. Pliner, L. Christiansen, K. L. Gunderson, F. J. Steemers, C. Trapnell, and J. Shendure, Science, 2015, 348, 910.
7. D. B. Seligson, S. Horvath, T. Shi, H. Yu, S. Tze, M. Gronstein, and S. K. Kurdistani, Nature, 2005, 435, 1262.
8. N. Hattori, T. Niwa, K. Kimura, K. Helin, and T. Ushijima, Nucleic Acids Res., 2013, 41, 7231.
9. A. Y. Chang and W. F. Marshall, J. Cell. Sci., 2017, 130, 819.
10. J. Aryaman, I. G. Johnston, and N. S. Jones, Front. Genet., 2018, 9, 718.
11. M. P. King and G. Attardi, Cell, 1988, 52, 811.
12. M. P. King and G. Attardi, Science, 1989, 246, 500.
13. T.-H. Wu, T. Teslha, S. Kalim, C. T. French, S. Moghadam, R. Wall, J. F. Miller, O. N. Witte, M. A. Teitell, and P. Y. Chiou, Anal. Chem., 2011, 83, 1321.
14. T.-H. Wu, E. Sagullio, D. Case, X. Zheng, Y. Li, J. S. Hong, T. Teslha, A. N. Patananan, J. M. McCaffery, K. Niazi, D. Braas, C. M. Koehler, T. G. Graebner, P. Y. Chiou, and M. A. Teitell, Cell Metab., 2016, 23, 921.
15. G. Poste and P. Reeve, Nat. New Biol., 1971, 229, 123.
16. A. Rustom, R. Saffrich, I. Markovic, P. Walther, and H. H. Gerdes, Science, 2004, 303, 1007.
17. X. Ou, P. Chen, and B. F. Liu, Anal. Sci., 2019, 35, 609.
18. M. Gel, S. Suzuki, Y. Kimura, O. Kurosaawa, B. Techau, H. Oana, and M. Washizu, IEEE Trans. Nanobiosci., 2009, 8, 300.
19. Y. Kimura, M. Gel, B. Techau, H. Oana, H. Kotera, and M. Washizu, Electrophoresis, 2011, 32, 2496.
20. K.-I. Wada, K. Hosokawa, E. Kondo, Y. Ito, and M. Maeda,
21. K.-I. Wada, K. Hosokawa, Y. Ito, and M. Maeda, Biotechnol. Bioeng., 2015, 112, 2334.
22. M. Okanojo, K. O. Okeyo, H. Hanzawa, O. Kurosawa, H. Oana, S. Takeda, and M. Washizu, Biomicrofluidics, 2019, 13, 034115.
23. K.-I. Wada, K. Hosokawa, Y. Ito, and M. Maeda, Biol. Open, 2017, 6, 1960.
24. C. B. Brocard, K. K. Boucher, C. Jedezsko, P. K. Kim, and P. A. Walton, Traffic, 2005, 6, 386.
25. A. F. Miranda, G. C. Godman, and S. W. Tanenbaum, J. Cell Biol., 1974, 62, 406.
26. K. Arai, S. Ohkuma, T. Matsukawa, and S. Kato, FEBS Lett., 2001, 507, 181.
27. E. A. C. Wiemer, T. Wenzel, T. J. Deerinck, M. H. Ellisman, and S. Subramani, J. Cell Biol., 1997, 136, 71.
28. Y. Eura, N. Ishihara, S. Yokota, and K. Mihara, J. Biochem., 2003, 134, 333.
29. E. H. Ball and S. J. Singer, Proc. Natl. Acad. Sci. U. S. A., 1982, 79, 123.
30. M. H. Heggeness, M. Simon, and S. J. Singer, Proc. Natl. Acad. Sci. U. S. A., 1978, 75, 3863.
31. L. V. Johnson, M. L. Walsh, and L. B. Chen, Proc. Natl. Acad. Sci. U. S. A., 1980, 77, 990.
32. M. Wu, A. Kalyanasundaram, and J. Zhu, Int. J. Nanomed., 2013, 8, 4033.
33. D. Dietrich, F. Seiler, F. Essmann, and G. Dodt, Biochim. Biophys. Acta, 2013, 1833, 3013.
34. S. M. Kim, K.-I. Wada, M. Ueki, K. Hosokawa, M. Maeda, Y. Sakai, and Y. Ito, Biochem. Biophys. Res. Commun., 2019, 520, 257e262.