ABSTRACT
In this report, we demonstrate a new approach to assemble micro-tissues with precise cell positioning based on the integration of a powerful cell-to-cell adherence system, avidin-biotin binding system (ABBS), optical tweezers and microdevices. Interestingly, avidinylated and biotinylated cell adherence occurred within 1 second using laser trapping, enabling single cell manipulation. We showed precise, direct single-cell-based tissue assembly using ABBS, optical tweezers and microdevices, followed by damage-free tissue culture. This approach to make micro-tissue has considerable potential for use in application such as tissue engineering, regenerative medicine, and drug screening system.

KEYWORDS
Avidin-biotin binding system, Quick and direct cell-to-cell adherence, Optical tweezers, Tissue engineering

INTRODUCTION
Well-controlled and efficient organization of micro-tissues showing normal tissue functions is very important in micro systems that enable various biological tests like drug screening. The major requirement for these micro-tissues is to have accurate alignment of cells, since most tissues in our body have their own layered structures. We have been investigating the feasibility of bottom-up tissue engineering, a new methodology to form small tissues, by assembling cells using ABBS as “instant glue” for cell-to-cell adherence. In this report, we demonstrated direct and precise assembly of micro-tissues by combining ABBS and single cell manipulation technologies such as optical tweezers in microfluidic devices.

THEORY
Figure 1A shows the principle of ABBS. Both avidin (protein) and biotin (vitamin) are widely applied in the biochemical field like immunostaining because of their strong affinity ($K_d = 10^{-15} \text{ M}$). This system was shown to be useful in attaching cells to the surfaces of scaffolds [1, 2]. In this study, we applied this system to direct cell-to-cell adherence to make micro-tissues in combination with optical tweezers and microfluidic devices.

EXPERIMENTAL

Cell culture, biotinylation, and avidinylation
The human hepatoma cell line Hep G2 was from the Japanese Center Research Bank (JCRB). The culture medium was Dulbecco’s modified minimum essential medium (DMEM; Sigma-Aldrich, St. Louis, MO, US) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, US), 25 mM hydroxyethylpiperazine-N’ 2-ethanesulfonic acid (HEPES; Doino, Kumamoto, Japan), 100 units/ml of penicillin (Wako Pure Chemicals, Osaka, Japan), 100 µg/ml of streptomycin (Wako), and 0.25 µg/ml of amphotericin B (Sigma). Cells were cultured in a CO$_2$ incubator (5% CO$_2$ 37°C) and biotinylated with EZ-Link® Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, US) based on instructions. Subconfluent Hep G2 cells in a 10 cm dish were treated with 2 ml biotinylation reagent (0.5 mM in phosphate-buffered saline, PBS) for 30 minutes in a CO$_2$ incubator, then washed with PBS, recovered with trypsin, and used as biotinylated cells. An aliquot of biotinylated cells was incubated with 2 ml of avidin (A9275, isolated from chicken egg white, Sigma)-PBS solution (1.5 mg/ml) for 30 minutes on ice, washed, and used as avidinylated cells. These biotinylated and avidinylated cells were stained with PKH26 (Sigma) or PKH67 (Sigma), as necessary. Assembled cells were observed by confocal laser microscopy (FV5-COL, Olympus, Tokyo, Japan) or fluorescence microscopy (IX71, Olympus).

Optical tweezers
Two-laser micromanipulation (Sigma Koki, Saitama, Japan) was connected to a phase-contrast microscope (Nikon, Tokyo, Japan). We used laser power of 1.2 A, 0.8 W.

2-methacryloyloxyethyl phosphorylcholine (MPC) coating
MPC reagent (Lipidure-CR1702, Nihon Yushi, Tokyo, Japan) was diluted 10 times with ethanol and overlaid on coverslips, followed by incubation at 75°C until dried up.

Fabrication of microdevices and loading of cells
A silicon wafer is surface-treated using a mixture of 30% hydrogen peroxide and sulfuric acid at a volume ratio of 1:2, and fluorinated acid. Negative photo-resist SU-8 (Micro Chem) is then spin-coated for ten seconds at 1,000 rpm, heated to 65°C for 60 minutes and to 95°C for 60 minutes, then cooled to room temperature. The SU-8-coated wafer is irradiated by ultraviolet (UV) light at 300 W/cm$^2$. UV light through a photo-mask for 80 seconds, and heated 95°C for 60
minutes. Followed by infiltration filtered in 1-methoxy-2 propylacetone for 60 minutes, SU-8 is removed from the non-irradiated part of the wafer, and a cast plate is obtained by coating the entire wafer with trifluorocarbon. A mixed solution of PDMS polymer: cross linker at a volume ratio 10:1 is poured on the cast plate, which is heated to 75 °C for 60 minutes to yield a solid PDMS. After the solid PDMS is peeled off the cast plate and the PDMS is surface treated with O2 plasma for 10 seconds followed by permanent bounding to the glass coverslips. A silicon tube with an internal diameter of 1 mm is installed on the two corners of the devices to make inlet and outlet. The chamber spaces were coated with MPC.

RESULTS AND DISCUSSION

The efficiency of cell-to-cell adhesion was checked using a combination of avidinylated and biotinylated human hepatoma Hep G2 cells as a model case. An avidinylated cell and a biotinylated cell were trapped by optical tweezers. Each cell was contacted and holed for 1 second, 5 seconds, 1 minute or 5 minutes. After holding, we tried to separate the contacted cells (Figure 2-A). Thirty pairs were tested in each condition (w/ and w/o ABBS). ABBS-dependent cell-to-cell adherence occurred within one second whereas just one pair was able to bind stably without ABBS (Figure 2-B). We needed to wait 5 minutes to bind intact cells firmly (Figure 2-B). ABBS increased the efficiency of cell adherence at least 30 times higher than the native cell-to-cell adhesion machinery. This result clearly shows that ABBS-dependent adherence is very useful in efficient cell assembly.

We demonstrated micro-tissue assembly in a microfluidic device, which consists of a chamber of 10 mm × 10 mm × 0.5 mm in size (Figure 3-A). The internal surfaces of the chamber were coated with MPC polymer to prevent cell adhesion to the glass or PDMS. We applied avidinylated and biotinylated cells sequentially into the device and distinguished them before permanent binding to the glass coverslips. As shown in Figure 3-B to D, we were able to make various micro-tissues having single line, double lines or circle. The most important thing is that the micro tissues did not adhere on the surface of device but they were floating in the devices. The micro-tissues can therefore be transferred or cultured as necessary.

Micro-tissues were cultured on a tissue culture plate to verify that manipulated and adhered cells were still alive. At least after 24 hours of culture, they adhered and spread onto the surfaces (Figure 4). These results clearly show that the combination of ABBS and optical tweezers in micro device is useful in precisely assembling micro-tissue having a well-controlled structure. This new method can be used for cell-based tests or fundamental biological research.

CONCLUSION

We have confirmed that ABBS is useful in adhering multiple cells directly. We also clarified that adherence between cells occurs within 1 second. Combined with ABBS, single cell manipulation such as is done with optical tweezers is a powerful tool in fabricating cell-based micro tissues having precisely arranged cells. These methods should be useful in cell-based applications in both basic and applied research.

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Figure 1 Schematic representation of the new method based on ABBS and optical tweezers
(A) An avidin protein consists of four subunits and each of them strongly binds one biotin molecule. (B) Avidin or Biotin molecules were introduced on the surface of cells to accomplish direct cell-to-cell binding.

Figure 2 Quick binding of cells by ABBS
(A) Single cell manipulation enabled us to check whether two cells (an avidinylated and a biotinylated cell or two intact cells) can bind each other within the indicated time. (B) ABBS helped cell-to-cell direct binding within 1 sec., whereas intact cells needed 5 min to adhere each other.

Figure 3 Assembled cells in the microfluidic device
(A) The microfluidic device which consists of a chamber 10 mm × 10 mm × 0.5 mm in size. (B), (C) and (D) Avidinylated and biotinylated cells were assembled with optical tweezers in the microfluidic device.

Figure 4 Cultivation of assembled cells
(A) Floating assembled cells on a tissue culture plate just before cultivation. (B) After 24 hours, assembled cells were able to attach to the culture plate.