**TITLE:**

Manipulating Living Cells to Construct Stable 3D Cellular Assembly without Artificial Scaffold

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**KEYWORDS:**

Manipulation; Polymer Solution; 3D Cellular Assembly; Optical Tweezers; Remote Control; Regenerative Medicine; Depletion Effect

**SHORT ABSTRACT:**

We demonstrate a novel method for constructing a single-cell-based 3-dimensional (3D) assembly without an artificial scaffold.

**LONG ABSTRACT:**

Regenerative medicine and tissue engineering offer several advantages for the treatment of intractable diseases, and several studies have demonstrated the importance of 3-dimensional (3D) cellular assemblies in these fields. Artificial scaffolds have often been used to construct 3D cellular assemblies. However, the scaffolds used to construct cellular assemblies are sometimes toxic and may change the properties of the cells. Thus, it would be beneficial to establish a non-toxic method for facilitating cell–cell contact. In this paper, we introduce a novel method for constructing stable cellular assemblies by using optical tweezers with dextran. One of the advantages of this method is that it establishes stable cell-to-cell contact within a few minutes. This new method allows the construction of 3D cellular assemblies in a natural hydrophilic polymer and is expected to be useful for constructing next-generation 3D single-cell assemblies in the fields of regenerative medicine and tissue engineering.

**INTRODUCTION:**

While human tissues are composed of several assemblies of cells and can help to maintain homeostasis of the body, single cells by themselves also play important roles *via* cell-to-cell　interaction. Therefore, it is important to elucidate how single cells can be stimulated by external signals and how they transfer such signals to other adherent cells. For this purpose, several methods have been established for the construction of single-cell-based 3-dimensional (3D) assemblies1-8. However, the materials that are used to construct cellular assemblies can still be improved. For example, synthetic gels and polymers including polyethylene glycol (PEG) possess certain chemical physicochemical properties and may affect target cells (*e.g.,* toxicity).

We recently reported a novel system that could generate a single-cell-based 3D assembly of cells using dextran (DEX) by establishing stable cell–cell contact9. We considered that this technology could be useful in several research fields, including regenerative medicine and even cancer biology. In this report, we describe how we manipulate single cells and construct 3-dimensional (3D) cellular assemblies in the presence of various hydrophilic biomacromolecules including DEX without an artificial scaffold.

**PROTOCOL:**

1. **Preparation of Cells**
   1. Maintain NAMRU mouse mammary gland epithelial cells (NMuMG cells) with 5 mL of D-MEM containing 10% (v) fetal bovine serum (FBS) and 1% (v) Penicillin-Streptomycin (P/S) in a 25 cm3 flask. Remove Dulbecco’s modified Eagle’s medium (D-MEM), containing 10% FBS and 1% P/S.
   2. Add 3-5 mL of 37 °C phosphate-buffered saline (PBS) (-) to the flask. Note that the pH of PBS is 7.1-7.3.
   3. Remove all the PBS from the flask using an aspirator.
   4. Add 1.5 mL of 37 °C trypsin (0.25%, w/v) to the flask.
   5. Incubate the flask for approximately 5 minutes at 37 °C in a CO2 incubator.
   6. Add 3.5 mL of D-MEM containing 10% FBS and 1% P/S to the flask. Pipette to mix.
   7. Transfer the cell suspension to a 15 mL centrifuge tube and centrifuge it for 3 min at room temperature. Note that the rotation radius and rotation speed of the centrifuge are 16.6 cm and 1500 rpm, respectively. Under this condition, the relative centrifugal force is 417 x g.
   8. Add 5 mL of fresh D-MEM (10% FBS and 1% P/S) to the flask after aspirating the medium, and then cryopreserve the cells with cryopreservationsolution, following the manufacturer's directions.
2. **Preparation of Dextran (DEX)**
   1. Prepare 80 mg/mL of DEX solution by mixing 10 mL of D-MEM (10% FBS, 1%P/S) and 0.8g of DEX. Note that the DEX solution can be filtered with a syringe filter (0.22 µm) after the cells have cultured for a sufficient time.
   2. Prepare a cell suspension containing 40 mg/mL DEX medium by mixing 200 μL of DEX solution and 200 μL of the cell suspension prepared in 2.1. Note that the number density of cells in the solution is approximately 2.3×105 cells/mL.
3. **Preparation for Laser and Microscopy**
   1. Turn on the laser (continuous wave, 1064 nm wavelength) (**Figure 1a**). Note that the use of a laser beam with a wavelength in the red to near-infrared region is most effective; this wavelength region is called the diagnostic and therapeutic window10 since it is minimally absorbed by cells.
   2. Double-click the software icon.
   3. Double-click the icons for the ① camera, ② light-emitting diode (LED), ③ focus adjust, and ④ moving stage. The displays corresponding to ①-④ will show up (**Figure 1b**).
4. **Cell Manipulation using the Laser Trapping System**
   1. Place 20 μL of the sample prepared in step 2.2 on the bottom cover glass (0.17 mm thickness, size = 30 mm × 40 mm), and cover it with the top cover glass (0.17 mm thickness, size = 18 mm × 18 mm), with separation provided by two spacers (0.17 mm thickness, size = 10 mm × 24 mm), as shown in **Figure 2**. Note that these glasses do not require a special coating.
   2. Place the sample cell prepared in step 4.1 on the lower objective Lens (water immersion, magnification = 60X, working distance = 0.28 mm, numerical aperture = 1.2) *via* distilled water (ca. 10 μL).
   3. Attach the upper objective lens (water immersion, magnification = 60X, working distance = 2 mm, numerical aperture = 1.0) at the top of the sample cell *via* distilled water (ca. 10 μL).
   4. Turn on the LED light by clicking icon 2 (**Figure 1b**).
   5. Adjust the distance between the sample and the lower objective lens by clicking the icons on panel 3 (**Figure 1b**) until it is in focus.
   6. Irradiate the laser beams at Position 1 and Position 2 (**Figure 1B**) of the sample by clicking icons I, II, and III (**Figure 1b**).
   7. Set the intensity of each laser beam to 1500 mW by entering this value at icon IV (**Figure 1b**).
   8. Move the sample stage by clicking icon 4 indicating directions (**Figure 1b**) until a cell is trapped at Position 1 (**Figure 1b**).
   9. Drag the cursor indicating Position 2 (**Figure 1b**) until another cell is trapped at Position 2.
5. **Construction of a 3-Dimensional (3D) Cell Structure**
   1. Manipulate a single cell so that it is in contact with another cell. Maintain this condition for 300 s; *i.e.,* each cell is exposed to a laser for 300 s.
   2. Record the X-Y axis shown in **Figure 1b**.
   3. Construct an arbitrary 2D cell assembly by trapping and transporting another cell to the cells.
   4. Construct a 3D cellular assembly by moving the stage up and down.
   5. Confirm that the assembly remains stable after the laser is switched off.

**REPRESENTATIVE RESULTS:**

**Figure 1** shows the microscope and software used in this study. **Figure 2** is a schematic representation of the procedure for placing the sample solution containing cells. **Figure 3** demonstrates the formation of a pyramid structure using double-beam optical tweezers. If the experiment is successful, these cellular assemblies remain stable even after the laser is switched off.

**Figure 1: (a)** The control system for the Laser Trapping System (NanoTracker2 (11)). The system is activated by turning on the laser switch following steps ①-③. **(b)** The software for controlling the Laser Trapping System. The camera, LED light, focus adjust, and moving stage are activated by clicking icons ①, ②, ③, and ④, respectively. The microscopic image is displayed in panel 1. The on/off control for the LED is in panel 2. The focus is controlled in panel 3. The laser beams are irradiated at Positions 1 and 2 by clicking icons I to IV. The details of this Laser Trapping Systemare provided in Ref. (12).

**Figure 2:** Representative schematic for placing the slide glass. 20 μL of the sample (cell suspension containing dextran) is placed on the slide and used for laser manipulation.

**Figure 3: a)** Assemblies of epithelial cells (NMuMG) of an intended shape in a medium with DEX (40 mg/mL): a pyramid is shown as an example of a 3D cluster. **b)** A schematic figure of the pyramid-shaped 3D cellular assembly is also shown.

**DISCUSSION:**

The present study shows a concrete application of our recent reports9,11 on the use of soluble polymers for the construction of 3D single-cell assemblies. Such assemblies are stably formed in the bulk solution when the number of cells is up to 10, and can be held by a single laser beam. Assemblies precipitate on the glass surface when there are more than 10 cells. Although the experiments are still in a primitive stage, we expect that the novel methodology could be a powerful tool for the construction of next-generation 3D single-cell assemblies, which are indispensable for progress in the fields of cell biology and regenerative medicine.

In a solution containing no polymer, cells repel each other due to the electrostatic repulsion arising from the surface charge, the hydration repulsion force, the glycocalyx repulsion effect, and membrane undulation. Our previous study showed that cell pairs can be stable for a long time when the cells are treated with PEG. More importantly, the successful transport of a cell pair to a region without PEG, after the cells had been held in contact for 5 minutes in PEG, suggests that cellular contact is maintained in a stable manner. This is well explained in terms of the depletion effect11, and essentially the same mechanism applies to the cellular assemblies generated using DEX9. Our current results suggest that other kinds of natural macromolecules could also be used to construct stable 3D cellular assemblies.

For the prompt transport of cells, the concentration of polymer is important. Generally, the viscosity of the solution drastically increases when the polymer is dissolved above the overlap concentration. Under this condition, it is difficult to manipulate cells using optical tweezers. Hence, the experiment should be performed below the overlap concentration. For a DEX solution, the overlap concentration is ca. 50 mg/mL (the kinetic viscosity is 5.5 mm2/s). As shown in Ref. 9, a stable cellular assembly was observed when the concentration of DEX was 10 mg/mL to 40 mg/mL. This result suggests that the depletion effect is sufficiently large to maintain stable cell-cell contact even when the DEX concentration is lower than the overlap concentration. It has been shown that the addition of DEX does not affect cell viability up to 40mg/mL 9.

The establishment of a method for the construction of 3D cellular assemblies is important in the field of regenerative medicine, since mimicking an *in vivo* cellular microenvironment by structuring single cells may facilitate stem cell-derived tissue formation. So far, we have used the present protocol to construct cellular assemblies using Neuro2A cells9 in addition to NMuMG cells. We hope to establish an experimental methodology for constructing 3D cellular assemblies of a larger number of cells of various morphologies. The optical tweezers system developed by Ichikawa *et al.*13 seems to be suitable for this purpose since the orientation of the cells can be controlled. Further trials along these lines should be promising.

**ACKNOWLEDGMENTS:**

The authors thank Shu Hashimoto, Aoi Yoshida, and Taeko Ohta at Doshisha University for their generous assistance with the experimental setup. This work was supported by KAKENHI (15H02121, 15K05400, 25103012, 50587441) and by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities. This study was also supported by a Polish grant from the KNOW (Leading National Research Centre) Consortium "Healthy Animal - Safety Food" (MS&HE; Decision No. 05-1 / KNOW2 / 2015).

**DISCLOSURES:**

The authors have nothing to disclose.

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