Journal of Visualized Experiments

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

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57815R1
Full Title:	Manipulating Living Cells to Construct Stable 3D Cellular Assembly without Artificial Scaffold
Keywords:	manipulation; polymer solution; 3D cellular assembly; Optical tweezers; remote control; Regenerative Medicine; depletion effect
Corresponding Author:	Koichiro Sadakane, Ph.D. Doshisha University Kyotanabe, Kyoto JAPAN
Corresponding Author's Institution:	Doshisha University
Corresponding Author E-Mail:	sadakanekoichiro@gmail.com
First Author:	Shoto Tsuji
Other Authors:	Shoto Tsuji
	Hiroaki Taniguchi
	Shiho Sato
	Takehiro Yamazaki
	Takahiro kenmotsu
	Kenichi yoshikawa
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

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

AUTHORS:
Shoto Tsuji[#,1]
t.shoto.bmm1109@gmail.com

Hiroaki Taniguchi[#2]
h.taniguchi@ighz.pl

Shiho Sato[1]
limmip6613@gmail.com

Takehiro Yamazaki[1]
t.yamazaki13lifephsy@gmail.com

Takahiro Kenmotsu[1]
tkenmots@mail.doshisha.ac.jp

Kenichi Yoshikawa[1]
keyoshik@mail.doshisha.ac.jp

Koichiro Sadakane[1]
ksadakan@mail.doshisha.ac.jp

1: Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Japan
2: The Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Postepu, Jastrzebiec, Poland

#These authors are contributed equally

CORRESPONDING AUTHOR:
Koichiro Sadakane

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) assemblies[1-8]. 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 contact[9]. 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

1.1. 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 cm^3 flask. Remove Dulbecco's modified Eagle's medium (D-MEM), containing 10% FBS and 1% P/S.

1.2. 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.

1.3. Remove all the PBS from the flask using an aspirator.

1.4. Add 1.5 mL of 37 °C trypsin (0.25%, w/v) to the flask.

1.5. Incubate the flask for approximately 5 minutes at 37 °C in a CO_2 incubator.

1.6. Add 3.5 mL of D-MEM containing 10% FBS and 1% P/S to the flask. Pipette to mix.

1.7. 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.

1.8. 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 cryopreservation solution, following the manufacturer's directions.

2. Preparation of Dextran (DEX)

2.1. 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.

2.2. 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 2.3×10^5 cells/mL.

3. Preparation for Laser and Microscopy

3.1. 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 window[10] since it is minimally absorbed by cells.

3.2. Double-click the software icon.

3.3. 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

4.1. 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.

4.2. 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).

4.3. 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).

4.4. Turn on the LED light by clicking icon 2 (**Figure 1b**).

4.5. 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.

4.6. Irradiate the laser beams at Position 1 and Position 2 (**Figure 1B**) of the sample by clicking icons I, II, and III (**Figure 1b**).

4.7. Set the intensity of each laser beam to 1500 mW by entering this value at icon IV (**Figure 1b**).

4.8. Move the sample stage by clicking icon 4 indicating directions (**Figure 1b**) until a cell is trapped at Position 1 (**Figure 1b**).

4.9. 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**

5.1. 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.

5.2. Record the X-Y axis shown in **Figure 1b**.

5.3. Construct an arbitrary 2D cell assembly by trapping and transporting another cell to the cells.

5.4. Construct a 3D cellular assembly by moving the stage up and down.

5.5. 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 System are 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 reports[9,11] 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 effect[11], and essentially the same mechanism applies to the cellular assemblies generated using DEX[9]. 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 mm^2/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 cells[9] 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.

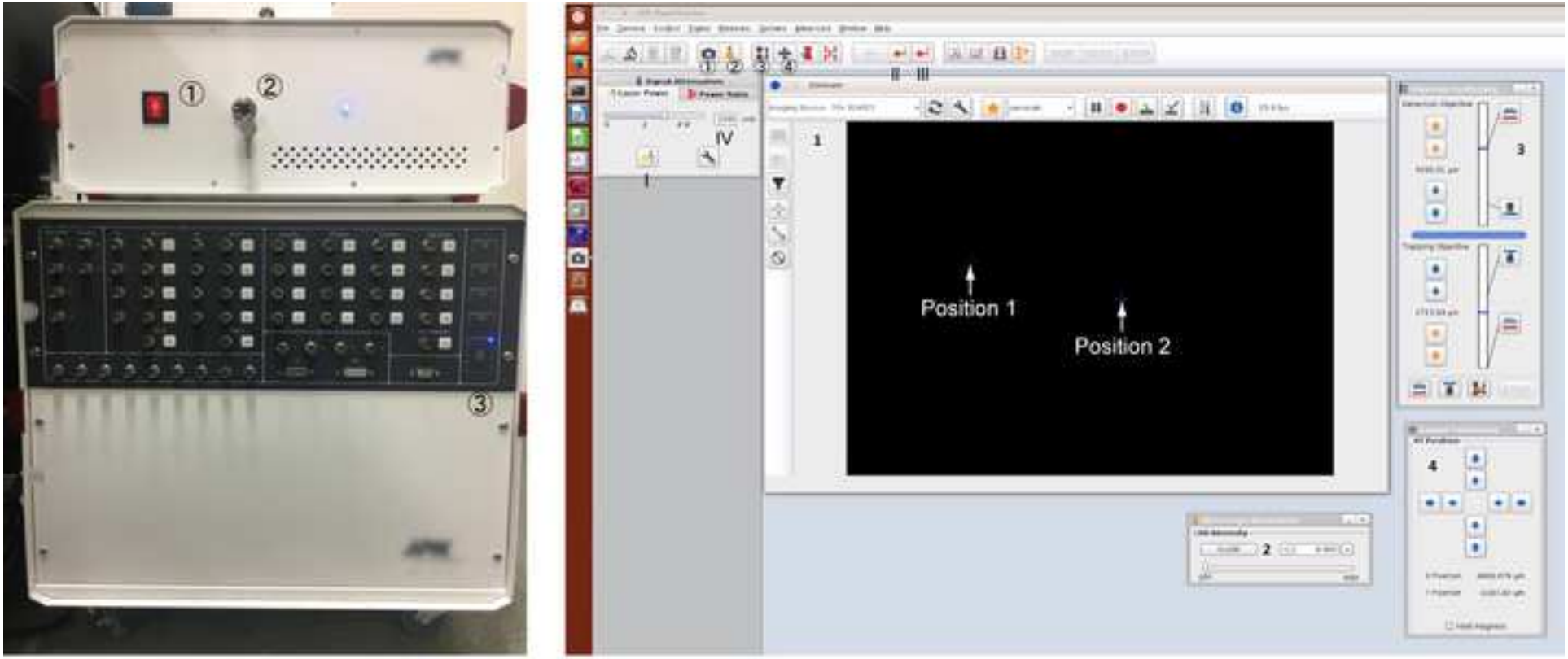
REFERENCES

1. Aloysious, N. & Nair, P.D. Enhanced survival and function of islet-like clusters differentiated from adipose stem cells on a three-dimensional natural polymeric scaffold: an *in vitro* study. *Tissue Engineering Part A* **20** (9-10), 1508-1522 (2014).
2. Fatehullah, A., Tan, S.H. & Barker, N. Organoids as an in vitro model of human development and disease. *Nature Cell Biology*. **18** (3), 246-254 (2016).
3. Kirkham, G.R., *et al.* Precision assembly of complex cellular microenvironments using holographic optical tweezers. *Scientific Reports*. **5**, 8577 (2015).
4. Liu, Y., Rayatpisheh, S., Chew, S.Y. & Chan-Park, M.B. Impact of endothelial cells on 3D cultured smooth muscle cells in a biomimetic hydrogel. *ACS Applied Materials & Interfaces*. **4** (3), 1378-1387 (2012).
5. Liu, Z.Q., *et al.* Dextran-based hydrogel formed by thiol-Michael addition reaction for 3D cell encapsulation. *Colloids and Surfaces B*. **128**, 140-148 (2015).
6. McKee, C., Perez-Cruet, M., Chavez, F. & Chaudhry, G.R. Simplified three-dimensional culture system for long-term expansion of embryonic stem cells. *World Journal of Stem Cells*. **7** (7), 1064-1077 (2015).
7. Sadovska, L., *et al.* A novel 3D heterotypic spheroid model for studying extracellular vesicle-mediated tumor and immune cell communication. *Biochemical and Biophysical Research Communications* (2017).

8. Srinivasan, P.P., *et al.* Primary Salivary Human Stem/Progenitor Cells Undergo Microenvironment-Driven Acinar-Like Differentiation in Hyaluronate Hydrogel Culture. *Stem Cells Translational Medicine*. **6** (1), 110-120 (2017).
9. Yoshida, A., *et al.* Manipulating living cells to construct a 3D single-cell assembly without an artificial scaffold. *Polymers*. **9,** 319 (2017).
10. Anderson, R.R, & Parrish, J.A., Selective photothermolysis: precise microsurgery by selective absorption of pulsed radiation. *Science*. **220**, 524-527 (1983).
11. Hashimoto, S., *et al.* Formation of Stable Cell-Cell Contact without a Solid/Gel Scaffold: Non-invasive Manipulation by Laser under Depletion Interaction with a Polymer. *Chemical Physics Letters*. **655**, 11–16 (2016).
12. Rauch, P. & Jähnke, T. Optical Tweezers for Quantitative Force Measurements and Live Cell Experiments. *Microscopy Today*. 22, 26-30 (2014).
13. Ichikawa, M., et al. Tilt control in optical tweezers. *Journal of Biomedical Optics*. **13**, 010503 (2008).

Figure1

Click here to download Figure Figure1.tif



(a) (b)

Figure2

Click here to download Figure Figure2.tif

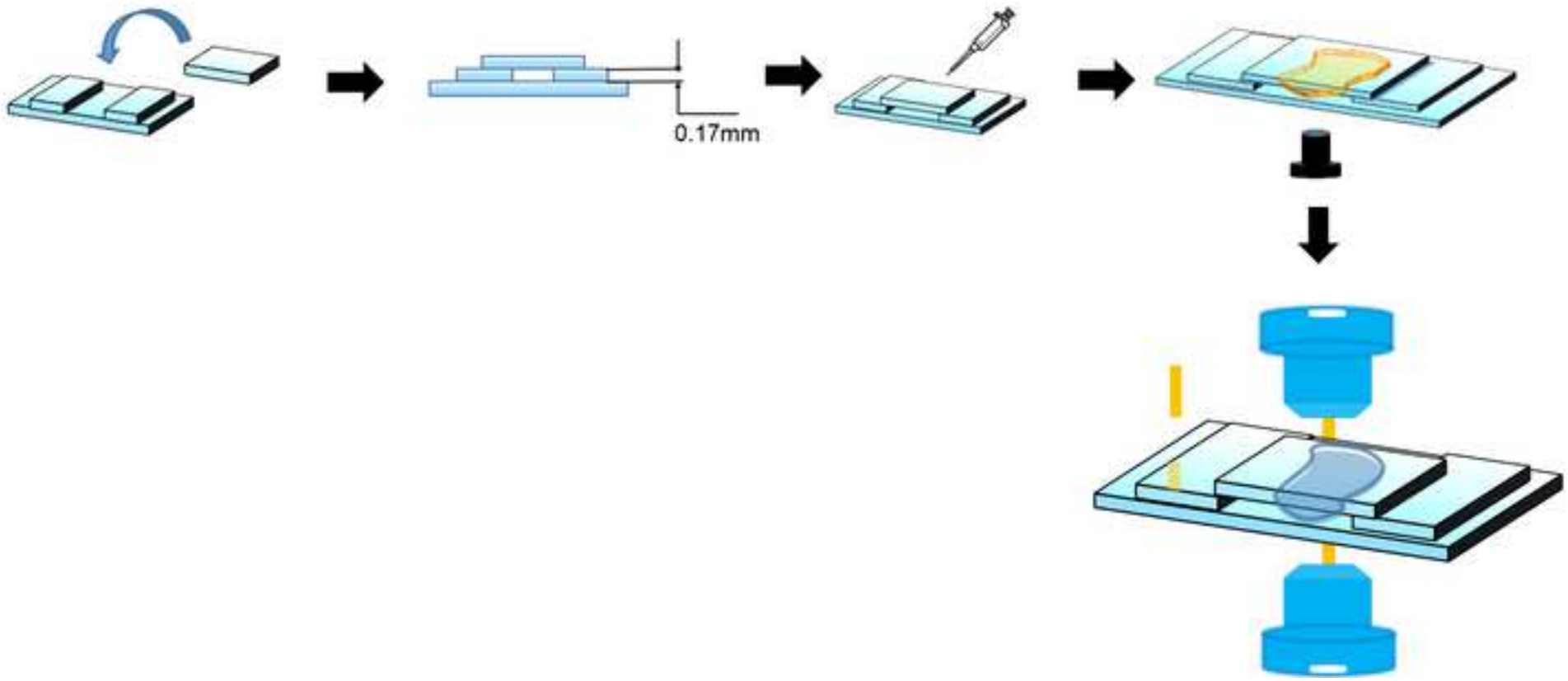


Figure3

Click here to download Figure Figure3.tif

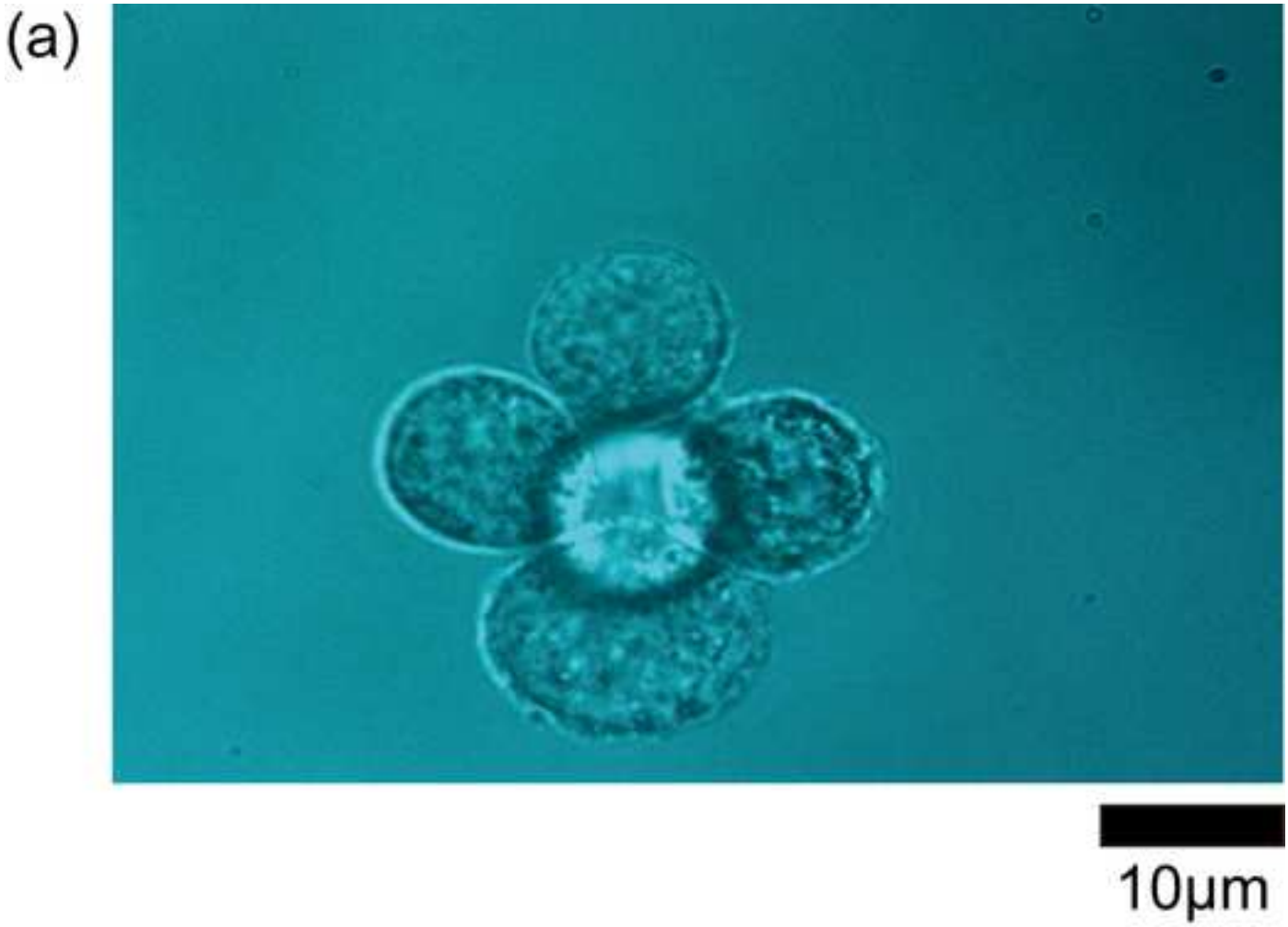


Table of Materials

Click here to download Table of Materials Materials.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
Microscope IX71	Olympus	IX71
Dextran(200,000; molecular biology-grade)	Wako	CAS.NO 9004-54-0
Laser Trapping System (NanoTracker 2)	JPK Instruments	S/N T-05-0200
Upper Objective Lens	Olympus	LUMPLFLN60XW
Lower Objective Lens	Olympus	UPLSAPO60XW
Top Cover Glass	MATUNAMI	C022401
Intermediate Cover Glass (Spacer)	MATUNAMI	-
Bottom Cover Glass	MATUNAMI	C030401
Camera	The Imaging Source	DFK 31AF03
		NanoTracker2 PFM
Software	JPK Instruments	software
NMuMG cells	RIKEN BRC	RCB2868
PBS	Wako	166-23555
	Nippon Zenyaku	
Cell banker	Kogyo	ZR621
	Wako Pure Chem.	
D-MEM	Ind., Japan	044-29765
	Cell Culture Biosci.,	
	Nichirei Biosci. Inc.,	
FBS	Japan	172012-500ML
	Thermo Fisher	
Trypsin	Scientific	25200056
	Wako Pure Chem.	
Penicillin-Streptomycin	Ind., Japan	161-23181

	Comments/Description
custom-made (size = 10mm×10mm, thickness = 0.17mm)	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Manipulating Living Cells to Construct Stable 3D Cellular Assembly without Artificial Scaffold

Author(s): Shoto Tsuji, Hiroaki Taniguchi, Shiho Sato, Takehiro Tamazaki, Takahiro Kenmotsu, Kenichi Yoshikawa, Koichiro Sadakane

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☐ Standard Access ☑ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Koichiro Sadakane

Department: Faculty of Life and Medical Sciences

Institution: Doshisha University

Article Title: Manipulating Living Cells to Construct Stable 3D Cellular Assembly without Artificial scaffold

Signature: Koichiro Sadakane Date: 31/12/2017

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dear Editor, Reviewer#2 and Reviewer#3,

Thank you very much for your fruitful comments. We summarize the revisions in response to your suggestions as follows.

Reply to the Editorial Comments

1-1
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have revised the spelling and grammar issues with the aid of the English editing service.

1-2
2. Please use SI units, e.g. please use “mL” instead of “ml”, “°C” instead of “C”. Please leave a white space between the values and the units.

We have corrected these points.

1-3
3. Please define all abbreviations before use.

We have defined all abbreviations before use.

1-4
4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

We have removed all commercial language from our manuscript, and sufficiently referenced in the table.

1-5
5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

In the protocol section, we have corrected all sentences to imperative form.

1-6
6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol steps should contain only 2-3 actions per step and a

maximum of 4 sentences per step.

We have confirmed that there is no problem.

1-7

7. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have added the Ref. (12) following this suggestion.

1-8

8. For steps that involve software or analyzing tools, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. This is the level of detail we’re looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.

We have corrected these points.

1-9

9. Please leave a blank line between all protocol steps as well as Notes.

We have left a blank line between all protocol steps.

1-10

10. Protocol: 1.1: How did you obtain and maintain NMuMG cells?

We have described the details in the protocol 1.1 and the table.

1-11

11. Protocol: 1.2: Please clearly describe this step. What is the container? How much is the pH of the buffer? How much is the volume of the buffer? Please use the SI units.

We have corrected the sentence to clarify this procedure. Additionally, we have added the details of PBS to the table.

1-12

12. Protocol: 1.3: Please rephrase this step for more clarity. How this step is done?

We have corrected the sentence to clarify this procedure.

1-13

13. Protocol: 1.4-1.7: Please use the SI units as instructed in comment 2.

We have corrected the sentences in 1.4-1.7 using SI units.

1-14
14. Protocol: 1.5: Please use the imperative tense for all actions in the protocol.

We have corrected the sentence to imperative form.

1-15
15. Protocol: 1.6: Please clearly describe this step (i.e., Add to what?)

We have corrected the sentence to clarify this procedure.

1-16
16. Protocol: 1.8: Please use the imperative tense for all protocol steps.

We have corrected the sentence to imperative form.

1-17
17. Protocol: 2, 3, 4: Please use the imperative tense for all steps in the protocol. Please follow the instructions in comment 2 for the units.

We have corrected the sentences to imperative form

1-18
18. Protocol: 5.1: Please clearly describe this step. How it is done? Which instruments are used?

Details on the operation of the laser unit have been described in the protocol 3.1-5.5. The instrument's name in the present study is "NanoTracker2". We have specified this in the caption of Fig. 1. Additionally, we have cited the Ref. (11) for more detailed information on NanoTracker2.

1-19
19. Protocol: 5.2-5.4: Please use the imperative tense for all the sentences in protocol steps. If a sentence is a note please indicate it as Note.

We have corrected the sentences to imperative form.

1-20
Representative results:
20. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

We have clarified this point in the protocol 5.5 and the last part of REPRESENTATIVE RESULTS.

1-21
21. Please upload each Figure individually to your Editorial Manager account as a .png, .pdf, or a .tiff file.

We have divided all figures (tiff format) one by one.

1-22
22. Figure 1: Please blur the brand name in panel (A). Please combine all panels of one figure into a single page.

We have applied a mosaic to the brand name. Additionally, we have combined all panels of one figure into a single page.

1-23
23. Figure 3: Please add the scale bar to this figure.

We have added the scale bar in Fig. 3.

1-24
24. If you are reusing figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [AUTHOR] et al.[REFERENCE]".

We have confirmed that there is no problem.

Discussion:
1. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have confirmed that there is no problem.

1-25
25. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

We have revised the table following your suggestions.

Reply to the comments by Reviewer #2:

Manuscript Summary:

The authors present a method for 3D assembly of cells by bringing them in stable cell-cell contact in presence of hydrophilic bio-macromolecules such as dextran. They use optical tweezers for the manipulation of cells to form an assembly. Though, the construction and operation of optical tweezers as a single particle manipulation tool has been well described in the literature, it is still necessary to provide a much more detailed information about optical tweezers instrumentation and its operation in the context of the current experiment. I would recommend at least the following changes be made in the manuscript to make it suitable for publication in this method journal.

2-1
Minor Concerns:
Comment1: What laser wavelength and power was used for the experiment? Mention suitable range of wavelength and laser power that can be used for the experiments?

The laser power and the wavelength have been shown in the protocol 4.7 and 3.1, respectively.

2-2
Comment 2: Specification of some important components of optical tweezers such as the objective lens should be mentioned.

We have specified the important components of the instrument in the protocol 4.2, 4.3, and the table.

2-3
Comment 3: The assembly of the glass slide containing the cell should be presented in more detail. More specific information on glass slide thickness and spacers used in the experiment should also be presented.

We have described the specific information on the slide glass and the spacer in the protocol 4.1 and the table.

2-4
Comment4: What is the physical size of the trapped cells? Provide a scale bar in figure 3a.

We have added the scale bar in Fig. 3.

2-5
Comment 5: What is the range of polymer concentrations that can be used to form stable cellular contacts without causing any problem to laser manipulation.

We have confirmed that the cellular contacts are formed stably when the concentration of DEX is 10 – 40mg/mL. We have described this point in DISCUSSION, as “As shown in Ref. (9), a stable cellular assembly was observed when the concentration of DEX was 10mg/mL to 40mg/mL.”.

2-6
Comment 6: The author mentions they use optical tweezer for manipulation of cells. Explain in detail how the laser tweezer was built. Was it a commercial system or customized in the laboratory for the experiments?
How are the two cells brought in contact and for how long they are kept in contact before they adhere to each other.

The optical tweezer system (NanoTracker2) used in the present study is manufactured by JPK Instruments. Instead of describing the details of the instrument, we have cited the Ref. (12).

2-7
Comment 7: How is the twin laser beam created?

As mentioned above, the details are shown in the Ref. (12).

2-8
Comment 8: Can the authors comment on the viability of the cells using this technique?

The viability of the cell under the present experimental condition has been described in the Discussion, as “It has been shown that the addition of DEX does not affect cell viability up to 40mg/mL (9).”.

2-9
Comment 9: Is the entire assembly of cells held by a single focused laser beam or attached to a surface?

We have clarified this point in DISCUSSION, as “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.”.

Reply to the comments by Reviewer #3:

Manuscript Summary:
The manuscript describes a protocol for assembling single cells to three-dimensional cell clusters by applying optical tweezers. It is briefly described how single cells are assembled in an artificial environment consisting of dextran and growth medium. The protocol is based on the research

article "Manipulating living cells to construct a 3D single-cell assembly without an artificial scaffold by Yoshida et al., 2017". The research article is very interesting, however the protocol described in this manuscript is rather on a lower scientific level. Therefore, it can't be recommended for publication without major revision.

3-1
Major Concerns:
The here described protocol is related to a highly specialized application. It would be helpful to see more general applications, by using different cell lines, different lasers (wavelength) and shapes.

As for the general applications and using different lasers, we have described in the last paragraph of DISCUSSION. As for the wavelength of laser, near infrared rays are suitable for the present experiment since near-infrared rays have high permeability to cell membranes. We have clarified this point in the protocol 3.1.

3-2
Figure 1: I don't see how this figure provides supporting information. Please provide an overview of more essential information, which allow the reader to repeat the assay. Which laser power, objective lenses, wavelength, exposure time?

The laser power, the details of the objective lens, wavelength, and exposure time have been shown in the protocol 4.7, 4.2-4.3, 3.1, and 5.1, respectively.

3-3
Line: 188-189: The term "organoids "is not correct in this context. Organoids are defined to be capable to undergo self-renewal and self-organization and do not require the assembly by optical tweezers.

We have deleted the term “organoids” in the manuscript.

3-4
Line 137-140: "Manipulate a single cell and adhere it to another cell." A figure showing how the cell is manipulated and how the cell is trapped would be beneficial for a deeper understanding of the procedure.

We have explained how the cell is trapped in the protocol 4.8 and 4.9.

3-5
In the discussion and introduction, regenerative medicine and tissue engineering are mentioned as a motivation for three-dimensional cell biology and in particular the application of the here described method. However, the reasoning behind this motivation is missing. It would be nice to give the reader a vision of how this method improves regenerative medicine, e.g. building tube-like structures from endothelial cells to form blood vessel-like structures in-vitro.

Following this suggestion, we have added the sentence in the last paragraph of DISCUSION, as “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.”.

Minor Concerns:

3-6

Line 115-117: The preparation of the dextran solution is described. Please state whether a sterilization step is required. Please provide a detailed information regarding the viscosity.

The DEX solution can be filtrated with a syringe filter (0.22μm) when the cells are cultured for a long time. As for the viscosity, we have described in DISCUSSION, as “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. 50mg/mL (the kinetic viscosity is 5.5 mm^2/s).”.

3-7

Line 111, 112 and 117: The preparation of the cell suspension is described. Please provide further information regarding the final cell number/concentration.

We have shown the number density of the cells in the solution (2.3×10^5 cells per 1mL) in the protocol 2.2.

3-8

Line 128: Does the glass slides require further treatment? Thickness, quality, pre-treatment?

We have clarified these points in the protocol 4.1.

3-9

Line 130, line 131: How is the "focus of the microscope" adjusted? A figure, showing the adjusted focus would provide a more detailed understanding of the procedure.

We have explained how the focus is adjusted in the protocol 4.5.

3-10

-An explanation why dextran and not only medium is needed for this assay would be helpful.

In the absence of polymers (DEX), stable cell assemblies cannot be formed since cells repel each other. We have described this point in DISCUSSION, as “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.”.

3-11
Figure 3: A scale bar is missing.

We have added the scale bar in Fig. 3.