**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 mm2/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 (2.3×105 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.