**Editorial comments:**

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*We thank the editor for giving us the opportunity to go through our manuscript again. We have carefully checked the manuscript for any grammatical errors and spelling mistakes and revised it accordingly.*

2. Summary: Please write the Summary in complete sentences.

*We have revised our short summary and re-written it in a full sentence.*

3. Please spell out each abbreviation the first time it is used.

*We have checked all the abbreviations and spelled it out when it has been used for the first time.*

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

*We have checked all the SI units and made changes at places where they were not according to the convention.*

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sylgard, Thinky, Pico Surf, Eppendorf, Cell Trace, etc.

*We understand this concern and have removed all the commercial names in the manuscript and updated it in the Table of Materials and Reagents.*

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

*We thank the editor for highlighting the use of aforementioned phrases. We have checked and edited the protocol accordingly and also, reduced the number of notes.*

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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. Some examples:

1.5: Does the pre-cured PDMS mixture refer to the content in the cup?

*Yes pre-cured PDMS refers to the content in the cup and we have mentioned it in the protocol. (Protocol step 1.5)*

1.6: How large is the petri dish?

*Theoretically, a petri dish of any size can be used as long as it fits the silicon wafer, fabricated for the replica molding process. We use a large circular petri dish and have mentioned its dimension in the protocol. (Protocol step 1.4)*

3.1.2, 3.1.6: What volume of PBS is used to wash? Please specify throughout.

*The volume of PBS used is 1 mL, but the amount of PBS used varies depending on the sample solution. We have mentioned this in the protocol step 3.1.2 and protocol step 3.1.6..*

3.1.5: What volume of ice-cold FCS is added?

*The volume of ice-cold FCS used is 1 mL, but the amount of PBS used varies depending on the sample solution. We have mentioned this in the protocol step 3.1.5.*

3.1.7: Please specify the media used.

*The media used is RPMI and we have updated this information in step 3.1.7*

3.2.2: How many concentrations are prepared?

*We have mentioned in step 3.1.1 that two samples of Jurkat T cells were harvested. Using this we have prepared in step 3.2.2, an agarose solution to yield an agarose concentration of 2%. For both the harvested cell samples, the yielded agarose concentration was 2%.*

3.3.4: Please describe how to analyze using flow cytometry.

*We feel that the large variety of flow cytometers and analysis software that are available make it hard to make a standardized protocol. In the main text and in the figure legend we have added a more balanced description how the analysis was performed.*

8. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

*We have made sure that all the steps are clear and each additional step is mentioned separately. We have also combined some of the smaller steps which are a continuation of the previous step.*

9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

*We have highlighted the parts that we would like to demonstrate in the video including notes, sub-steps and other necessary and relevant information.*

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

*We have highlighted the parts that we would like to demonstrate in the video including notes, sub-steps and other necessary and relevant information.*

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

*We have highlighted the parts that we would like to demonstrate in the video including notes, sub-steps and other necessary and relevant information.*

12. Figures 3-5: Please include a space between numbers and their corresponding units (i.e., 1 M/mL, 2 M/mL, 1.07 mm, etc.). Please define the error bars in the figure legend.

*We thank the editor for highlighting the unit conventions in the figures. We have modified our figures according to the new convention where we have mentioned cell concentration as follows: 1.0E6 cells/mL, etc. We added in the figure legend that the error bars represent the standard error of the mean (SEM).*

13. Figure 6: What does 10M cells mean? Please define the scale bar of panel C in the figure legend.

*In our figure, 10M means actually 10 million cells. As mentioned above, we have updated the writing convention in which we write the cell number and cell concentration (10.0E6 cells). Furthermore, we thank the editor for pointing out the missing scale bar information. We added that the scale bar represents 150 uM.*

14. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

*As suggested, we have removed all the trademark and registered symbol from the table.*

**Reviewers' comments:**

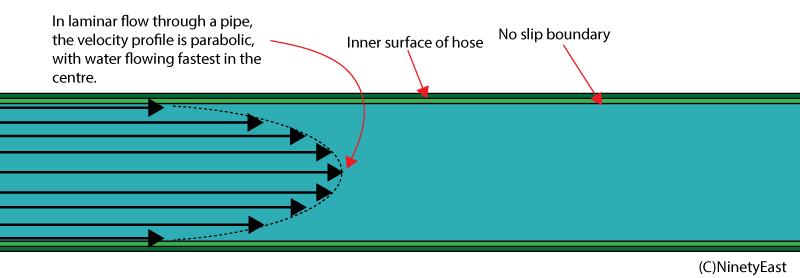
**Reviewer #1:**

Manuscript Summary:

This manuscript has introduced a protocol for droplet microfluidic-based cell seeding which is the most fundamental step for droplet single cell technologies; this is highly valuable in the field. It utilized pipette tips sealed with mineral oil to prevent quick cell sedimentation, which has led to a more uniform cell distribution through the sample injection process. Compared with traditional methods, the reported one is efficient as it can achieve cell encapsulation ratio near that predicted by Poisson distribution. Thus, it is recommended for publication on JoVE. But before acceptance, we have several suggestions:

Major Concerns:

1. The cells are different in density with the cell culture media; thus with the setting shown in figure 3A, it is highly possible that cells distribution within the tube would change gradually with time, leading to nonuniform encapsulation efficiencies over time. We suggest the authors provide more information in this respect.

*We thank the reviewer for the thorough reading of our manuscript and we agree that this point deserves more explanation. The tubing used for cell seeding is of very small dimensions and can be compared to microchannels. The flow in the tubing is pressure driven with a parabolic velocity profile.*

*This implies that the maximum flow velocity is at the center of the tubing and minimum velocity is at the edges of the tubing. When only one cell flows through the channel, the cell should be located somewhat at the center where the velocity is highest. However, when a population of cells flows through the through the tubing, the velocity gradient causes the cells to be pushed towards the edges where they settle or slow down, because the velocity at the boundary is close to zero. Thereby, the sedimentation or settlement of cells in tubing reduces the encapsulation efficiency. This theory of reduced cell encapsulation is supported by the observation that large aggregates of cells enter the device upon reaching the final microliters of the loaded cell suspension. We have updated this information in the manuscript as well.*

Minor Concerns:

1. There are several grammatical mistakes, please double check.

*We thank the reviewer for highlighting this concern. We have cross-checked our manuscript and made sure that the manuscript is free from grammatical mistakes.*

2. Some of the descriptions for the experiment steps are vague to the readers. Please improve them:  
a. In 2.3.8 the total volume of the sample is not clear.

*We thank the reviewer for the concern. We have updated this information in the protocol. Note: This protocol step is now 2.3.7 .We have also updated this information in step 2.3.9 Furthermore, we have checked all protocol steps again to decrease potential ambiguity.*

b. In 3.2.4 note, the speed can be expressed in ul/h. Also, it is quite fast. Thus the total volume of the oil phase might be mentioned.

*As suggested, we have expressed the flow rates in µL/h. For our experiments we use 1-mL syringes. We typically fill the entire syringe with oil. The volume of the oil phase used, however, varies during the experiment and is dependent on the flow rates and the time duration for stable droplet production. With higher flow rates for the oil phase and longer duration for droplet production, the consumption of oil will be higher and vice-versa.*

c. In 3.2.20, the viability of cells incubated under such condition should be testified.

*We agree that this information is important, however the viability and biocompatibility was thoroughly tested in the paper by Chokkalingam, V. et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. Lab Chip 13, 4740 (2013). We have made a reference to this paper in the section that concerns the use of low-melting agarose for cell encapsulation.*

**Reviewer #2:**

Manuscript Number: JoVE57848

A pipette-tip based method for seeding cells to droplet microfluidic platforms

Remarks on protocol:

Protocol  
The protocol steps are clearly written and easy to understand. I have only two small remarks on them that are listed below.

1.14: It is useful to mention the settings of the plasma asher, but you should also reveal the company and model name of the plasma asher used. It is not in the materials and equipment table.

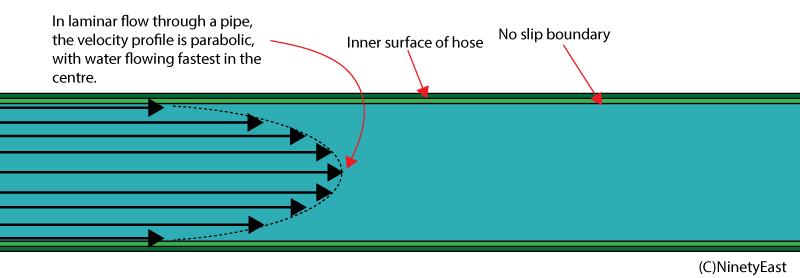
*We have given the most important settings of the plasma asher used here. The other settings are as default and never get modified. (We have also updated the company and model name of the plasma asher in the material list).*

2.2.8. What is your recommended PDMS plug diameter for a 1000 µl pipette tip?

*For 1000 µL pipette tip, plugs of diameter ranging between 5 mm and 7 mm can be used. With a plug of diameter 5 mm, a sample volume of around 400 µL can be aspirated in the pipette tip. If a plug of larger diameter is used (7 mm), more sample volume can be aspirated (around 900 µL). We have added this information to our manuscript.*

Representative Results

The results of the setup in Figure 4 are not completely comprehensible to me. In my understanding the cells should sediment to some extend towards the chip inlet, when they are injected in the tubing on top of the tripod. Thus, the approach is not too different from your presented pipette-tip based technique. However, you seem to have low encapsulation efficiency. Do you have an explanation for this? Have you tried different tube lengths? If you have sticky cells, which tend to the surface of the tubing you could also try to coat the walls with e.g. BSA as an alternative to Teflon tubing. And do you have any explanation for the increased efficiency in your pipette-tip based loading method?

*We agree with the reviewer that this point deserves more explanation. The tubing used for cell seeding is of very small dimensions and can be compared to microchannels. The flow in the tubing is pressure driven with a parabolic velocity profile. This implies that the maximum flow velocity is at the center of the tubing and minimum velocity is at the edges of the tubing. When we have only one cell flowing through the channel, the cell should be located somewhat at the center where the velocity is highest. However, when a population of cells flows through the through the tubing, the velocity gradient causes the cells to be pushed towards the edges where they settle down because the velocity at the boundary is close to zero. The sedimentation or settlement of cells in tubing reduces the encapsulation efficiency. This theory of reduced cell encapsulation is supported by the observation that large aggregates of cells enter the device upon reaching the final microliters of the loaded cell suspension. Previously, we have tried loading cells via tubing either positioned horizontally or vertically and also tried various tube lengths. In these experiments there was no clear benefit in both the tube position and length. Furthermore, the length of the tubing that we used depended on our experimental setup and loaded cell suspension.*

*A pipette-tip, unlike a tubing, has variable dimension with larger diameter at the top and it converges at the bottom, resembling a nozzle. This means that the average velocity of the sample will be smaller at the top that gradually increases towards the narrower side. When the velocity is low at the top, it makes sure that all the cells get pushed towards the end (small changes in velocity from the center towards the end, smaller velocity gradient). As the flow reaches the end of the tip, the increased velocity pushes the cells out of the tip and the cells immediately enter the microfluidic chip. This variable flow velocity across the length of the pipette tip makes sure that the cells do not sediment to the edges of the tip and we obtain a much higher encapsulation efficiency as a smaller number of cells are lost.*

*In previous studies we have opted to use different types of tubing but also there have not observed significant or noteworthy differences.*

Figures  
I have no negative remarks on Figure 1 and Figure 2. But Figure 3 and 4, showing two conventional setups for droplet generation, need to be improved. Figure 3A has a poor quality and the caption is not very explanatory. The legend in Figure 3B has an untypical unit writing. Before I read the text, it was not clear to me that "1M/mL" means "1\*10^6 cells/mL". Maybe you should change it in the figure itself or write a better caption. Figure 4A is not sufficiently explained in the caption. It does not show how and where exactly the cells are injected in the tubing. Furthermore you do not explain what tube length you chose and why. Is there a reason why you have such an inconsistent number of replicates per concentration, e.g. in Figure 4C "2 M/mL (n=26)" and "12 M/mL (n=1)"?

Figure 5A is nicely illustrated. Therefore Figure 5B is redundant and can be left out because no new information is shown. What is the cell concentration for the results shown in Figure 5F?  
Figure 6D contains much information, which is not mentioned or explained in the caption or text at all.

*We thank the reviewer for highlighting the concerns with the figures. We have improved the resolution of the figure that illustrate the different setups. Also, we have changed the convention of writing the units in all the figures and used the following format: 1.0E6 cells/ML etc. In Figure 4A, we have provided better description in the figure itself specifying where the cells are injected from in the tubing. For this method, we use the tube length depending on the setup. What is important is that the cell concentration should only be filled in one half of the tube length and this length can vary depending on the sample volume. We would like to apologize for the typo in the replicate number. We have updated this information in the figure. Finally, as suggested by the reviewer we omitted Figure 5B from the figure, mentioned the cell concentration in 5F and have added a more balanced description of figure 6 in the figure legend.*

Discussion  
Did you use Teflon tubing for all your presented experiments? If so, you should mention it in the protocol and representative results part and not only in the discussion.

*We have used Teflon tubing for all the experiments, second method onwards. For all our subsequent experiments, including the ones in protocol, we adapted our methods to work only with Teflon tubing. We have updated this information in the manuscript.*

**Reviewer #3:**

Manuscript Summary:

The authors present a new methodology that uses pipette-tips to load cells to droplet-based microfluidic devices without the significant loss of cells.

Major Concerns:

1. If different type of cells are loaded together, will it still work?

*The microfluidic chip design used in our laboratory is a three-inlet device where the inner two inlets are used for seeding cell samples and media and the outer inlet is used for oil phase. For single-cell encapsulation, we use only one inlet and flush media or stimuli from another inlet, or split the cell concentration and flush same cell population from both the inlet to achieve desired encapsulation rate. Also, the two inlets can be used for loading two different cell types to probe cellular communication between two different cell types at single-cell level. We have addressed this concern in our discussion section and also highlighted that we can use this method to pair multiple cells in single droplet for understanding the influence of cellular microenvironment on different cell types. Overall, yes this method will work when different cell types are loaded together given both the loading techniques use pipette-tip based approach.*

2. Can this method apply to microparticles loading rather than cell?

*We believe that it is possible to use this method for seeding microparticles, that face similar loading statistics as cells, to microfluidic devices and we have mentioned it as a potential application in the introduction section.*

3. Cells always precipitate down to bottom of tip, how to solve this problem?

*We understand the concern of the reviewer and agree that cells do precipitate down to the bottom of the tip. This is the reason why we have to optimize the cell concentration to make sure that the amount of cells that precipitate to the bottom of the tip is minimum and interfere very less with the seeding procedure. This is the reason why work with a maximum cell concentration of 13 million cells per mL to have a high encapsulation efficiency. With increase in cell concentration, more cells will start to precipitate and settle at the bottom of the tip interfering with our experimental procedure.*