Editorial Comments:

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.
Response: Grammar issues were resolved.

2. Please place the superscripted numbered references before the punctuation.
Response: Corrected.

3. JoVE cannot publish manuscripts containing commercial language. 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.
Response: Corrections were made.

4. Please add more details to your protocol steps.
Response: More detailed explanations were added.

5. 5.2.2: What happens after centrifugation? Aspiration?
Response: This goes along with comment 4. We have added more detailed explanations to the protocol.

6. Please remove the embedded figure(s) from the manuscript.
Response: Figures were removed from the manuscript.

7. 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
Response: Additional information was added to the "Discussion" section of the paper to elaborate on the requested information.

Reviewer 1:

MAJOR CONCERNS
Comment 1: *The work makes claims that the setup allows "minimal effects from matrix molecules" (page 3, line 82) to be observed. However, there is only one mass spectrum presented showing the cellular metabolite peaks. It would be good to present a "background" signal for the system during non-cell MS acquisition in the matrix (i.e. cell culture media) to compare with the cellular content. A chronogram could also be presented for a cellular metabolite to get an idea of how the signals for each cell changes during acquisition. These can be presented in the supporting information.*
Response: We appreciate detailed and very helpful suggestions from the reviewer. We added additional figures (**Figure S1**) showing the ion signal change during the data acquisition (i.e., before, during, and after detecting cellular species). We also added mass spectra of "background" (cell culture medium and PBS) compared with cellular species (mass range 50-1000) tested on the same day. In addition, we provided a zoomed-in region (mass range 750-760) showing the overlapped regions of representative ions (**Figure S3**).

Comment 2: *References to some of the earlier live SCMS were lacking, specifically the paper by Mizuno et al. 2008 (live single-cell video-mass spectrometry for cellular and subcellular molecular detection and classification) where a video system was used for positioning of the probe. Some references to these papers would be good to have in the introduction to give better background information on the field. The sensitivity and the coverage of the metabolites and lipids are not very good. Maybe it is hard to do MS/MS since the data acquisition time from one cell is limited.*

Response: References were added to the introduction to give readers a better variety of single-cell methods. The sensitivity of detecting cellular species is primarily limited by small amounts of cellular contents from a cell, potential ion loss through the extended ion transfer tubing, and possible suppressed ionization efficiency due to matrix effect. In addition, the relatively short acquisition time limits MS/MS of ion signals obtained from one cell. We have added the corresponding contents in the "Conclusion" of the revision.

MINOR CONCERNS

Page 5, line 149- since the Single-probe used here is modified from a previous design, it will be good to have a photograph of the unit presented in the text.

Response: A figure (now **Figure 2**) was added to show a comparison between the two probes.

Page 7, line 263- the drug molecules mentioned (gemcitabine, taxol, and OSW-1) do not have their masses presented, and it is not clear in figure S2 which masses belong to which molecule. It will also be good to present the mass difference in ppm for each of the drugs relative to their theoretical monoisotopic masses.

Response: **Table 1** was added, which gives the tentative names of the compound, the *m/z* values, and mass error (ppm) compared with the reported values in the database.

Reviewer 2:

MINOR CONCERNS

Title: "Integrated Cell Manipulation Platform Coupled with Single-probe Mass Spectrometry for the Analysis of Single Suspension Cells." Even though the focus of the paper is the so-called "Single-probe platform", it is not clear which classes of biomolecules the authors look at. The proof of principle experiment shows that they are aiming for the analysis of metabolites (They consistently identify lipids) or small drug molecules they treated the cells with. The authors should specify in the title as well as in the text that they aim for the identification of metabolites, but in theory, the system could also be used for the identification of other biomolecule classes in the future.

Response: We really appreciate all constructive suggestions. Page 1, line 3: The title was changed to "Integrated Cell Manipulation Platform Coupled with the Single-probe for Mass Spectrometry Analysis of Drugs and Metabolites in Single Suspension Cells".

Lines 25-26: "An integrated cell manipulation platform is developed for use in conjunction with the Single-probe mass spectrometry setup for the real-time analysis of individual suspension cells under ambient conditions." The authors have to emphasize more thoroughly that the paper is about the real-time isolation of cells, not about the real-time analysis via mass spectrometry, otherwise the key message of the paper is misleading.

Response: The cell is alive while being selected, and there is signal within seconds of lysing,

creating a near real-time analysis. Due to this technicality, “real-time analysis” has been changed to “on-line, rapid analysis” throughout the paper.

Lines 40-42: “This integrated setup is capable of SCMS analysis of targeted patient-isolated cells present in body fluids samples (e.g. urine, blood, saliva, etc.), allowing for potential applications of SCMS analysis to human medicine and disease biology.” The authors statement might be correct, because of the principle they are selling, but they only show experimental data of an in vitro cultivated cancer cell line that was treated with three drugs compared to untreated identifying eight metabolites. Therefore, the statement of the authors that this setup can be used for isolated patient cells is far-fetched.
Response: This integrated setup is designed to ultimately analyze patient cells. In our ongoing studies, we have successfully utilized this setup to measure cells isolated from the urine of bladder cancer patients and obtained preliminary results. However, studies of patient samples are beyond the scope of the current work, so they are not included in this manuscript. Nevertheless, we rephrased these sentences to indicate this technique can be potentially used to analyze patient cell samples in future studies.

Lines 48-49: “Human biology, especially disease biology, is increasingly understood to be the result of activities on the level of individual cells, but the traditional analytical methods, such as liquid chromatography mass spectrometry (LCMS), can only analyze lysates prepared from large numbers of differing cells.” The authors claim that LCMS can only analyze lysates prepared from large numbers of differing cells. There are already, for example, proteomics papers published, which show the analysis of as low as 10 cells that were prepared and analyzed in a 10-cell “bulk.”
Response: We rephrased the corresponding sentences to indicate that the traditional methods such as LCMS are generally used to analyze populations cells. The corresponding sentence now reads “…, but the traditional analytical methods, such as liquid chromatography mass spectrometry (LCMS), are generally used to analyze samples prepared from populations of cells, which cannot represent the process on the individual-cell level. These standard, traditional methods are unable to discern the effects of cellular heterogeneity.”

“K562 cells are incubated with gemcitabine (1 μM) and taxol (1 μM) for 1 h and OSW-1 (100 nM, 1 μM) for 4 h and 2 h, respectively.” “All three drug compounds are detected using the ICMP/Single-probe MS setup. These results suggest this method can be used to study intracellular lipids, drugs, and metabolites on the single-cell level from cells in solution in real time.” The authors are “washing off” the small molecules from the cells, which is not enough to state that the signals of the small molecules reside from intracellular molecules-They can still reside from leftovers on the extracellular membrane after washing. Therefore, the authors cannot state that this method can now be used to study intracellular lipids, drugs, and metabolites on the single-cell level. To be able to state this, the authors have to show quantitative changes of pathways that are affected by the drugs compared to an untreated control. Also, the mass spectrometry analysis itself is not happening in real time, it is the single-cell isolation from a single-cell suspension that is happening in real time.
Response: Wording was changed from “avoid” detection of extracellular compounds to “minimize” detection of extracellular compounds throughout the paper. **Figure S1** is also added to clarify that the measured peaks are not from sampling media. “Real-time analysis” was changed to “on-line, rapid analysis” throughout the paper.

Lines 294-297: "In addition, internal standards (e.g. isotopically-labeled drug compounds) can be added into the acetonitrile for quantification of molecules of interest (e.g. drug molecules) from individual cells, including rare cells such as stem cells and circulating tumor cells (CTCs), which can play a key role in revolutionizing personalizing drug treatments in the future." The statement of the authors goes too far. The quantification of molecules of interest from individual cells seems possible, but from "rare cells such as stem cells and circulating tumor cells" is too far-fetched-First, they have to show that the quantification of molecules from individual cells is possible with their method.
Response: We have published a paper of single cancer stem cells (purchased cell samples) using our previously established Single-probe SCMS technique (reference 51). We are currently conducting studies to quantify the amount of drug compound from individual suspension cells, including patient-derived cells, using this integrated new setup. In principle, many types of cells (including rare cells) can be analyzed if samples are provided. However, this new system has not been tested with cancer stem cells or CTS, so we decided to remove these specific examples. These sentences now read as "Potentially, internal standards (e.g. isotopically-labeled drug compounds) can be added into the sampling solvent for quantification of molecules of interest (e.g. drug molecules) from individual cells, including those that can play a key role in revolutionizing personalizing drug treatments in the future".

Reviewer 3:
MINOR CONCERNS

1. There are some typo/grammar issues throughout the manuscript. For example, i) abstract: "in vitro fertilization. This The integrated cell"; ii) introduction "Thermo LTQ Orbitrap XL mass spectrometer" should be LTQ Orbitrap XL mass spectrometer (Thermo); protocol: and iii) "Structure conformation is confirmed using MS/MS analysis" should be "the chemical structure was confirmed using MS/MS analysis." Please carry out a careful read.
Response: We thank reviewer for detailed suggestions. Corrections were made.

2. *More details are required in the protocol with clarification:*
i) What is its size and shape of the heating coil and at what temperature is it used (authors state to use the manufacturer's conditions, but it is best if they could provide a temperature?
Response: The length of the heating coil has been added. However, the other parameters are manufacturer's units.
ii) What size or thickness is the glass slide?
Response: The only glass slide utilized in the current study is a standard microscope glass slide used to secure the Single-probe for easy coupling to the flexible arm clamp. The protocol for this is previously published and cited in the "NOTE" section of this step.
iii) What size of culture well were used to grow the cells?
Response: Line 225 was changed to "The day before analysis (~18-24 h), seed out cells for testing in a T25 cell culture flask."
iv) Is there a step to count cells?
Response: Standard protocols were used to count cells, so they are not included.
v) What is the composition of each buffer used (e.g. RPMI and PBS)?
Response: Line 228 now reads "Heat 1X phosphate buffered saline (PBS) and Roswell Park Memorial Institute (RPMI) medium…"
vi) Provide a schematic representation (drawing) of the setup to complement Figure 1. Include all connections to enhance adaption by other labs.

Response: **Figure 1 (b)** was modified to accommodate the remaining connections.
vii) Provide in table form all identified m/z species.
Response: This is similar to a comment from Reviewer 1. **Table 1** was added, which gives the identified compound, it's *m/z* ratio, and ppm error.

3. Managing expectations. This is an important technical platform that has great potential. Perhaps for clarity the authors could include in the title the specific types of molecules analyzed. As well, add in the abstract and introduction the type of molecules analyzed the dynamic range provided (and they can include what they envision in the future). Finally, the authors state that this method can be used in patient's circulating cells, yet they only show a few MS spectra for a suspension cell culture. This should be mentioned early on in the paper.
Response: This resembles a comment from Reviewer 2. On page 1, line 3, the title was changed to "Integrated Cell Manipulation Platform Coupled with the Single-probe for Mass Spectrometry Analysis of Drugs and Metabolites in Single Suspension Cells".