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Corresponding Author:	Zhibo Yang, Ph.D. University of Oklahoma Norman, OK UNITED STATES
Corresponding Author's Institution:	University of Oklahoma
Corresponding Author E-Mail:	zhibo.yang@ou.edu
Order of Authors:	Zhibo Yang, Ph.D.
	Shawna J. Standke
	Devon H. Colby
	Ryan C. Bensen
	Anthony W. G. Burgett
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TITLE:

Integrated Cell Manipulation Platform Coupled with the Single-probe for Mass Spectrometry Analysis of Drugs and Metabolites in Single Suspension Cells

AUTHORS AND AFFILIATIONS:

Shawna J. Standke[1], Devon H. Colby[1], Ryan C. Bensen[1], Anthony W. G. Burgett[1], Zhibo Yang[1]

[1]Department of Chemistry and Biochemistry; University of Oklahoma; Norman, OK, USA

E-mail addresses of co-authors:

Shawna J. Standke	Shawna.J.Standke-1@ou.edu
Devon H. Colby	Devon.Colby@ou.edu
Ryan C. Bensen	RCBensen@ou.edu

Corresponding Authors:

Anthony W. G. Burgett	Anthony.Burgett@ou.edu
Zhibo Yang	Zhibo.Yang@ou.edu

KEYWORDS:

Single cell mass spectrometry, suspension cells, integrated cell manipulation platform, Single-probe, ambient ionization, microscale sampling

SUMMARY:

An integrated cell manipulation platform is developed for use in conjunction with a single-probe mass spectrometry setup for the on-line analysis of individual suspension cells under ambient conditions.

ABSTRACT:

Single cell mass spectrometry (SCMS) enables sensitive detection and accurate analysis of broad ranges of cellular species on the individual-cell level. The single-probe, a microscale sampling and ionization device, can be coupled with a mass spectrometer for on-line, rapid SCMS analysis of cellular constituents under ambient conditions. Previously, the single-probe SCMS technique was primarily used to measure cells immobilized onto a substrate, limiting the types of cells for studies. In the current study, the single-probe SCMS technology has been integrated with a cell manipulation system, typically used for in vitro fertilization. This integrated cell manipulation and analysis platform uses a cell-selection probe to capture identified individual floating cells and transfer the cells to the single-probe tip for microscale lysis, followed by immediate mass spectrometry analysis. This capture and transfer process removes the cells from the surrounding solution prior to analysis, minimizing the introduction of matrix molecules in the mass spectrometry analysis. 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.

INTRODUCTION:

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), are generally used to analyze samples prepared from populations of cells, whereas the acquired molecular information cannot accurately represent the chemical processes on the individual-cell level. These standard, traditional methods are unable to discern the effects of cellular heterogeneity on an analytical measurement, and the process of destroying and mixing the cells to prepare the lysate potentially leads to the alteration or loss of cellular components[1,2]. These limitations of traditional methods are especially important in the analysis of patient cells, in which the obtained samples can contain a complex mixture of many different cell types. To overcome these deficiencies, single cell molecular analysis methods, including single cell mass spectrometry (SCMS) methods, are increasingly being developed and applied to bioanalysis, especially of cellular metabolites and low molecular weight biomolecules[3,4].

The first SCMS techniques developed used vacuum-based techniques to perform the analyses under non-ambient conditions[2,5–11]. Non-ambient SCMS techniques are capable of analyzing cellular lipids and metabolites, but require sample pretreatment under artificial conditions, and therefore are not suitable for real-time analysis. The sample preparation process for non-ambient analysis includes the addition of matrix components, and this preparation can alter cellular components from their natural environment[12]. Therefore, ambient mass spectrometry (MS) techniques, which do not require a vacuum for the sampling environment, are utilized to analyze cells in a near-native environment. Not having a vacuum environment allows for versatility in the experimental design; cameras can be added to monitor the cellular process and softer ionization techniques can be combined with separation techniques to receive better information from each single-cell experiment[4,12-42].

The single-probe SCMS method is an ambient technique that analyzes live, mammalian cancer cell lines in a near-native environment[21,43–46]. In addition, the single-probe device has been used for other mass spectrometry applications, including analysis of extracellular molecules in multicellular spheroids and MS imaging of tissues[47–52]. However, since cell immobilization on substrates is required for this method, suspension cells cannot be directly analyzed using this technique[3,53]. Therefore, the single-probe SCMS system could not be directly used to sample non-adherent single cells, such as non-adherent cell lines or suspension cells isolated from a patient's blood or other bodily fluids[54]. In this work, an integrated cell manipulation platform (ICMP) is coupled with the single-probe SCMS technique to analyze live, suspension cells on-line with minimal sample preparation (**Figure 1**)[46]. The ICMP consists of an inverted microscope to monitor cell selection, a glass cell-selection probe, a microinjector to capture individual floating cells, a heated plate to maintain cellular temperature, two cell manipulation systems to control spatial movements of both the glass cell-selection probe and single-probe, and a digital microscope to observe cell transfer from the cell-selection probe tip to the single-probe tip. The fabrication of the single-probe is detailed in previous publications and will not be addressed here[21,48]. The ICMP/single-probe system is coupled to a high resolution mass spectrometer. This integrated setup allows for the sampling of identified single cells from complex biological samples with minimal effects from matrix molecules.

PROTOCOL:

1. Glass cell-selection probe fabrication

1.1 Convert single-bore glass tubing into a tapered probe with a sharp tip.

1.1.1. Place a single-bore glass tube (ID: 0.3 mm, OD: 1.1. mm) into the clamps of a vertical pipette holder, centering the glass with respect to the heating coil and tighten to secure the tube in place. The heating coil is comprised of an 18-gauge nickel-chromium resistance wire (~60 mm in length) coiled around a metal rod (diameter = 3.90 mm) 2.5 times.

1.1.2. Set the glass tubing with temperature program 19.5 (manufacturer's unit). This parameter can be modified for a particular instrument.

1.1.3. Set the solenoid plunger at 4 (manufacturer's unit). This parameter can be modified for a particular instrument.

1.1.4. Trigger the solenoid to pull the glass tubing. This step creates two probes fused at the tip.

1.1.5. Use tweezers to cut ~1 mm away from the tip of each probe, creating an orifice of ~10 µm in diameter at the probe tip.

1.2. Bend the glass probe for easy coupling to the ICMP/single-probe SCMS setup.

1.2.1. Set a pulled glass probe into the microforge, positioning the tip ~3 mm above the platinum heating wire.

1.2.2. Turn the heat on the platinum wire to 30% of the maximum temperature.

1.2.3. Bend the probe ~45° from the original position (**Figure 2**).

2. Integrated cell manipulation platform assembly

2.1. Place the inverted microscope, microinjector, and two cell manipulation systems on a motorized table for easy coupling with the mass spectrometer.

2.1.1. Modify one of the cell manipulation systems to accommodate a single-probe by replacing the end with an arm clamp.

2.1.2. Use a plastic syringe with a needle to fill the microinjector with mineral oil. Avoid bubbles in the tubing as this will affect suctioning.

2.1.3. Replace the stage insert of the inverted microscope with the heated plate. Set the heated

plate at 37 °C prior to analysis.

2.2 Set up the glass cell-selection device.

2.2.1. Insert the glass cell-selection probe inside the metal holder of the microinjector by placing the long (non-bent) side into the capillary holder and tightening the screw to secure the probe in place. Position the probe tip's angle parallel to the heated plate.

CAUTION: The glass probe is very sharp and fragile, and it breaks easily. Protect your eyes and be extra cautious while inserting the probe into the microinjector.

2.2.2. Secure the metal holder of the microinjector into the cell manipulation system. Position the probe tip near the middle of the inverted microscope light.

3. Create an extended ion transfer tube for the mass spectrometer inlet

3.1. Use a metal cutter to cut a piece of stainless-steel tubing (OD: 0.0625 (1/16) in, ID: 0.021 in) ~250 mm in length.

3.2. Measure 135 mm from the end and place a metal feral so ~135 mm will be exposed to the atmosphere and ~115 mm will be inside the mass spectrometer. Secure the feral using two wrenches to tighten it.

4. Couple the ICMP with a single-probe setup

4.1. Secure the glass slide containing the single-probe into the arm clamp of the cell manipulation system.

NOTE: Single-probes are fabricated according to a previously-published protocol[48] with two minor changes in the current study: the nano-ESI emitter is made longer for easy coupling to the mass spectrometer, and the single-probes are glued to the glass side on the right-hand side to avoid interfering with the spatial movement of the glass cell-selection device (**Figure 2**).

4.2. Connect the solvent-providing capillary to a conductive union by placing the capillary into the sleeve (1/16 x .005 in) of the plastic ferrule and finger-tightening the fitting.

4.2.1. Connect the other side of the conductive union to a capillary (ID: 40 µm, OD: 150 µm), which is connected to a syringe containing the sampling solvent, by placing the capillary into the sleeve (1/32 x .007 in) and tightening the fitting. Use acetonitrile with 0.1% formic acid as the sampling solvent in these experiments.

NOTE: The sampling solvent is flexible, but it should primarily contain acetonitrile (or acetonitrile with formic acid for better ionization) for a rapid microscale cell lysis.

4.2.2. Secure the syringe into the syringe pump on the mass spectrometer.

4.2.3. Place the ionization voltage cord onto a copper wire attached to the conductive union.

4.3. Position the nano-ESI emitter ~1 mm to the orifice of the extended ion transfer tube.

4.3.1. Use the cell manipulation system to control the spatial movements of the Single-probe and position the nano-ESI emitter centrally in front of the extended ion transfer tubing.

5. Suspended cell sample preparation

5.1. The day before analysis (~18-24 h), seed out cells for testing in a cell culture flask (T25). K562 human myeloid leukemia cells are used as models in this study.

5.1.1. Heat 1x phosphate buffered saline (PBS) and Roswell Park Memorial Institute (RPMI) medium supplemented with 10% synthetic fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C for 30 min.

5.1.2. Seed ~1 x 10^6 cells in a total volume of 10 mL by combining cells with warm medium. In general, use a 10-mL pipette to place 8 mL of RPMI medium into a cell culture flask. Then, use a 2 mL pipette to put 2 mL of confluent K562 cells the medium for ~1 x 10^6 cells.

5.1.3. Incubate the cells at 37 °C and 5% CO_2 until analysis.

5.2. Prepare cells for analysis.

5.2.1. Pipette cells from the cell culture flask into a 15-mL centrifuge tube.

5.2.2. Spin cells down at 400 x *g* and 37 °C for 5 min and discard the supernatant.

5.2.3. Resuspend cells in 4 mL of RPMI medium containing the drug compound at the desired treatment concentration.

NOTE: For analysis of control cells, resuspend the cells in 4 mL of RPMI medium and skip to **Step 6**.

5.2.4. Incubate the cells for the duration of the treatment time at 37 °C and 5% CO_2.

5.2.5. Spin cells down at 400 x *g* and 37 °C for 5 min. Aspirate the supernatant.

5.2.6. Cells are resuspended in 10 mL of PBS, and centrifuge at 400 x *g* and 37 °C for 5 min. After spinning, discard the supernatant. Repeat this step 3 times to minimize detection of drug from extracellular constituents.

5.2.7. Resuspend cells in 4 mL of PBS for analysis.

6. Perform SCMS measurements using the ICMP/single-probe setup

6.1. Customize parameters for the mass spectrometer for the experiment.

6.1.1. Under the **Scan Mode** heading of the instrument software, select **Define Scan**. Use a resolution of 60,000 m/Δm at *m/z* 400, 1 microscan, 100 ms maximum injection time, and automatic gain control (AGC) on. A mass range (m/z) of 100-1000 was utilized for the experiments. Parameters can be modified based on the instrument model.

6.1.2. Under **Syringe Pump**, select a flow rate of 150 nL/min. Flow rate needs to be optimized for each experiment.

6.1.3. Select **NSI Source** and apply a voltage of ~4.5 kV. This parameter also needs to be optimized for each experiment.

6.2. Turn on the inverted microscope (with 40x magnification selected for both the top plate and bottom lens) and connect it to the USB-port of a laptop to capture live-video feeds. Turn on the heated plate and set it to 37 °C.

6.3. On the computer, go to the **Acquire Data** tab, and select **Continuously** under **Acquire Time**.

6.4. Prepare sample for analysis.

6.4.1. Pipette 2-3 mL of sample into the lid of a small Petri dish (35 mm x 12 mm).

6.4.2 Position the sample in the center of the light from the inverted microscope on top of the heated plate.

6.5. Prepare the glass cell-selection probe for analysis. Use the cell manipulation system to move the probe so its tip is focused under the inverted microscope in the same plane as the cells.

6.6. Select an individual cell for analysis.

6.6.1. Use the cell manipulation system to move the cell-selection probe tip to a targeted cell. This process is monitored using the inverted microscope.

NOTE: If the tip of the cell-selection probe cannot be focused in the same plane as the cells, it is possible that the bent part of the probe is not appropriately angled. Adjust the position of the cell-selection probe until both probe tips can be focused along with cells under the microscope.

6.6.2. Gently turn the handle of the microinjector to adjust the position of the mineral oil inside the tubing. A gentle suction is provided by the microinjector to secure the targeted cell to the

cell-selection probe tip.

NOTE: If the cell cannot be captured by the cell-selection probe through the suction force, check the cell-selection probe to ensure it is fully-inserted into the capillary holder. In addition, inspect the mineral oil levels in the microinjector and tubing, and expel air if there is any.

6.6.3. Use the cell manipulation system to move the cell at the cell-selection probe tip to the single-probe tip, using a digital microscope focused on the single-probe tip to monitor this process. When touching, a small acetonitrile droplet at the single-probe tip induces a rapid lyses of the cell, and then cell lysate is immediately ionized for on-line MS analysis.

NOTE: Because the selected cell is secured to the cell-selection probe tip through a gentle suction, this cell can be potentially detached during its transfer to the Single-probe tip. Therefore, if ion signals of typical cellular lipids (see representative results below) are not observed within 5 s, it is possible that the cell became unattached, and the selection of a different cell is needed.

REPRESENTATIVE RESULTS:

First, untreated K562 cells are used to establish the experimental method. In a typical SCMS experiment, obvious changes of mass spectra can be observed from transferring a cell, during the detection of cellular contents, and after finishing the measurement (**Figure S1**). Three common cellular lipid peaks (phosphatidylcholine, PC), including PC(34:4) (*m/z* 754.536), PC(36:4) (*m/z* 782.567), and PC(38:5) (*m/z* 808.583), are monitored to ensure the cell is successfully transferred and cellular contents are detected (**Figure S2**)[21,43,46,55,56]. If lipid peaks are not seen within 5 s, the mineral oil level in the microinjector is altered to reduce the suction holding the cell at the cell-selection probe tip; caution needs to be taken so that no mineral oil is pushed out from the cell-selection probe. The identity of many PC's in the mass range of *m/z* 750-850 are confirmed using MS/MS on untreated cell lysate samples (**Figure 3, Figure S2, Table 1**)[46].

K562 cells are also subjected to treatment with various drug compounds to expand the versatility of the method. 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. Cells are then washed with PBS to minimize the detection of drug compounds from extracellular content. The contribution of matrix (e.g., ions from cell culture medium, PBS, and solvent) to mass spectra of cellular contents can be eliminated through data subtraction, due to their significantly different ion signals (**Figure S3**). All three drug compounds are detected using the ICMP/single-probe MS setup (**Figure S4**)[46]. 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 a near-native environment.

FIGURE AND TABLE LEGENDS:

Figure 1. Experimental setup for single suspension cell MS experiments. (**A**) The integrated cell manipulation platform (ICMP) coupled with a mass spectrometer. (**B**) Schematic for analysis of suspended cells. (**C**) Experimental view of K562 cells to be selected using the cell-selection probe.

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Figure 2. Photos of a modified single-probe and a cell-selection probe utilized for single suspension cell MS experiments.

Figure 3. Zoomed-in mass spectrum from a single cell showing the representative species (*m/z* 750-850). Chemical structures are confirmed using MS/MS analysis (**Figure S1**). Reprinted with permission from Standke et al[46]. Copyright 2019 American Chemical Society.

Table 1. Identified cellular components using the ICMP/Single-probe setup. The detection of all drug compounds were confirmed by comparing the MS/MS results with standard compound.

DISCUSSION:

The integrated cell manipulation and analysis platform is constructed to expand the versatility of the single-probe MS method, allowing for on-line, rapid analysis of non-adherent cells in a near-native environment. A major advantage of the technique is that minimal sample preparation is required, so the cells are analyzed in conditions that mimic their standard state. Particularly, individual cells of interest can be visually identified and selected, minimizing the influence of matrix effect on MS ionization efficiency while maintaining cells in their natural environment, so the results are more representative cells' native status (**Figure S3**). This technique can be potentially used to study patient cells suspended in biofluids in future studies. Another advantage of this technique is the flexible selection of the sampling solvent. It is important to include acetonitrile as the main sampling solvent so that microscale lysis can occur rapidly. 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 can play a key role in revolutionizing personalizing drug treatments in the future[54].

Although this integrated system can be conveniently used to analyze broad ranges of cells, a limitation of the method is that neither the single-probe nor cell-selection probe is commercially-available; dictating the need for optimization of many parameters (e.g., flow rate, voltage, length between the nano-ESI emitter and ion transfer tubing, etc.) prior to each experiment. In addition, due to the smallness of the Single-probe and cell-selection probe, environmental perturbation (e.g., air flow) may result in difficulties establishing a junction between the two probes. A short-term solution is the bending of the cell-selection probe close to the end to minimize the length of tapering. Future work includes the development of a housing to enclose the critical parts of the setup to minimize environmental effects. Due to the limited amount of cellular contents and short acquisition time (~2-3 s) from a cell, MS/MS analysis can be only conducted for relatively abundant species. Other factors influencing the detection sensitivity include the suppressed ionization efficiency due to the introduction of matrix along with the cell and potential ion loss through the extended ion transfer tubing.

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DISCLOSURES:

The authors have nothing to disclose.

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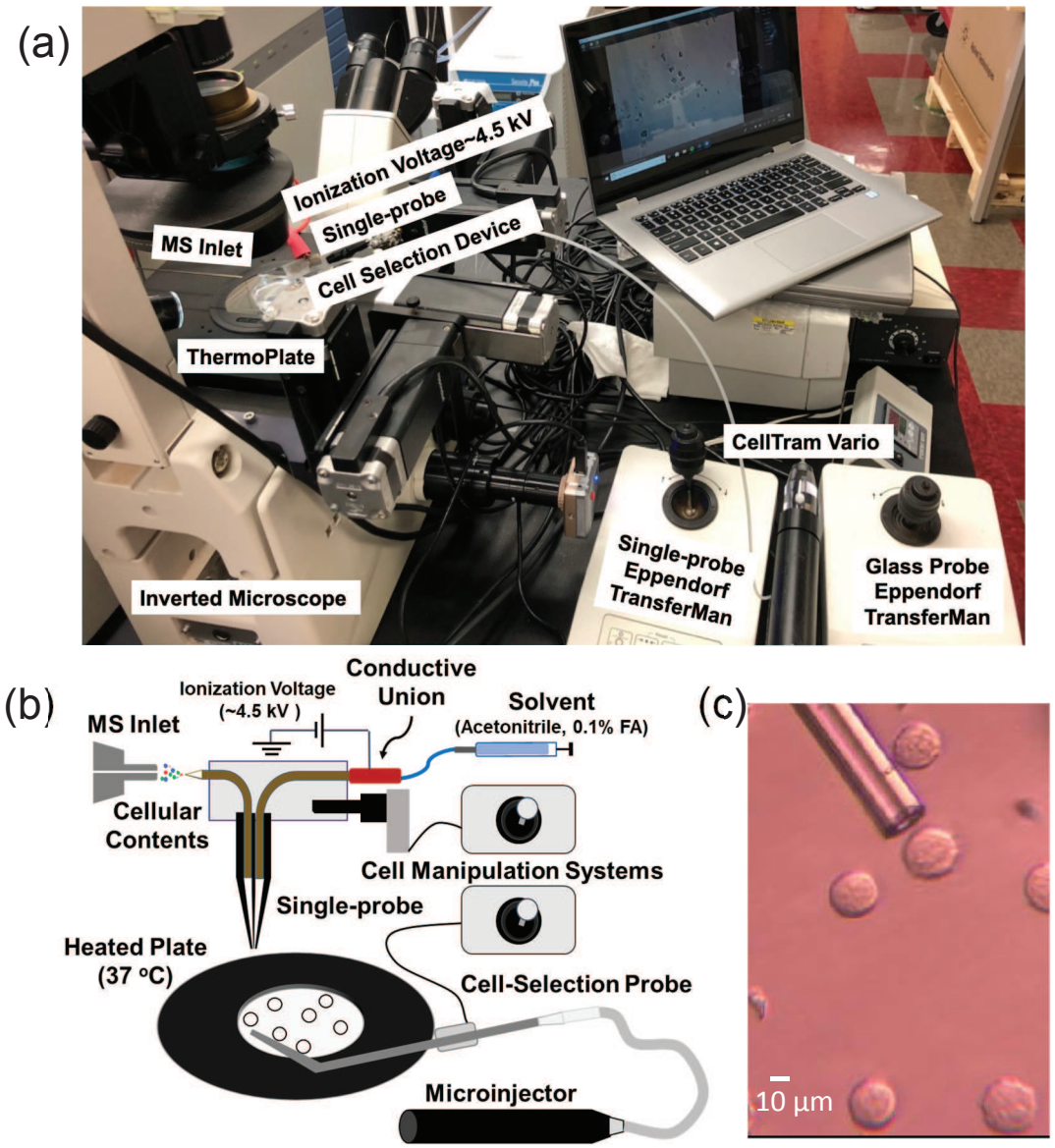
Figure 1



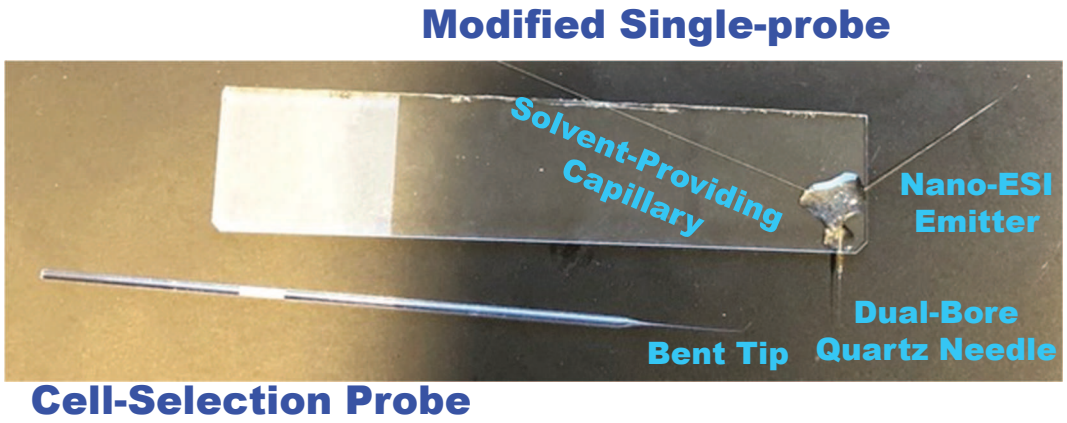
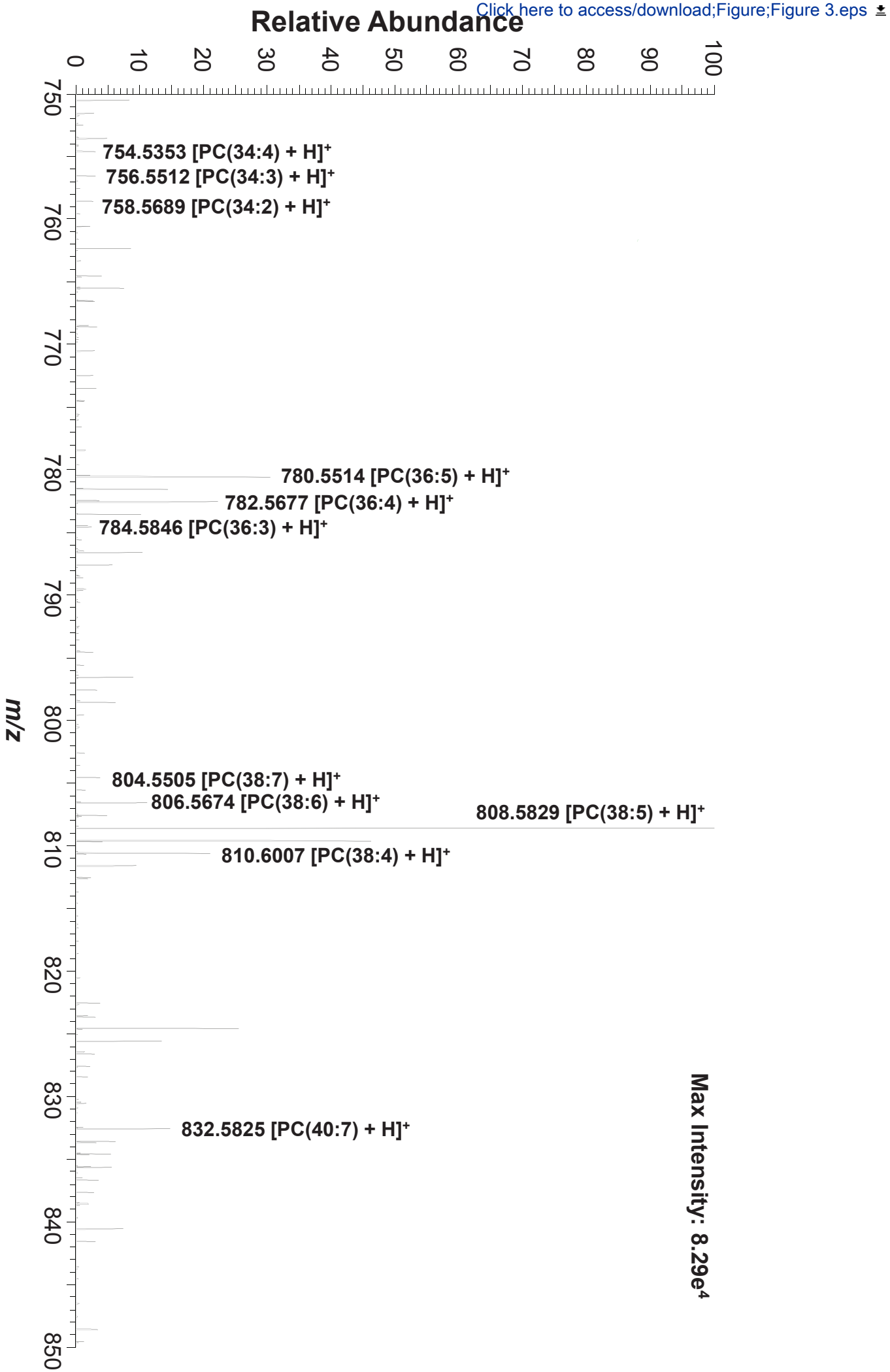


Figure 3



Table

Drug Molecule*	***m/z***	**Mass Error (ppm)**
$[Gemcitabine + H]^+$	264.076	11.32
$[Taxol + Na]^+$	876.3183	2.74
$[OSW\text{-}1 + Na]^+$	895.4448	0.89
Cellular Lipids		
$[PC(34:4) + H]^+$	754.5353	3.71
$[PC(34:3) + H]^+$	756.5512	3.44
$[PC(34:2) + H]^+$	758.5689	0.66
$[PC(36:5) + H]^+$	780.5514	3.07
$[PC(36:4) + H]^+$	782.5677	2.17
$[PC(36:3) + H]^+$	784.5846	0.64
$[PC(38:7) + H]^+$	804.5505	4.1
$[PC(38:6) + H]^+$	806.5674	2.48
$[PC(38:5) + H]^+$	808.5829	2.72
$[PC(38:4) + H]^+$	810.6007	0
$[PC(40:7) + H]^+$	832.5825	3.12

Name of Material/Equipment	Company	Catalog Number
Acetontrile	Millipore Co.	AX0145-1
CellTram Vario	Eppendorf	6221
Copper wire	stores.ebay.com/jewelerheaven	Dead soft, round, 20 guage, 25 ft
Digital stereomicroscope	Shenzhen D&F Co.	Supereyes T004
Disposable micropipette, 1-5 µL	Rochester Scientific	5065
Dual bore quartz tubing, 1.120"x0.005"x12"	Friedrich & Dimmock, Inc.	MBT-005-020-2Q
Epoxy resin	Devcon	Part No. 20945
Eppendorf cell manipulation system	Eppendorf	Transferman NK517800397-U.R.
External nut	VALCO*CHEMINERT	EN1
Formic acid	Sigma-Aldrich	399388-500ML
Fused silica capillary, ID: 40 µm, OD: 100 µm	Polymicro Technologies	TSP040105
Fused silica capillary, ID: 50 µm, OD: 150 µm	Polymicro Technologies	1068150015
HyClone Synthetic fetal bovine serum (FBS)	Fischer Sci	SH3006603
Inline MicroFilter	IDEX Health & Science LLC	M-520
Laser puller	Sutter Instrument Co.	Model P-2000
LED UV lamp	Foshan Liang Ya Dental Equipment	LY-C240
LTQ Orbitrap mass spectrometer	Thermo Scientific	LTQ Orbitrap XL
Microforge	Narishige, Co.	MF-9
Microunion	IDEX Health & Science LLC	M-539
PEEK tubing, 1/32x0.005x 5ft	IDEX Health & Science LLC	1576
PEEK tubing, 1/32x0.007x 5ft	IDEX Health & Science LLC	1577
Penicillin/Streptomycin	Gibco/Life Technologies	15140-122
Petri dish, 35x10 mm	VWR	25382-334
Phosphate Buffered Saline (PBS)	VWR	0780-50L
Platinum wire	Narishige, Co.	Model PT-A
Power supply	Nikon	PSM-2120
RPMI, 1X with Corning glutagro	Corning	10-104-CV
Single-bore tubes	Boralex	5065
Stainless steel ferrules, for 1/16" OD	IDEX Health & Science LLC	VHP-200-01x
Stainless steel tubing, 1/32x 205 µm x30 cm	IDEX Health & Science LLC	U-1128

Syringe, 250 µL	Hamilton	1725LTN250UL
T25 flask	CellStar	690160
Thermo LTQ XL ion source interface flange	New Objective	PB5500
ThermoPlate	TokaiHit	55R30N
TrypLE Express	Gibco	12605-010
Tube cutter, for 1/16" stainless steel	SUPELCO	58692-U
USB digital photography microscope	dx.com	SO2 25~500X
UV curing resin	Prime Dental	Item No. 006.030
Vertical pipette puller	David Kopf Instruments	Model 720
Voltage housing	PicoChip	PCH-A00120
Wire cutter	Craftsman	4 1/2 in end nipper

Comments/Description
Sampling solvent
ICMP
Conductive union setup
Analysis
Cell-selection probe fabrication
Single-probe fabrication
Single-probe fabrication
ICMP
Ion transfer tube fabrication
Sampling solvent
Single-probe fabrication, conductive union setup
Conductive union setup
Cell culture
Conductive union setup
Single-probe fabrication
Single-probe fabrication
Analysis
Cell-selection probe fabrication
Conductive union
Conductive union setup
Conductive union setup
Cell culture
Sample preparation
Cell culture
Microforge
ICMP
Cell culture
Cell-selection probe fabrication
Ion transfer tube fabrication
Ion transfer tube fabrication

Sampling syringe
Cell culture
Analysis
ICMP
Cell culture
Ion transfer tube fabrication
Analysis
Single-probe fabrication
Cell-selection probe fabrication
ICMP/MS interface
Conductive union setup

Title: Mass Spectrometry Measurement of Single Suspended Cells Using a Combined Cell Manipulation System and a Single-Probe Device

Author: Shawna J. Standke, Devon H. Colby, Ryan C. Bensen, et al

Publication: Analytical Chemistry

Publisher: American Chemical Society

Date: Feb 1, 2019

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Author(s):	Shawna J. Standke, Devon H. Colby, Ryan C. Bensen, Anthony W. G. Burgett, Zhibo Yang

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Institution:	University of Oklahoma
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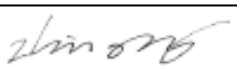
Signature:	
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Editorial Comments:

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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".

SUPPLEMENTAL FILES

TITLE:
Integrated Cell Manipulation Platform Coupled with the Single-probe for Mass Spectrometry Analysis of Drugs and Metabolites in Single Suspension Cells

AUTHORS:
Shawna J. Standke, Devon H. Colby, Ryan C. Bensen, Anthony W. G. Burgett*, Zhibo Yang*

CORRESPONDING AUTHORS:
Anthony.Burgett@ou.edu
Zhibo.Yang@ou.edu

University of Oklahoma
Department of Chemistry and Biochemistry
101 Stephenson Parkway
Norman, OK 73019, USA

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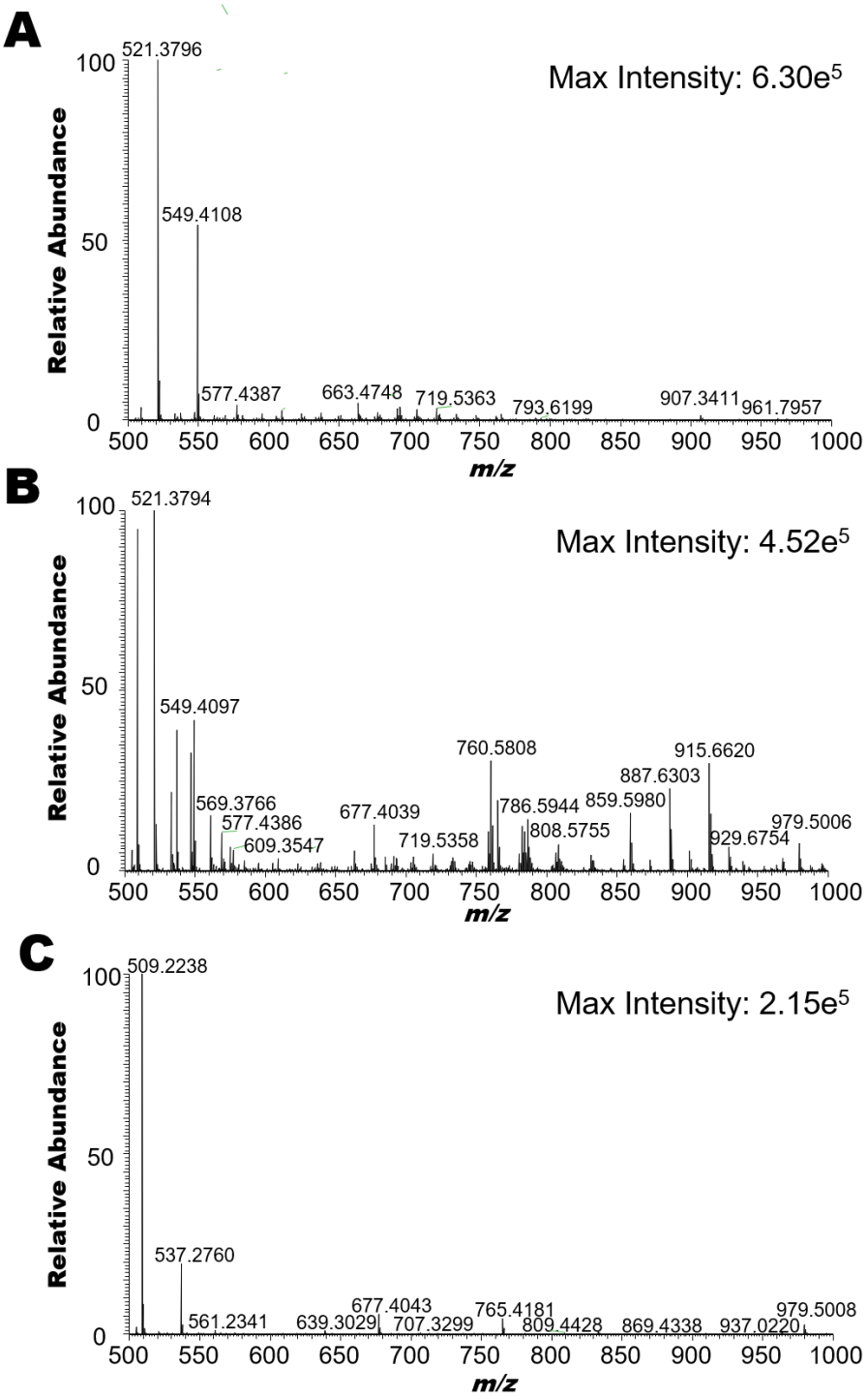
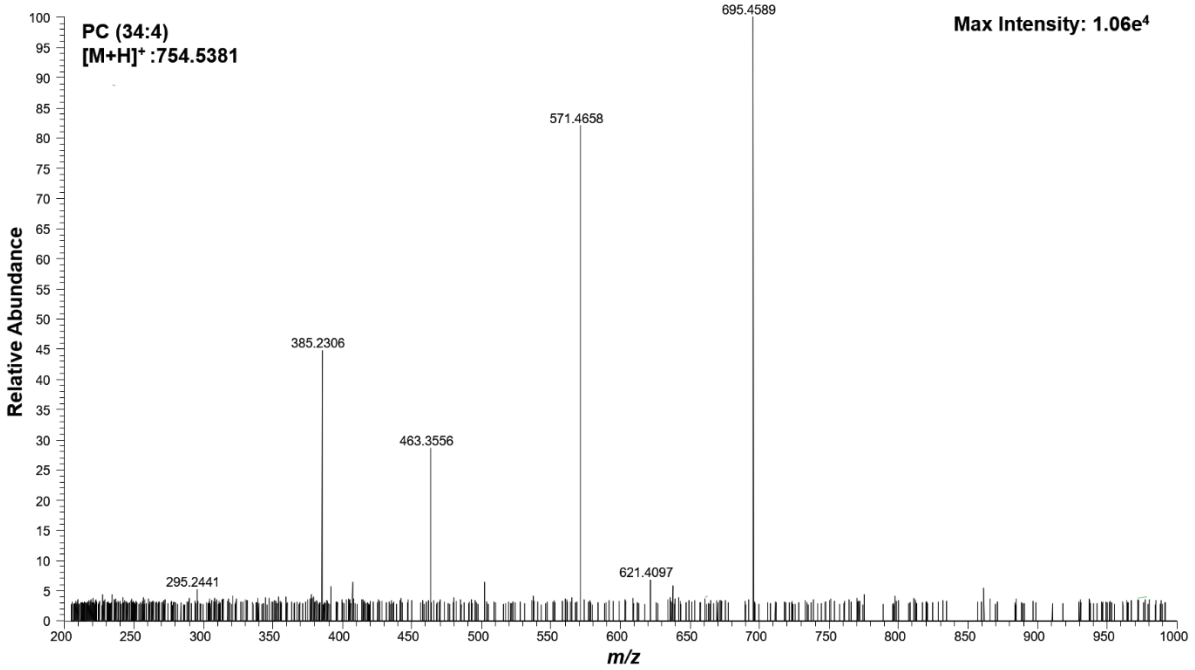


Figure S1. The zoomed-in mass spectra (*m/*z 500-1000) showing changes of ion signals (A) before analysis of a cell, (B) during acquisition of the cell, and (C) after cell analysis using the suspended cell platform.

(a)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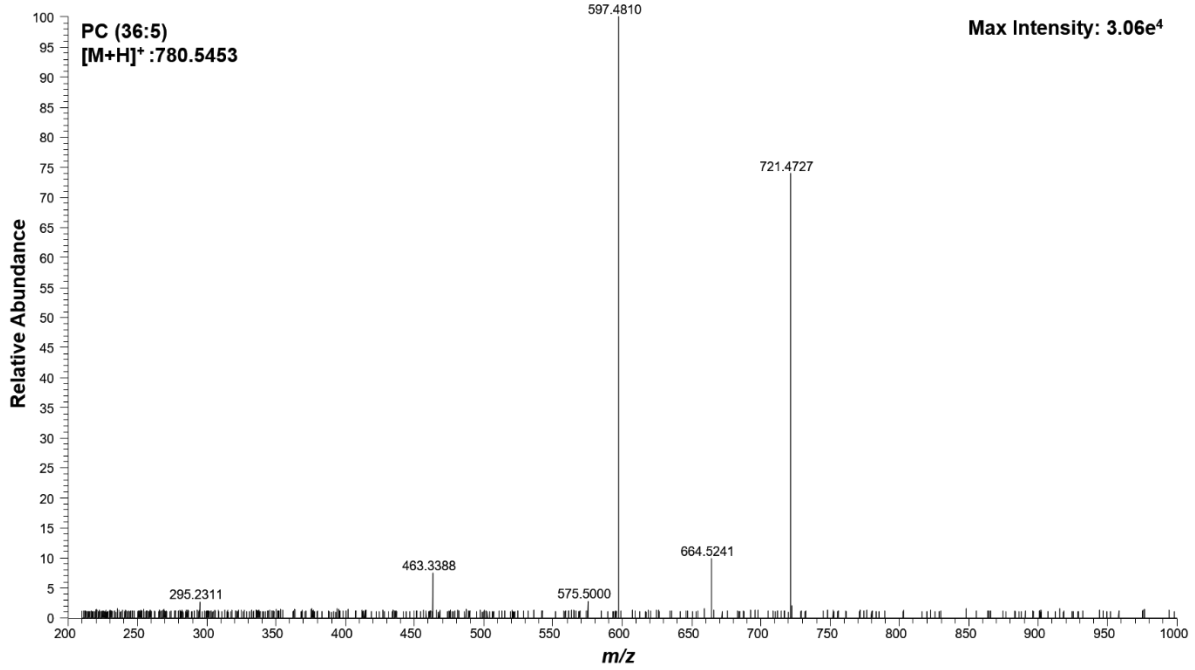
Relative Abundance
m/z

(b)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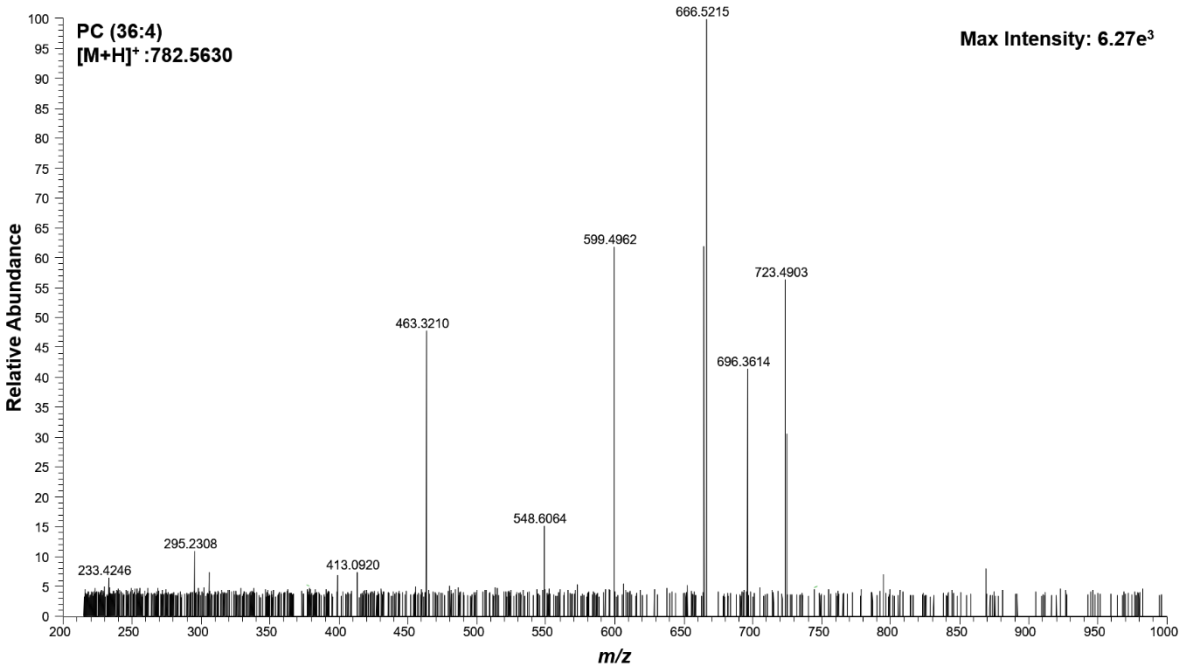
664.5241
463.3388
295.2311
575.5000
Relative Abundance
m/z

(c)


PC (36:4)
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Relative Abundance
m/z
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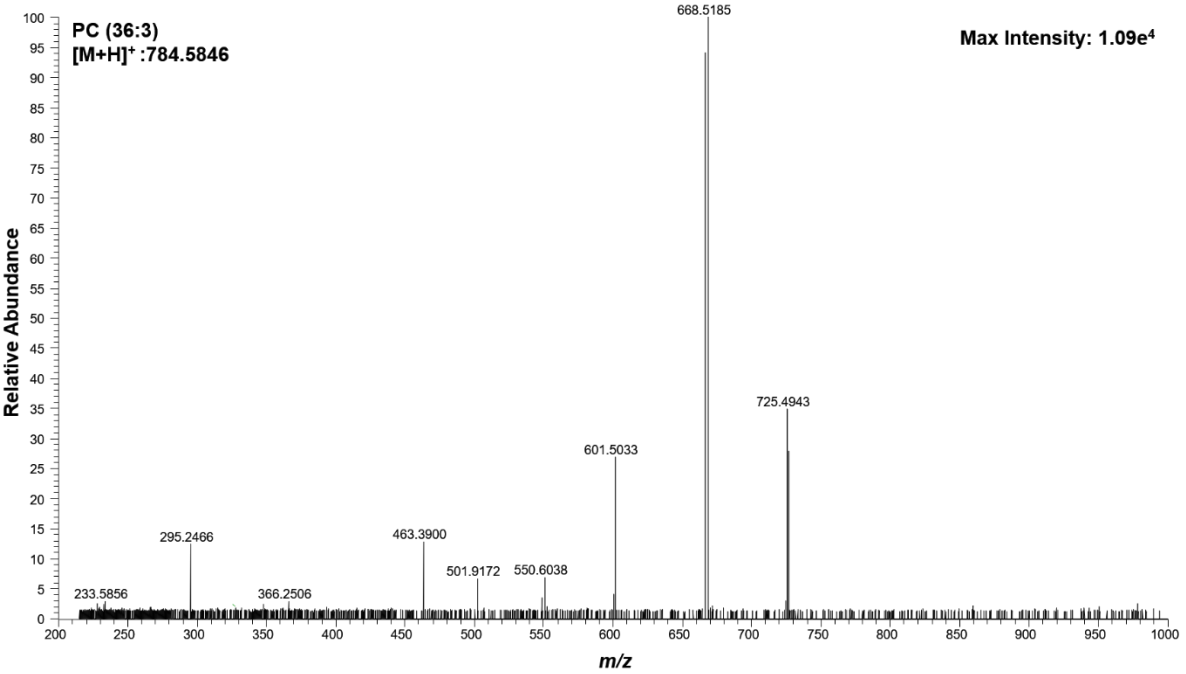
723.4903
463.3210
696.3614
548.6064
295.2308
413.0920
233.4246

(d)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Relative Abundance
m/z
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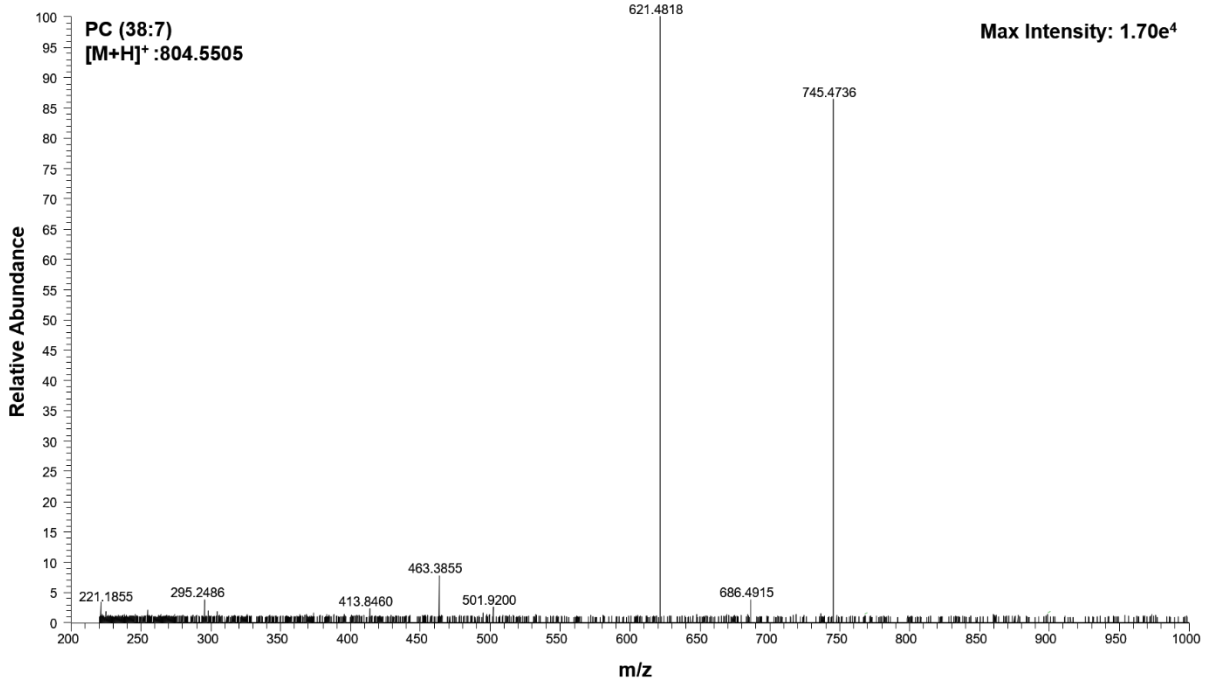
550.6038
233.5856
366.2506

(e)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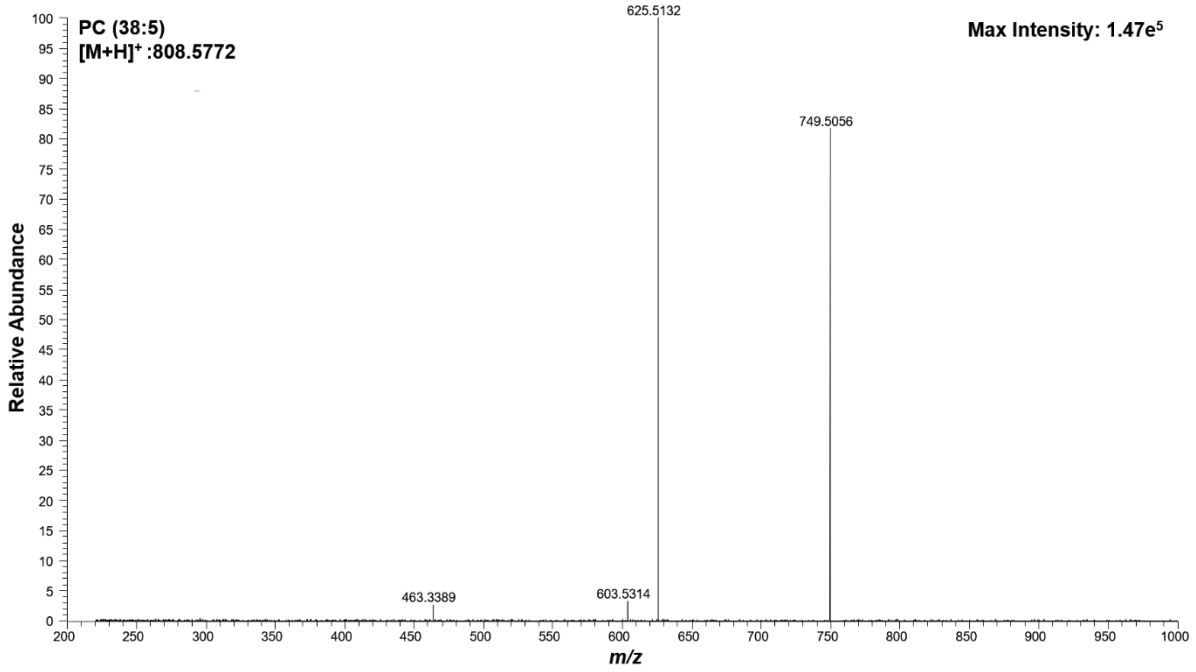
501.9200
686.4915
Relative Abundance
m/z

(f)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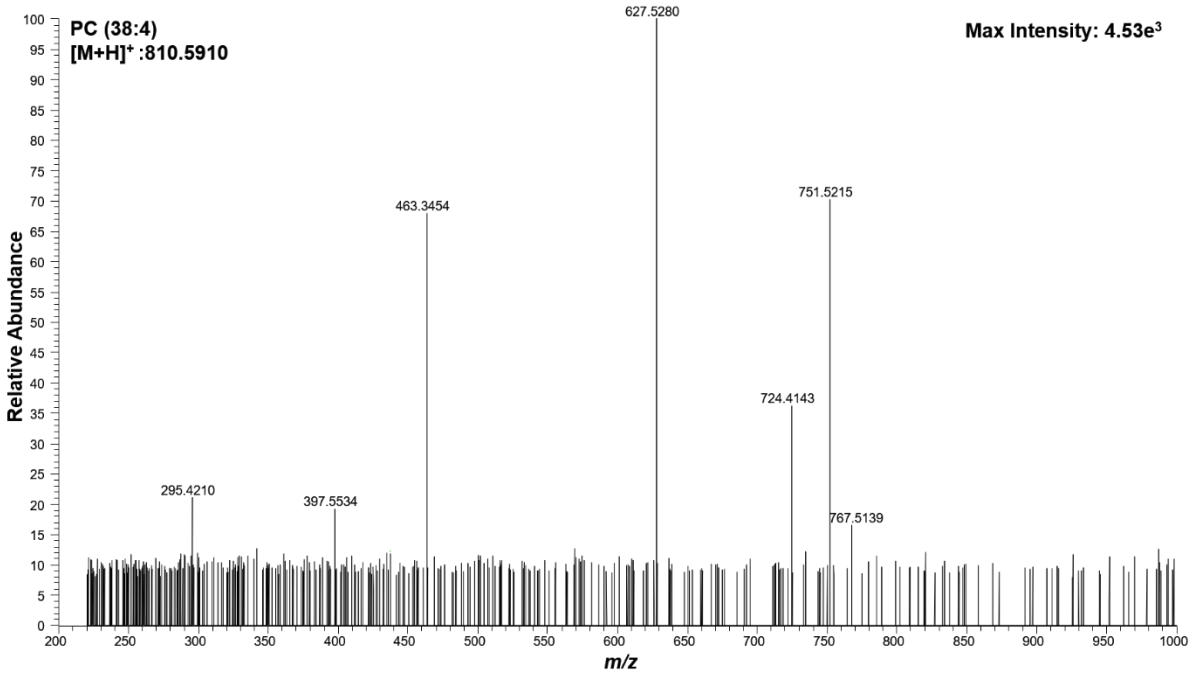
463.3389
603.5314
Relative Abundance
m/z

(g)



(h)

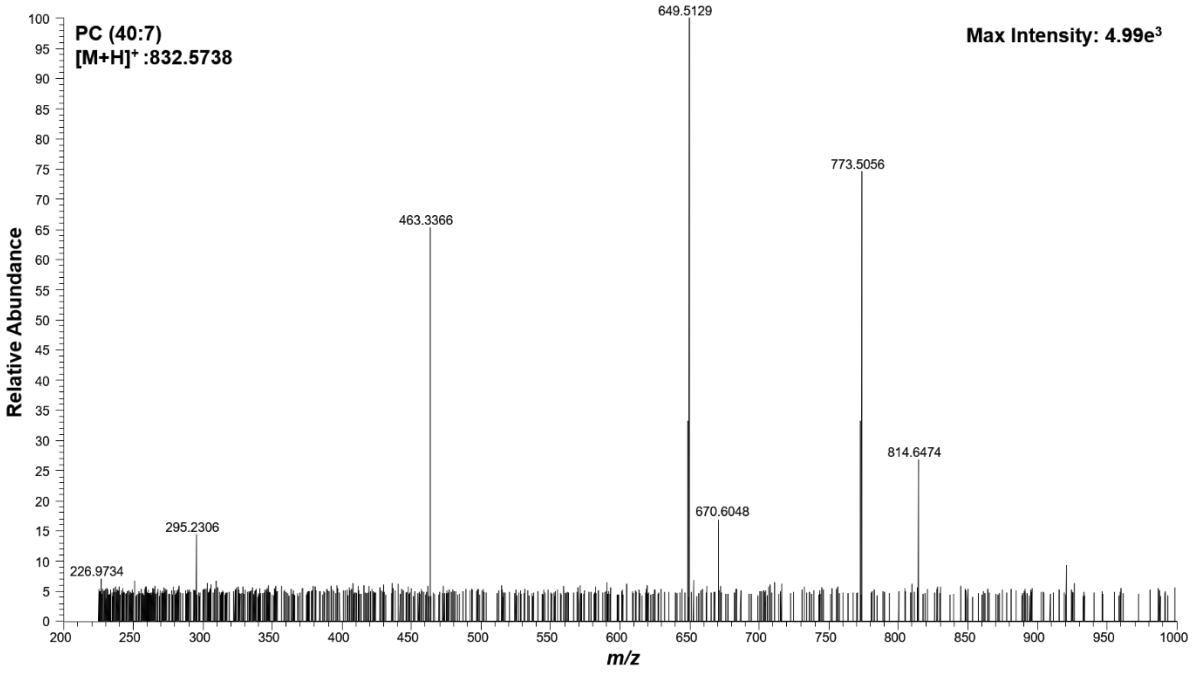


Figure S2. MS/MS verification of lipids with 10-40 manufacturer's unit energy at: (a) *m/z* 754.5 (b) *m/z* 780.5 (c) *m/z* 782.5 (d) *m/z* 784.5 (e) *m/z* 804.5 (f) *m/z* 808.5 (g) *m/z* 810.5 (h) *m/z* 832.5. Reprinted with permission from Standke et al[1]. Copyright 2019 American Chemical Society.

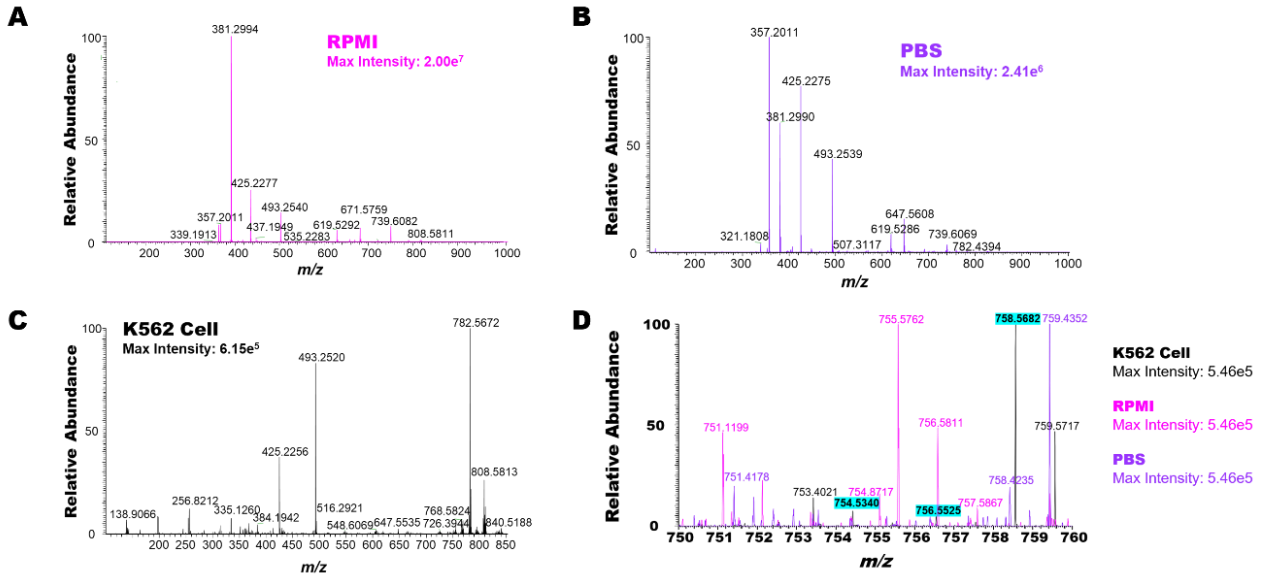


Figure S3. Mass spectra of (A) RPMI cell culture medium, (B) PBS, and (C) an individual K562 cell. (D) A zoomed-in region (m/z 750-760) of the combined three spectra (manually combined) from an individual K562 cell, RPMI media, and PBS showing the differences among them, indicating the identified PC species (highlighted) from the cell can be clearly distinguished.

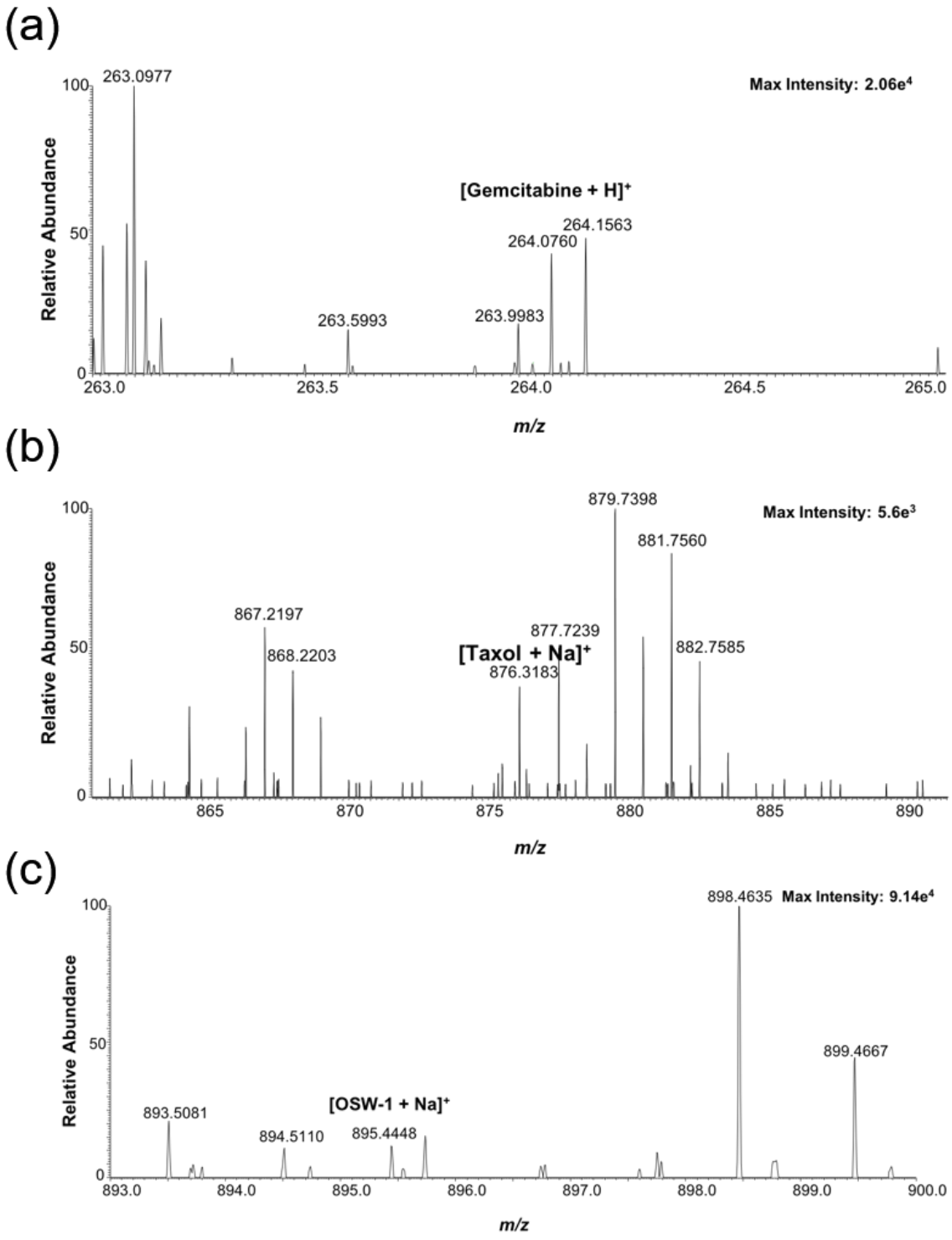


Figure S4. Mass spectra obtained from treating individual K562 cells with: (A) gemcitabine (1 µM, 1 hr) (B) taxol (1 µM, 1 hr) and (C) OSW-1 (100 nM, 4 hr). Reprinted with permission from Standke et al[1]. Copyright 2019 American Chemical Society.

REFERENCES

1. Standke, S.J., Colby, D.H., Bensen, R.C., Burgett, A.W.G., Yang, Z. Mass Spectrometry Measurement of Single Suspended Cells Using a Combined Cell Manipulation System and a Single-Probe Device. *Analytical Chemistry*. **91** (3), 1738–1742, doi: 10.1021/acs.analchem.8b05774 (2019).