Methods in Molecular Biology 2064

Springer Protocols

Bindesh Shrestha *Editor*

Single Cell Metabolism

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, UK

For further volumes:
http://www.springer.com/series/7651

For over 35 years, biological scientists have come to rely on the research protocols and methodologies in the critically acclaimed *Methods in Molecular Biology* series. The series was the first to introduce the step-by-step protocols approach that has become the standard in all biomedical protocol publishing. Each protocol is provided in readily-reproducible step-by-step fashion, opening with an introductory overview, a list of the materials and reagents needed to complete the experiment, and followed by a detailed procedure that is supported with a helpful notes section offering tips and tricks of the trade as well as troubleshooting advice. These hallmark features were introduced by series editor Dr. John Walker and constitute the key ingredient in each and every volume of the *Methods in Molecular Biology* series. Tested and trusted, comprehensive and reliable, all protocols from the series are indexed in PubMed.

Single Cell Metabolism

Methods and Protocols

Edited by

Bindesh Shrestha

Waters Corporation, Milford, MA, USA

Humana Press

Editor
Bindesh Shrestha
Waters Corporation
Milford, MA, USA

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-9829-6 ISBN 978-1-4939-9831-9 (eBook)
https://doi.org/10.1007/978-1-4939-9831-9

Chapter 2

Identification of Metabolites in Single Cells by Ion Mobility Separation and Mass Spectrometry

Linwen Zhang, Linda L. Allworth, and Akos Vertes

Abstract

Non-targeted metabolic analysis of single cells by mass spectrometry (MS) is important for understanding individual cell functions and characterizing cell-to-cell heterogeneity. However, identifying biomolecules in single cells presents significant challenges due to the low picoliter volume samples and the structural diversity of metabolites. Capillary microsampling electrospray ionization (ESI) MS with ion mobility separation (IMS) enables the analysis of single cells under ambient conditions with minimum sample pretreatment and improved specificity. Here, we describe a protocol for the analysis of the metabolic makeup, and the identification of ions produced from single cells by capillary microsampling ESI-IMS-MS.

Key words Single-cell analysis, Metabolomics, Mass spectrometry, Ion mobility separation, Isobaric ions, Collision cross-section

1 Introduction

Metabolic analysis of individual cells within an isogenic population enables the characterization of cellular physiological states and their phenotypic heterogeneity [1–3]. With high sensitivity and specificity, mass spectrometry (MS) is a valuable tool for the non-targeted analysis of a wide range of metabolites and lipids from single cells [4, 5]. High-performance liquid chromatography (HPLC) combined with MS is a routine method for the analysis of complex biological samples with high specificity [6, 7]. However, this method requires elaborate sample pretreatment and is not compatible with the metabolic analysis of single human cells with low picoliter volumes. Ion mobility separation (IMS) integrated with MS can be used to distinguish isobaric ions in the gas phase, based on their size, shape, charge, and mass, on a timescale of milliseconds [8–10]. The collision cross-section (CCS) values measured by IMS can enhance metabolite assignment confidence [11, 12]. In addition, the combination of collision-induced dissociation (CID)

Bindesh Shrestha (ed.), *Single Cell Metabolism: Methods and Protocols*, Methods in Molecular Biology, vol. 2064, https://doi.org/10.1007/978-1-4939-9831-9_2,

and IMS can improve specificity for the distinction and identification of isobars, isomers, and conformers [13].

Capillary microsampling and electrospray ionization (ESI) MS with IMS has been demonstrated for the metabolic analysis of single plant cells with improved molecular coverage [14]. This technique has been extended to subpopulations of human cells in particular stages of mitosis, and to the subcellular analysis of peptides in single snail neurons with identified function [15–17]. Here, we describe a protocol for analyzing small metabolites and lipids, and distinguishing isobaric ions in single adherent mammalian cells. Picoliter volumes of subcellular contents can be sampled and analyzed by this technique. The protocol does not require the detachment of the cells from the substrate surface or tissue for analysis, thereby avoiding the perturbation of metabolic state associated with scraping or trypsinization.

2 Materials

2.1 Reagents and Chemicals

1. Electrospray solution: HPLC grade methanol:water (*v/v* 4:1) with 1 mM ammonium formate (HPLC grade, Sigma-Aldrich, St. Louis, MO, USA).
2. For CCS determination, poly-DL-alanine (P9003, Sigma-Aldrich, St. Louis, MO, USA) was used as the calibrant and dissolved in the electrospray solution to reach a final concentration of 0.1 g/L.
3. One pouch of phosphate buffered saline (PBS) (Bioperformance certified grade, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1.0 L deionized water (18.2 MΩ·cm) to prepare 1× PBS solution.
4. 2-Deoxy-D-glucose (2-DG) (≥99.0% purity, Sigma-Aldrich, St. Louis, MO, USA) stock solution was prepared in 1× PBS solution to reach a final concentration of 500 mM.

2.2 Cell Culture

1. Adherent mammalian cell lines, such as HepG2/C3A (CRL-10741, ATCC, Manassas, VA, USA).
2. Cell culture medium for HepG2/C3A: Eagle's Minimum Essential Medium (ATCC, Manassas, VA, USA) supplemented with 10% (*v/v*) fetal bovine serum and 1% (*v/v*) penicillin-streptomycin (Invitrogen, Grand Island, NY, USA).
3. An automated cell counter (Countess, Invitrogen, Grand Island, NY, USA) was used for determining the viable cell numbers using trypan blue.
4. 2 mL of cell suspensions at a density of 2×10^5 cells/mL was seeded on a 35 mm Petri dish (Corning, Tewksbury, MA, USA). Cells were maintained at 37 °C and 5% CO_2 in an incubator (HERAcell 150i, Thermo Scientific, Waltham, MA, USA) for 12–16 h before MS analysis (*see* **Note 1**).

2.3 Capillary Fabrication

1. A micropipette puller (P-1000, Sutter Instrument, Novato, CA, USA) installed with a box filament (FB255B, Sutter Instrument, Novato, CA, USA) was used for capillary fabrication.
2. Thin-walled borosilicate glass capillary with a filament (TW100F-3, World Precision Instruments, Sarasota, FL, USA) was chosen to produce relatively large tip openings (~1 μm) for adherent mammalian cell sampling. The filament in the capillary is necessary for drawing the electrospray solution to the end of the tip and minimizing air bubbles in the sample (*see* **Note 2**).
3. Pipette storage box (Sutter Instrument, Novato, CA, USA).

2.4 Cell Sampling

1. An inverted microscope (IX71, Olympus, Tokyo, Japan) with a maximum magnification of 600× was used for the visualization of cells.
2. A micromanipulator (TransferMan NK2, Eppendorf, Hauppauge, NY, USA) was mounted on the microscope for performing cell sampling.
3. A capillary holder (IM-H1, Narishige, Tokyo, Japan) was attached with a syringe.

2.5 Single-Cell ESI-IMS-MS

1. Microloader tips (Cat No. 930001007, Eppendorf, Hauppauge, NY, USA) for backfilling the capillary with the electrospray solution.
2. A microelectrode holder (MEW-F10A, Warner Instruments, Hamden, CT, USA) with a platinum wire of 200 μm in diameter and ~5 cm in length (Alfa Aesar, Ward Hill, MA, USA) provided electrical connection to the electrospray solution in the capillary tip. High voltage was applied to the microelectrode by a regulated power supply (PS350, Stanford Research Systems Inc., Sunnyvale, CA, USA) to generate an electrospray (*see* **Note 3**).
3. A quadrupole time-of-flight (TOF) mass spectrometer equipped with a traveling wave (T-wave) IMS system (Synapt G2-S, Waters Co., Milford, MA, USA) was used to collect mass spectra. The commercial ion source was removed and the capillary assembly was installed for electrospray ionization in the ambient environment.
4. The parameter settings for the ion mobility mass spectrometer were optimized to achieve maximum separation at the highest ion transmission. All instrument settings were saved in a parameter file. The major parameters included mass range: m/z 50–995, acquisition mode: mobility-TOF, the sensitivity and polarity of ion modes, scan rate: 0.5 s/scan, IMS wave velocity: 650 m/s, and wave height: 40 V.

5. Nitrogen drift gas was supplied at a flow rate of 90 mL/min and a pressure of 3.25 mbar. When the mass spectrometer was operated in MS/MS mode, argon was supplied as a collision gas for performing CID.

2.6 Data Analysis

1. DriftScope 2.8 (Waters Co., Milford, MA, USA) software was used for automatic peak detection, the conversion of drift time (DT) to CCS, and CCS calibration.

3 Methods

To perform capillary microsampling ESI-IMS-MS, customized capillaries are pulled based on the characteristics of cell types and are used for sampling of individual adherent cells. This technique can also be applied for the analysis of suspended cells held by holding pipettes. The glass capillary held by a capillary holder is used to sample individual cells under the observation by an inverted microscope. The capillary is backfilled with electrospray solution and placed in a microelectrode holder. Applying high voltage to the electrical connection on the microelectrode holder produced an electrospray from the cell contents. The generated ions are separated in a T-wave IMS system according to their CCSs and then analyzed by a mass spectrometer based on their mass-to-charge ratios (*m/z*). Tandem MS is also performed for structural identification of the ions. Time aligned parallel (TAP) fragmentation, where CID is applied after ion separation by IMS, is performed to differentiate and identify isobaric ions with close to identical *m/z*. The resulting ionic *m/z* and CCS information are used for metabolite and lipid assignments.

3.1 Preparation of Capillaries for Cell Sampling

1. Initially the program settings of the micropipette puller were adjusted to produce capillaries that achieve optimal cell extraction and reproducible MS signal. A two-step pulling program was set as follows, Step 1: Heat = 574, Pull = 95, Velocity = 40 and Delay = 170, and Step 2: Heat = 564, Pull = 90, Velocity = 70 and Delay = 120 at Pressure = 500. The parameter settings should be varied for other capillary types and filament heating conditions. The optimized parameters were saved to repeatedly produce sampling capillaries on the micropipette puller.
2. Before an experiment, ~20 capillaries were pulled and stored in the pipette storage box to prevent breakage and contamination of the tips.

3.2 CCS Calibration

1. To perform CCS calibration, 1.0 μL poly-DL-alanine solution was backfilled into a capillary through a microloader tip. The capillary was tapped on the side to eliminate air bubbles.

2. The capillary was mounted in the microelectrode holder. This assembly was placed in front of the mass spectrometer inlet orifice with the capillary tip at a distance of ~5 mm.
3. A negative voltage of −1500 V was applied on the microelectrode by a high voltage power supply.
4. The acquisition parameters saved earlier were selected for the mass spectrometer.
5. The acquisition of IMS-MS data for poly-DL-alanine solution was initiated.
6. A CCS calibration curve fitted as a power function was generated using the DriftScope 2.8 software based on the poly-DL-alanine data. The reference CCS values for poly-DL-alanine were obtained from previous publications [8, 11]. The curve with an $R^2 \geq 0.99$ was accepted for the determination of CCS values for unknown ions.

3.3 Capillary Microsampling

1. 0.5× PBS buffer was prepared by mixing equal volume of 1× PBS solution and deionized water. The buffer was warmed to 37 °C in a beads bath for 15 min.
2. Before cell sampling, a 35 mm dish of adherent cells was obtained from the incubator and washed three times by 0.5 mL 0.5× PBS buffer. Then the dish containing 0.5 mL 0.5× PBS was placed on the center of the microscope sample stage.
3. A pulled capillary was inserted in the capillary holder, and they were attached to the micromanipulator at an angle of 45° relative to the microscope stage. The position of the capillary was adjusted to the center of the field of view and at ~4 mm above the cells.
4. The monolayer of cells was brought to the focal plane and a cell of interest was selected by moving the microscope sample stage. Figure 1a shows a microscope image of a HepG2/C3A cell before capillary sampling.
5. The capillary tip controlled by the micromanipulator was carefully moved over the targeted cell and lowered to approach it. In the meantime, the microscope focal plane was adjusted back and forth between the tip and the cell to monitor their relative distance and position.
6. When the tip approached the cell layer, the step size of the micromanipulator was switched from "coarse" to "fine" mode.
7. The capillary was further lowered to slightly touch the cell membrane, and at that point the height of the tip was set as *z*-axis limit for the micromanipulator to prevent tip damage (*see* Fig. 1b and **Note 4**). Then a negative pressure was applied

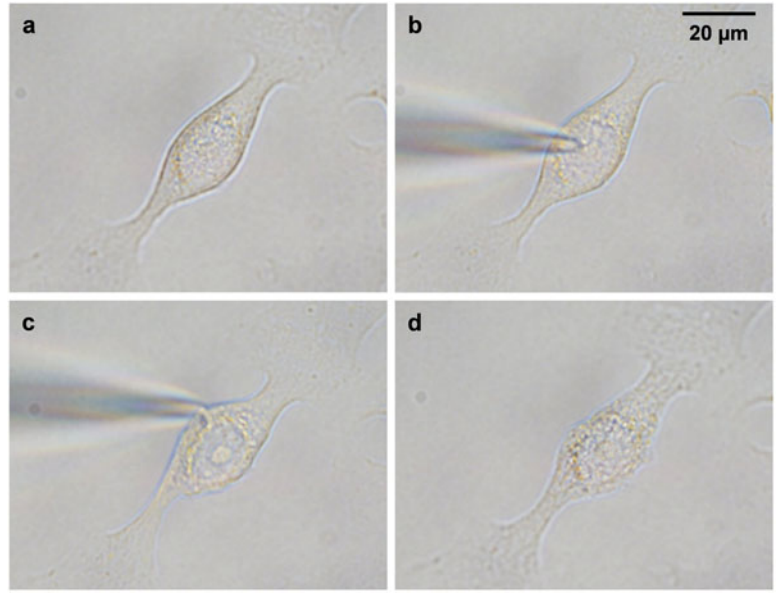


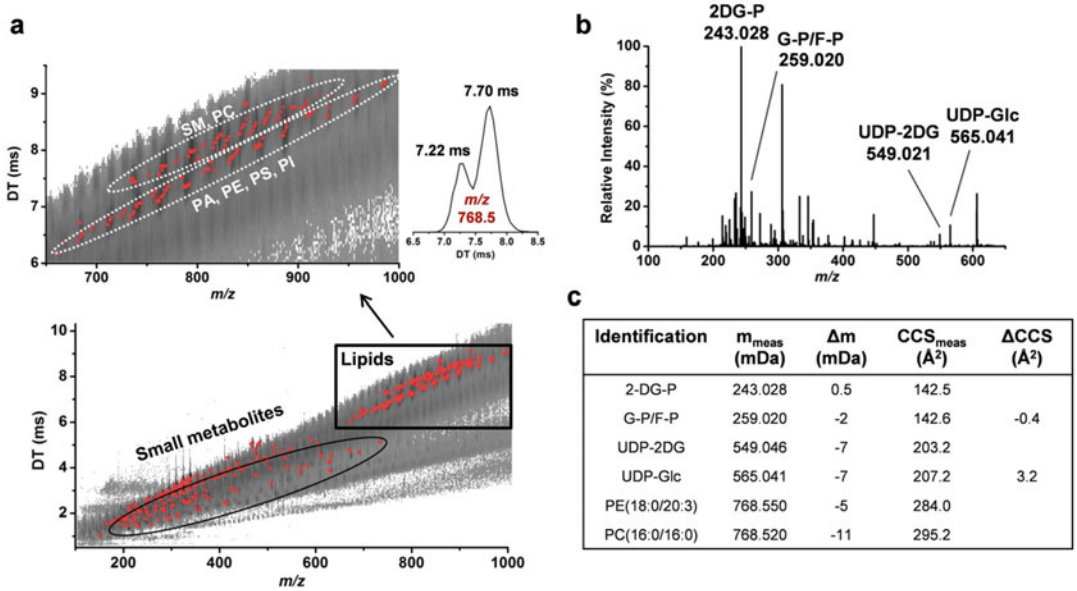
Fig. 1 (a) Cell of interest is brought in focus under the microscope. **(b)** Capillary tip is slightly touching the cell membrane before sampling. **(c)** Negative pressure is applied during cell sampling. **(d)** Capillary tip is removed from the cell after sampling

using a syringe attached to the back of the capillary holder to extract the cell contents (*see* Fig. 1c).

8. After sampling the cell contents, the tip was removed from the dish (*see* Fig. 1d).
9. The cells were returned to the incubator.

3.4 Single Cell ESI-IMS-MS

1. The capillary was removed from the capillary holder, and back-filled with 1.0 μL electrospray solution. To remove air bubbles, the capillary was gently flicked on the side.
2. The capillary was placed in the microelectrode holder and the platinum wire came in contact with the electrospray solution. The capillary tip was aligned with the orifice of the mass spectrometer at a distance of ~5 mm.
3. The data acquisition by the mass spectrometer was initiated using the previously saved parameters.
4. To generate ions from the sampled cell contents, a high voltage of −1500 V was applied to the microelectrode. Typically, during the first 5–10 s of the electrospray, cell related signal was collected, whereas the electrospray background was acquired for the following 10 s (*see* **Note 5**).



Identification	m_{meas} (mDa)	Δm (mDa)	CCS_{meas} (Å²)	ΔCCS (Å²)
2-DG-P	243.028	0.5	142.5	
G-P/F-P	259.020	-2	142.6	-0.4
UDP-2DG	549.046	-7	203.2	
UDP-Glc	565.041	-7	207.2	3.2
PE(18:0/20:3)	768.550	-5	284.0	
PC(16:0/16:0)	768.520	-11	295.2	

Fig. 2 (a) At the bottom, DT vs. *m/z* plot for a cell treated with 5 mM 2-DG for 1 h with the detected ions marked by red dots. Metabolite and lipid species are separated and highlighted in distinct regions. On the top, the zoomed DT vs. *m/z* plot in the lipid region shows sphingomyelin (SM) and phosphatidylcholine (PC) lipids separate from phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) lipid species spanning the same *m/z* range. A DT distribution of a pair of separated isobaric species with nominal *m/z* 768.5 is shown on the right. **(b)** Mass spectrum of the 2-DG-treated cell shows two new biotransformation products, 2-DG phosphate (2-DG-P) and UDP-2-deoxyglucose (UDP-2DG). **(c)** Table lists selected metabolites identified based on accurate mass measurement, CCS values, and tandem mass spectrometry (G-P/F-P = glucose phosphate or fructose phosphate, UDP-Glc = UDP-glucose). The Δm values indicate the deviation from calculated accurate mass, whereas ΔCCS stands for the difference from literature values

3.5 Data Processing and Metabolite Identification

1. A DT vs. *m/z* plot representing a three-dimensional dataset comprised of ion abundances, DT, and *m/z* information was collected for the analysis of each single cell. DriftScope 2.8 software was used to visualize and process the data. Figure 2a shows a DT vs. *m/z* plot of a cell treated by 2-DG at 5 mM for 1 h. The top panel shows that lipid classes are partially separated within an *m/z* range. An example of the DT distribution for isobaric ions with nominal *m/z* 768.5 is shown on the right.
2. Ions of interest in different regions on the DT vs. *m/z* plot were selected and exported to the MassLynx 4.1 software (Waters Co., Milford, MA) to generate and process the corresponding mass spectra. Figure 2b shows the exported mass spectrum corresponding to small metabolites. Two biotransformation products produced in the cell in response to 2-DG treatment, 2-DG phosphate (2-DG-P) and UDP-2-deoxyglucose (UDP-2DG) were detected.

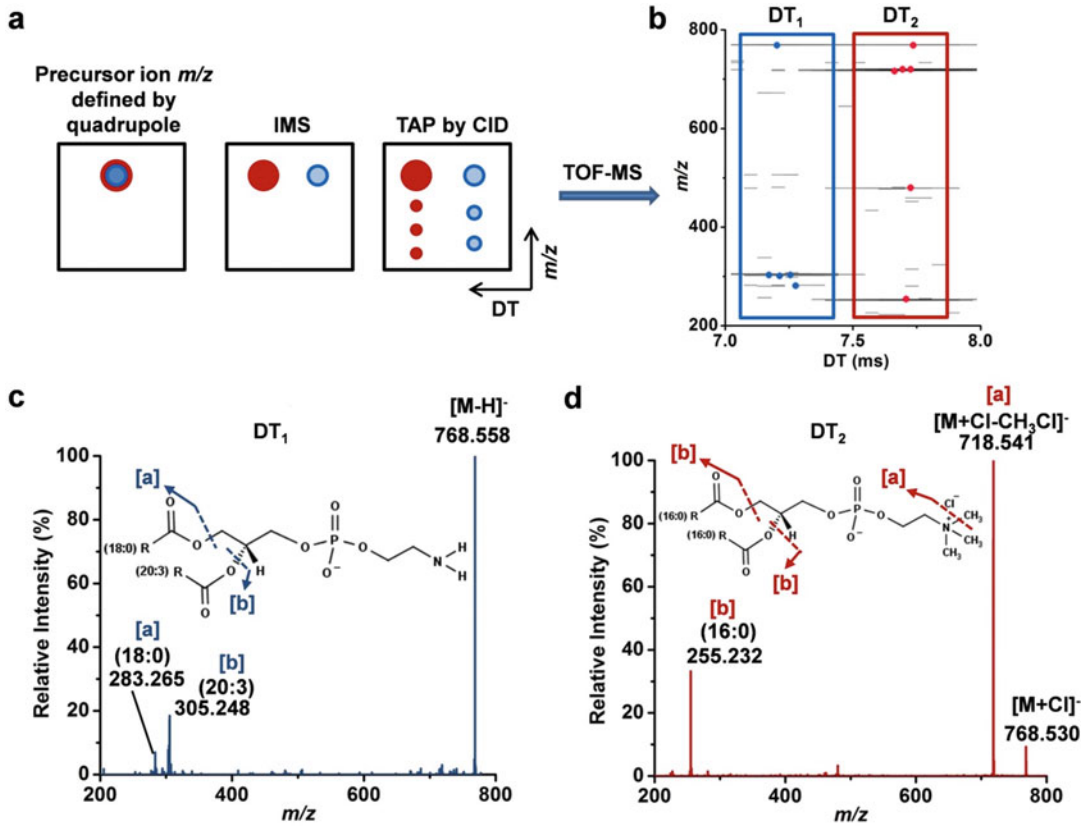


Fig. 3 (a) Schematic representation of the TAP fragmentation procedure for distinction and identification of isobaric ions. Precursor ion *m/z* is selected by quadrupole mass analyzer. The isobaric ions with defined *m/z* are resolved by IMS, followed by CID to produce drift time correlated fragment ions. **(b)** Single cell DT vs. *m/z* plot for TAP fragmentation of isobaric ions with nominal *m/z* 768.5. The precursor ion at $7.0 < DT_1 < 7.4$ ms and its corresponding fragment ions are marked by blue dots and framed in a blue rectangle, whereas the ion at $7.5 < DT_2 < 7.9$ ms and its fragment ions are marked by red dots and framed in a red rectangle. Tandem mass spectra for **(c)** the DT_1 ion and **(c)** the DT_2 ion with the fragmentation patterns are shown in the insets. DT_1 and DT_2 ions are identified as $[PE\ (18:0/20:3)\text{-}H]^-$ and $[PC\ (16:0/16:0) + Cl]^-$, respectively

3. The determination of CCS values for the unknown ions was based on the CCS calibration curve for poly-DL-alanine produced by the DriftScope 2.8 software. The measured *m/z* and CCS values were used for tentative assignment of the unknown metabolites by searching metabolomics databases, e.g., Human Metabolome Database (http://www.hmdb.ca/), and previously published CCS values, respectively [11, 12] (*see* Fig. 2c).
4. For further structural identification of the unknown ions, TAP fragmentation was performed and tandem mass spectra were acquired in mobility-TOF MS/MS mode. Before IMS, the nominal *m/z* of the unknown ion was set as the precursor mass. Following IMS, the ions were subjected to CID with the collision energy scanned between 10 and 40 eV. Figure 3a

shows a representation of TAP fragmentation to distinguish and identify two isobaric ions. Figure 3b shows an example of a DT vs. *m/z* plot for TAP fragmentation of a pair of isobaric ions with nominal *m/z* 768.5. Distinct tandem mass spectra for individual isobaric ions were derived (*see* Fig. 3c and d). According to the fragmentation patterns, they were assigned as $[PE\ (18{:}0/20{:}3)\text{-}H]^-$ and $[PC(16{:}0/16{:}0) + Cl]^-$, respectively.

4 Notes

1. Depending on the cell line and growth rate, an appropriate density of cells should be seeded on the Petri dish to make sure that individual cells could be easily distinguished for sampling.
2. Direct contact with the sharp end of the capillary tip can cause injury and should be avoided. The capillaries should be disposed in sharp containers.
3. Direct contact with the high voltage connections can cause electric shock or death. All the electrical components should be insulated and shielded. Do not touch the electrical components when the high voltage power supply is turned on.
4. When touching the dish bottom, the capillary tip might break. On such occasions, a large amount of PBS solution enters the capillary. To avoid PBS interference in the mass spectra, a new tip should be used.
5. While approaching the cell, the PBS solution may enter the capillary tip and introduce interference during analysis. Mass spectra of the PBS solution are separately acquired for background subtraction.

Acknowledgements

The authors acknowledge the financial support from the U.S. National Science Foundation under Grant No. CHE-1152302.

References

1. Walker BN, Antonakos C, Retterer ST, Vertes A (2013) Metabolic differences in microbial cell populations revealed by Nanophotonic ionization. Angew Chem Int Ed 52:3650–3653
2. Ibanez AJ, Fagerer SR, Schmidt AM, Urban PL, Jefimovs K, Geiger P, Dechant R, Heinemann M, Zenobi R (2013) Mass spectrometry-based metabolomics of single yeast cells. Proc Natl Acad Sci U S A 110:8790–8794

3. Zenobi R (2013) Single-cell metabolomics: analytical and biological perspectives. Science 342:1243259
4. Shrestha B, Vertes A (2009) In situ metabolic profiling of single cells by laser ablation electrospray ionization mass spectrometry. Anal Chem 81:8265–8271
5. Svatos A (2011) Single-cell metabolomics comes of age: new developments in mass spectrometry profiling and imaging. Anal Chem 83:5037–5044
6. Wilson ID, Plumb R, Granger J, Major H, Williams R, Lenz EA (2005) HPLC-MS-based methods for the study of metabonomics. J Chromatogr B 817:67–76
7. Pitt JJ (2009) Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. The Clinical biochemist Reviews / Australian Association of Clinical Biochemists 30:19–34
8. Bush MF, Campuzano IDG, Robinson CV (2012) Ion mobility mass spectrometry of peptide ions: effects of drift gas and calibration strategies. Anal Chem 84:7124–7130
9. Lanucara F, Holman SW, Gray CJ, Eyers CE (2014) The power of ion mobility-mass spectrometry for structural characterization and the study of conformational dynamics. Nat Chem 6:281–294
10. Shrestha B, Vertes A (2014) High-throughput cell and tissue analysis with enhanced molecular coverage by laser ablation electrospray ionization mass spectrometry using ion mobility separation. Anal Chem 86:4308–4315
11. Paglia G, Williams JP, Menikarachchi L, Thompson JW, Tyldesley-Worster R, Halldorsson S, Rolfsson O, Moseley A, Grant D, Langridge J, Palsson BO, Astarita G (2014) Ion mobility derived collision cross sections to support metabolomics applications. Anal Chem 86:3985–3993
12. Paglia G, Angel P, Williams JP, Richardson K, Olivos HJ, Thompson JW, Menikarachchi L, Lai S, Walsh C, Moseley A, Plumb RS, Grant DF, Palsson BO, Langridge J, Geromanos S, Astarite G (2015) Ion mobility-derived collision cross section as an additional measure for lipid fingerprinting and identification. Anal Chem 87:1137–1144
13. Fenn LS, McLean JA (2011) Structural resolution of carbohydrate positional and structural isomers based on gas-phase ion mobility-mass spectrometry. PCCP 13:2196–2205
14. Zhang L, Foreman DP, Grant PA, Shrestha B, Moody SA, Villiers F, Kwak JM, Vertes A (2014) In situ metabolic analysis of single plant cells by capillary microsampling and electrospray ionization mass spectrometry with ion mobility separation. Analyst 139:5079–5085
15. Zhang L, Sevinsky CJ, Davis BM, Vertes A (2018) Single-cell mass spectrometry of subpopulations selected by fluorescence microscopy. Anal Chem 90:4626–4634
16. Zhang L, Khattar N, Kemenes I, Kemenes G, Zrinyi Z, Pirger Z, Vertes A (2018) Subcellular peptide localization in single identified neurons by capillary microsampling mass spectrometry. Sci Rep 8, 12227
17. Zhang L, Vertes A (2018) Single-cell mass spectrometry approaches to explore cellular heterogeneity. Angew Chem Int Ed 57:4466–4477