Chapter 3

Relative Quantitation in Single-Cell Metabolomics by Laser Ablation Electrospray Mass Spectrometry

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Abstract

Single-cell analysis of metabolites by mass spectrometry (MS) is challenging due to the very limited volume and inherent molecular complexity of the sample. Quantitative metabolomic analysis of individual cells provides information on the metabolic heterogeneity of cells unattainable by aggregate analysis of multiple cells. Depending on the ionization method, MS can offer quantitative analysis for a broad class of metabolites exhibiting both high sensitivity and selectivity. Laser ablation electrospray ionization (LAESI) has been successfully exploited to analyze metabolites from broad range of biological samples, including single cells and small cell populations. In this work, we describe a protocol for the relative quantitation of metabolites in single cells by LAESI-mass spectrometry.

Key words Single-cell analysis, Metabolomics, Metabolites, Laser ablation electrospray ionization, LAESI, Mass spectrometry

1 Introduction

Exploring phenotypical heterogeneity within a population of isogenic cells requires quantitative metabolomic analysis of single cells [1, 2]. Mass spectrometry (MS) enables the multispecies analysis of numerous metabolites simultaneously [3]. Cell lysates of single human erythrocytes and ruptured neurons of the snail nervous system were successfully analyzed by matrix-assisted laser desorption ionization (MALDI) MS [4, 5]. Electrospray ionization (ESI) was utilized to analyze metabolites in brown rat mast cells by using the electrospray emitter as a micropipette to extract the cellular contents. Coupled with capillary electrophoresis ESI was used to detect metabolites from a single neuron of a California sea slug [6, 7]. Direct metabolic analysis of single cells and small cell populations can be performed by laser ablation electrospray ionization (LAESI)-MS in the ambient environment without sample extraction [8–13]. In LAESI-MS, water-rich biological samples are

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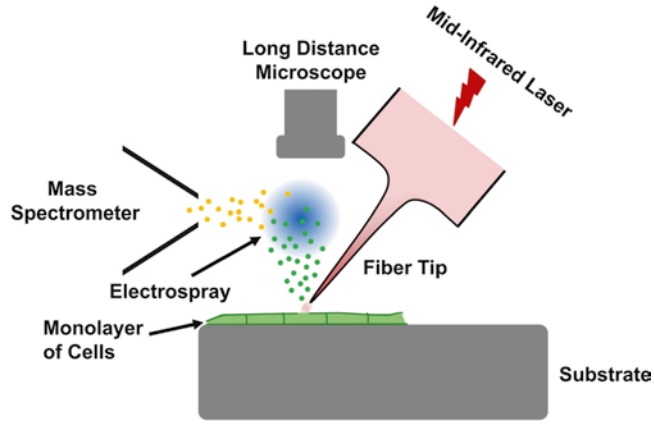

Fig. 1 Conceptual representation of a fiber-LAESI ion source for the analysis of adherent single cells or cellular monolayer tissue placed on a substrate. A single cell is ablated with a mid-infrared (IR) laser pulse through a sharpened GeO_2-based glass fiber. The resulting ablation plume (*green dots*) is intercepted by electrospray (*blue plume*) delivered perpendicular to the plane of the drawing. The produced LAESI ions (*yellow dots*) are analyzed by a mass spectrometer. This figure appears in color in the online version of this chapter (color figure online)

ablated by a mid-infrared (IR) laser pulse followed by the ionization of the ablation plume by an electrospray [14]. In single-cell LAESI-MS, the laser energy is deposited into the cell by a sharpened GeO_2-based optical fiber [8]. The conceptual representation of the fiber-based LAESI-MS ion source is shown in Fig. 1. Multivariate statistical analysis can be employed to infer metabolic differences between cells by LAESI-MS as well as capillary electrophoresis (CE) coupled to MS [13, 15].

Quantitative comparison of metabolite concentration is necessary for assessing the differences between individual cells. Mass spectrometric quantitation of small molecules frequently utilizes liquid chromatography (LC) ESI-MS or CE ESI-MS [16, 17]. MS quantitation requires that the mass spectrometric peak intensities are proportional to the concentrations of the analyzed metabolites and the amount of the consumed sample. Relative quantitation relies on measuring peak intensity ratios for each analyzed cell and comparing them over the studied population. This approach works best if the reference ion intensity does not change significantly for the studied cells. As cell volumes can differ substantially within a population, either correction has to be achieved or only a fixed subcellular volume has to be analyzed. Assuming a constant cell thickness, measuring the observed cell surface area in a microscope image can be used to account for cell volume differences. Using laser ablation sampling, for a well-defined ablation geometry fixing the number of delivered laser pulses can assure that the same subcellular volume is removed for analysis.

In quantitation by multiple reaction monitoring (MRM), intensities of a metabolite fragment produced by tandem MS are compared [18]. This approach discounts the interfering effect of other chemical species with an indistinguishable mass ensuring accurate quantitation. Absolute quantitation of known metabolites can be performed by introducing known amounts of isotope-labeled internal standards. Quantification is achieved by comparing the signal intensities between the known standard and the indigenous metabolites. Some ambient ionization methods, for example desorption electrospray ionization (DESI), utilized isotopically labeled internal standards to perform quantitative analysis of small molecules [19]. In the current contribution, we discuss a protocol for the relative quantitation of metabolites in adherent cells and cells in a monolayer by LAESI-MS.

2 Materials

2.1 Optical Fiber Preparation

1. Remove the coatings on both ends of a germanium dioxide (GeO_2)-based optical fiber (Infrared Fiber Systems, Silver Spring, MD) before its use as follows.
   (a) Heat 1-methyl-2-pyrrolidinone in a beaker to ~150 °C inside a fume hood. The depth of this softening agent should be the same as the desired length of coating that is removed.
   (b) Insert a fiber end into the heated solvent for ~1 min leading
   (c) Immerse the softened coating into isopropanol for a minute, and then remove and peel off any remaining coating with a lint-free tissue. Rinse with the isopropanol solvent.
   (d) Repeat the last two steps for the other end of the fiber.
2. If reduction of the fiber length is necessary, score and gently snap the uncoated fiber with a sapphire blade (e.g., KITCO Fiber Optics, Virginia Beach, VA, USA).
3. Chemical etching of a fiber end to produce a sharpened tip is performed as follows.
   (a) Dip one of the uncoated fiber ends into 1 % (*v*/*v*) reagent-grade nitric acid solution. After the initial contact, insert the fiber vertically 0.3–0.5 mm into the diluted acid. The acid should form concave meniscus around the fiber tip.
   (b) In ~15 min, the tip thins at the meniscus and the lower segment spontaneously detaches leaving a sharpened tip.
   (c) Rinse with deionized water to remove the acid residues.

2.2 Targeting Microscopes

Two long-distance microscopes (a top-view and a side-view device) are utilized to align the sharpened optical fiber tip for the ablation of single cells. Both homebuilt microscopes are based on 7× precision zoom video microscope units (Edmund Optics, Barrington, NJ, USA) and 5× or 10× infinity corrected long working distance objective lenses (M Plan Apo; Mitutoyo Co., Kanagawa, Japan). The images are captured by CCD cameras (Marlin F131, Allied Vision Technologies, Stadtroda, Germany) attached to a personal computer via FireWire connection.

2.3 Laser Ablation and Mass Spectrometry

1. Laser pulses at 2.94 μm wavelength are produced by the combination of Nd:YAG laser with an optical parametric oscillator. Pulses of 5 ns length emitted with a repetition rate between 5 and 20 Hz (Opolette 100, Opotek Inc., Carlsbad, CA, USA).
2. Mount the sharp end of the fiber on a micromanipulator (e.g., MN-151, Narishige, Tokyo, Japan).
3. Hold the blunt end of the fiber with a bare fiber chuck (BFC300, Siskiyou Corporation, Grants Pass, OR, USA), and mount the chuck on a five-axis translator stage (BFT-5, Siskiyou Corporation, Grants Pass, OR, USA). This aids the alignment of the fiber with the laser beam for efficient coupling.
4. Laser pulses of ~1 mJ initial energy are steered by gold-coated mirrors (PF10-03-M01, Thorlabs, Newton, NJ, USA) to a plano-convex calcium fluoride lens (Infrared Optical Products, Farmingdale, NY, USA) that focuses the beam onto the blunt end of the optical fiber.
5. The laser beam is delivered through the etched end of the fiber that is moved close to the cell surface for efficient energy deposition and ablation.
6. The sample is held on a substrate, e.g., a pre-cleaned microscope slide mounted on a plate holder (FP01, Thorlabs Inc., Newton, NJ, USA) and positioned by a manual or a motorized three-axis translation stage (Thorlabs Inc, Newton, NJ, USA).
7. To minimize artifacts due to sample drying, an environmental chamber with humidity control might be necessary.
8. In case of delivering a set number of laser pulses, a high-performance optical shutter (SR470, Stanford Research Systems Inc., Sunnyvale, CA, USA) can select the pulse count for each cell.
9. A mass spectrometer with an atmospheric pressure interface, e.g., designed for electrospray analysis, can acquire the LAESI mass spectra (Q-TOF Premier, Wasters Co., Milford, MA, USA).

2.4 Electrospray

1. The following components can be used to fabricate an electrospray assembly: a metal union with a conductive perfluoroelastomer ferrule, fittings, tubing sleeve, needle port (U-435, M215, F-331Nx, F-242x or 9013; IDEX Health & Sciences, Oak

Harbor, WA, USA), fused silica tubing, metal taper tips (CT360-100-50-5 or MT320-50-5-5, New Objective Inc., Woburn, MA, USA), blunt 22 gauge syringe (Kel-F hub 90134, Hamilton Company, Reno, NV, USA), 500 μl syringe (81222, Hamilton Company, Reno, NV, USA).

2. Supply the electrospray solvent at 200–300 nl/min flow rate by a syringe pump (Harvard Apparatus, Holliston, MA, USA) to the emitter.
3. Apply a high voltage (~3.0 kV) to the metal union by a regulated power supply (e.g., PS350, Stanford Research Systems Inc, Sunnyvale, CA, USA).

2.5 Samples and Chemicals

1. Obtain a single layer of adherent cells or a cellular monolayer of tissue, such as the epidermal tissue of an *Allium cepa* bulb.
2. Use HPLC-grade methanol and water (Acros Organics, Geel, Belgium) (1:1 ratio) as the electrospray solution. Acidify the solution with glacial acetic acid (0.1 %, v/v) (Fluka, Munich, Germany).

3 Methods

To perform single-cell analysis by LAESI-MS, cells are selected by moving the sample using the translation stage and observing the cells with the top-view microscope. A single cell is chosen for ablation by locating it below the apex of the fiber tip.

1. Assemble the single-cell LAESI-MS experimental setup as described in Subheading 2.
2. Couple the laser beam into the optical fiber, and fill the syringe with the electrospray solution.
3. Bring the mass spectrometer to operational mode.
4. Mount the sample on the substrate in front of the mass spectrometer inlet orifice.
5. Run the syringe pump, and turn on the high voltage connected to the electrospray emitter. Adjust the flow rate and the high voltage to achieve a stable spray.
6. Switch on both of the long-distance microscopes, and focus them for clear observations. The side-view microscope is used to adjust the distance between the etched fiber tip and sample surface for optimal ablation, whereas the top-view microscope visualizes the cells and helps to align the fiber tip over the cell selected for ablation.
7. Optimize the LAESI ion source geometry by adjusting the position of the fiber tip and the sample with respect to the electrospray emitter and the mass spectrometer orifice.

8. Using the side-view microscope, initially set the distance between the fiber tip and the cell surface to 200–300 μm.
9. Observing through the top-view microscope, move the sample laterally so that the outline of the fiber tip is over the cell selected for ablation.
10. Lower the fiber tip to a distance of 20–30 μm above the cell surface. Utilize the side-view microscope with a video feedback during this alignment.
11. Start the acquisition of mass spectra.
12. Set repetition rate of laser to produce the highest signal-to-noise ratio in the mass spectrum without disrupting the neighboring cells.
13. Set the shutter speed to obtain the desired number of laser pulses for each analysis. Keep the number of pulses per cell constant throughout the analysis.
14. Activate the mid-IR laser and open the electronic shutter. Due to the production of LAESI ions, the delivered laser pulses should result in a peak in the total ion chromatogram (*see* Fig. 2a). Selected ion chromatograms of cell-related ions show similar peaks (*see* Fig. 2b), whereas the average signal for background ions remains unchanged during the ablation process (*see* Fig. 2c). Successfully ablated single cells will exhibit an ablation mark as shown in the inset of Fig. 3.
15. Repeat the single-cell analysis for other cells.
16. End the data acquisition, and turn off the LAESI system by stopping the laser pulses, disabling the high voltage, switching off the syringe pump and the microscopes, and setting the mass spectrometer to standby mode.
17. Process the acquired data to obtain peak areas for the ions related to the metabolites of interest for each cell. Representative mass spectra from two single cells are shown in Fig. 3. The two spectra exhibit similar peak areas (889 and 831) for the *m/z* 381.082 potassiated disaccharide (sucrose ion), whereas they show a high intensity (1,585) for the *m/z* 535.113 cyanidin malonyl glucoside ion in the purple cell and no signal in the nonpigmented cell.
18. Normalize the peak areas for the metabolites of interest with reference to the peak area for the reference ion with relatively constant peak areas. In case the entire cell content was ejected for analysis, the cell volume variations have to be factored in.
19. The normalized peak area counts provide relative quantitation for the metabolites of interest.

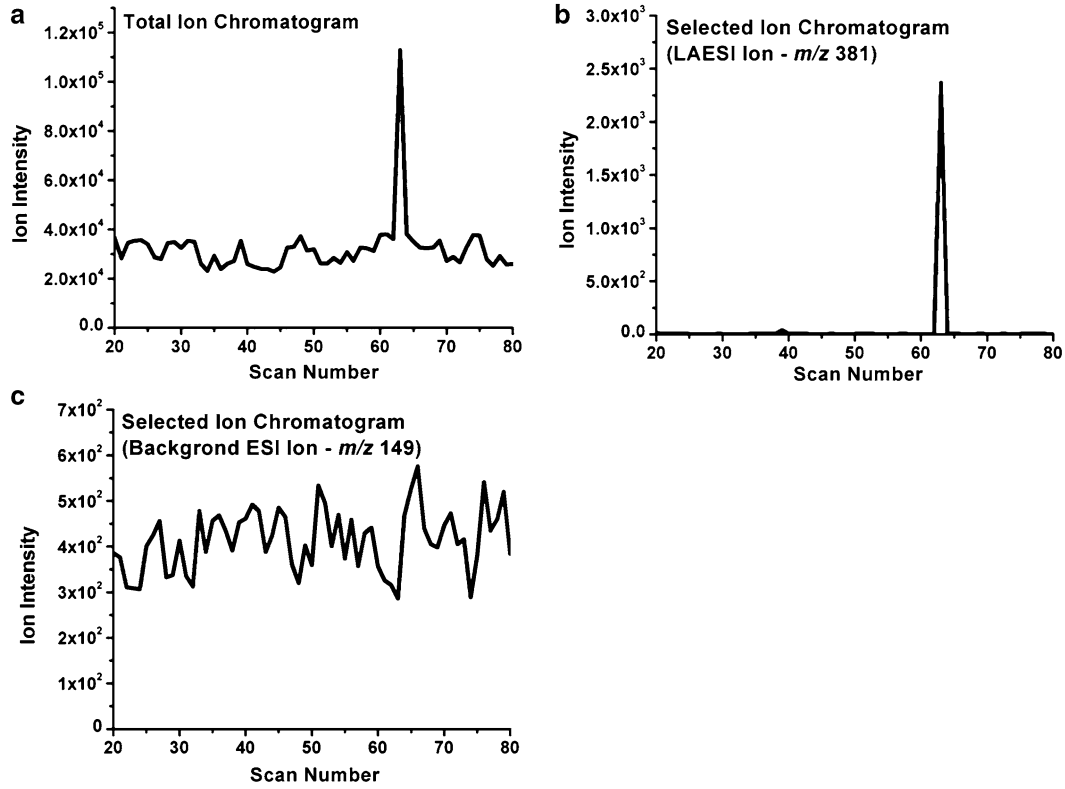

Fig. 2 An LAESI mass spectrum from a single cell was acquired on scan number 63. (**a**) The total ion chromatogram (TIC) shows a spike during the interrogation of the cell. (**b**) The selected ion chromatogram of the *m/z* 381.082 ion displays a sharp peak in the same scan range indicating that this ion originates from the cell. (**c**) The selected ion chromatogram for a background ion (e.g., *m/z* 149) produced solely from the electrospray solution remains unchanged throughout the analysis

4 Notes

1. The mid-IR laser used in these experiments is a class IV laser that may cause severe and permanent damage to the eyes or the skin. The diffuse reflections of the laser beam can also be hazardous to the eyes or the skin. During the operation of the laser wear appropriate eye protection and avoid skin exposure.
2. A direct contact with the high voltages applied in these experiments can result in electric shock or death. Make sure that all the electrical connections are properly insulated and shielded. Do not touch the electrified components until the voltage is turned off and the charges dissipated.
3. In the absence of an environmental chamber, acquire the mass spectra within a couple of minutes following the preparation of the sample to avoid its dehydration.

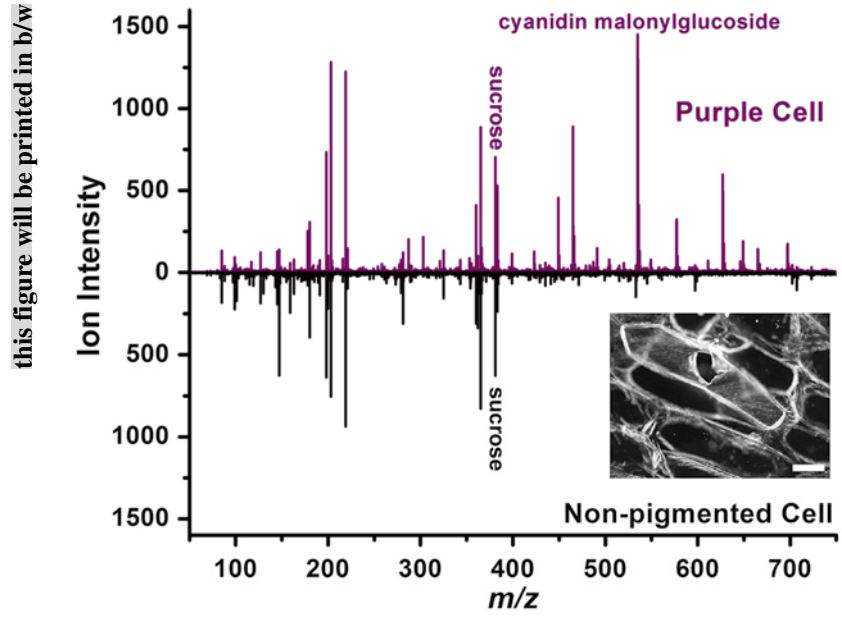

Fig. 3 Single-cell LAESI mass spectra for a purple cell (*top*; appears in *purple* in the online version of this chapter) and nonpigmented cell (*bottom*) from *A. cepa* epidermis in its bulb. The spectra show similar ion intensities for the sucrose ions but significantly higher intensity for the cyanidin malonyl glucoside in the purple cell. The *inset* displays a microscope image of the ablation mark on an *A. cepa*

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