Methodologies for Metabolomics

EXPERIMENTAL STRATEGIES AND TECHNIQUES

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6 Direct Metabolomics from Tissues and Cells: Laser Ablation Electrospray Ionization for Small Molecule and Lipid Characterization

Akos Vertes, Bindesh Shrestha, and Peter Nemes

The systematic study of metabolites and their related pathways in various organisms is experiencing a renaissance. In the first half of the twentieth century, studying select metabolic processes played a central role in biochemistry and brought about major discoveries, such as the ornithine and the citric acid cycle (Kornberg, 2000). From the middle of the century, increasingly powerful tools used in metabolism research included efficient separation methods (gas chromatography [GC] and high-performance liquid chromatography) combined with spectrometers aimed at small molecules (nuclear magnetic resonance [NMR] and mass spectrometry [MS]). In the 1970s and early 1980s, genetics took center stage, aided by the introduction of DNA sequencing, polymerase chain reaction (PCR), and recombinant DNA technologies.

In the late 1980s, with the introduction of soft ionization methods in MS, the systematic investigation of peptides and proteins became possible, and proteomics was born. During the 1990s and the first decade of the twenty-first century, researchers in genomics accomplished the sequencing of genomes in multiple species. The development of microarray techniques gave us powerful tools to study the transcriptome, and, with the help of new techniques in MS, investigators in proteomics began the challenging task of mapping the corresponding proteomes.

Metabolomics – the systematic investigation of the metabolites and metabolic processes in an organism – is lagging behind owing to multiple challenges. In contrast to nucleic acids and proteins, metabolites are structurally diverse. As a consequence, their separation and spectroscopic identification require a wide variety of protocols. Metabolite concentrations also vary extensively in space and time; for example, the temporal changes are more rapid than in the case of biopolymers. Typical changes in the transcriptome can take minutes, hours, or longer (Ye et al., 2009), whereas protein and metabolite concentrations vary in the range of minutes and seconds. Conventional metabolomic protocols rely on rapid sampling that includes quenching of the biochemical processes and often derivatization before separation and spectrometric analysis (Smart et al., 2010). Because of these complex steps, it is always questionable how close to its native state the studied system has remained.

To explore the native metabolome, new methods are needed that respond to various small organic molecules on a time scale of seconds. Ideally, these techniques have to be capable of local analysis and exhibit sufficiently high sensitivity. Because metabolite molecules are much smaller than nucleic acids or proteins, they are more

prone to spatial redistribution during sample preparation. The local analysis and molecular imaging of metabolites in tissues and cells also require special considerations (see Chapters 18, 21, and 22 for noninvasive and nondestructive in vivo metabolic imaging by NMR techniques).

The advent of atmospheric pressure ionization methods for MS has enabled the direct analysis of tissues and cells with little or no sample preparation. This rapidly expanding group of technologies has been used extensively to analyze biological samples directly and includes ambient desorption electrospray ionization (DESI) (Takats et al., 2004), direct analysis in real time (DART) (Cody et al., 2005), extractive electrospray ionization (EESI) (Chen et al., 2006a, 2007), atmospheric pressure infrared matrix-assisted laser desorption ionization (AP IR-MALDI) (Li et al., 2007; Vertes et al., 2008), electrospray-assisted laser desorption ionization (ELDI) (Shiea et al., 2005), and laser ablation electrospray ionization (LAESI) (Nemes and Vertes, 2007). Many of these techniques can be characterized as in situ or, in some cases, in vivo methods (Nemes and Vertes, 2012). Although the analysis sacrifices some cells of the studied organism, it is less obtrusive than biopsies. Ultimately, sampling can be arranged so that a living organism can be viewed unperturbed, as long as the sampled volume is significantly smaller than the volume of the organism. This chapter focuses on the applications of LAESI MS for the direct analysis of tissues and cells with two-dimensional and three-dimensional imaging and depth profiling of small metabolites and lipids.

1. Direct Tissue Analysis and Imaging with Laser Ablation Electrospray Ionization

In the field of biomedical analysis, LAESI MS has exhibited considerable success in interrogating the small molecule composition of biological fluids, such as blood plasma and urine as well as plant, animal, and human tissues and single cells. There are numerous fundamental and unique aspects that place LAESI MS among the enabling methodologies in direct metabolomic investigations.

In LAESI MS, the sampling step is spatially independent from that of ion generation. First, the native water molecules of the sample are excited by a focused mid-infrared laser light. At a wavelength of 2,940 nm, rapid energy deposition occurs through the excitation of the vibrational modes of water molecules. Provided that sufficient energy is coupled into the sample, the result of the process is phase explosion–driven ablation that expels particulate matter within a few hundred microseconds (Apitz and Vogel, 2005; Chen et al., 2006b; Chen and Vertes, 2008), and the momentum gained by the ejectiles transports them to several tens of millimeters above the sample surface (Nemes and Vertes, 2007).

An electrospray source, preferably operated in the cone-jet mode, efficiently generates a cloud of small charged droplets (Nemes et al., 2007) that intercepts the particles ablated from the sample (Nemes and Vertes, 2007). As the droplets coalesce with the particulate matter, the metabolites of the sample are transferred into a charged solution–phased microenvironment. Ultimately, metabolites are converted to soft ions via processes similar to ion production during conventional electrospray ionization (ESI) (Nemes et al., 2012). Correspondingly, with LAESI MS, samples can be analyzed within seconds, providing a snapshot of the fast-evolving metabolite

composition. This forms the basis of high-throughput and large-scale investigations using LAESI MS.

1.1. Label-Free Metabolite Identification

The ions generated in LAESI can be analyzed by any mass spectrometer that is equipped with an atmospheric pressure interface. Most commonly, a commercial ESI or atmospheric pressure chemical ionization source is replaced by a custom-built LAESI ion source. More recently, the introduction of commercial LAESI ion sources has been announced (Protea Biosciences, Inc., Morgantown, WV). The LAESI system has been successfully implemented on time-of-flight and ion trap instruments that feature quadrupole and hexapole ion guides for enhanced ion transfer and collisional activation. The mass spectrometer can be operated in single-stage or tandem modes to facilitate chemical identification (Nemes and Vertes, 2007).

The application of LAESI MS in metabolomics follows different workflows for metabolite discovery than for the structural identification of known compounds. The latter typically employs a multistep MS-based approach. Accurate mass-to-charge ratio (m/z) measurement is the starting point in the structure identification by LAESI MS. Ions are measured with m/z accuracies such as less than 5 ppm, although for targeted compounds lower accuracies can also yield satisfactory results. The measured m/z values are compared against metabolomic databases for prokaryotic, plant (e.g., http://www.plantcyc.org/), and animal species as well as human samples (e.g., http://www.hmdb.ca/). There is a growing number of multiorganism pathway databases integrated with genomic and proteomic data (e.g., http://metacyc.org/, http://www.genome.jp/kegg/pathway.html) that can also help identification.

Isotope distribution patterns can also be determined to narrow the list of putative compounds further. The remaining positive matches are evaluated in tandem MS experiments, whereby the fragmentation behavior of the unknown ion is compared with that of chemical standards. In certain cases, a reactant can be added to the electrosprayed solution to transform the analytes chemically. Here the objective is to improve the ion yield or facilitate structure-specific fragmentation. The latter can be beneficial for lipids that are known to yield a very limited number of fragments in collision-activated dissociation from protonated precursors. Introducing lithium ions via the electrosprayed solution leads to the generation of lithiated lipid species that readily fragment into numerous characteristic product ions that help to decipher the structure of the parent lipid molecule (Shrestha et al., 2010a). This multistep elucidation scheme for known metabolites usually yields identifications with high analytical confidence for biomedical samples.

In the metabolite discovery mode (i.e., in cases where a particular metabolite has not been described in the literature for a given species), the use of databases is of limited help. In these situations, tandem MS–based techniques are mandatory, and orthogonal analytical techniques are needed to enhance the confidence of identification further. The latter include offline experiments using classic separation methods (e.g., liquid chromatography with MS detection). An appealing yet unused alternative is coupling LAESI with ion mobility spectrometry (Bohrer et al., 2008), which could separate structural isomers. In this experiment, the time of LAESI ion

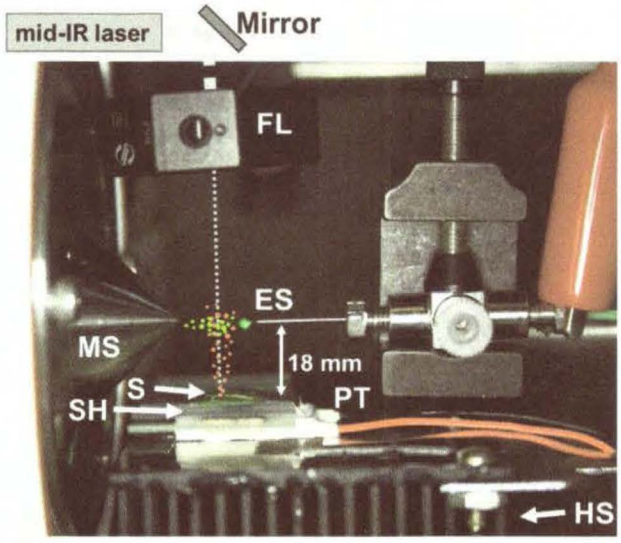

Figure 6.1. Schematic of the LAESI MS apparatus for metabolic investigations directly from tissues. The sample (S), positioned on a sample holder (SH), is ablated by a mid-infrared laser focused by a focusing lens (FL), and the ablation plume (*red dots*) is intercepted by charged droplets (*green dots*) generated by an electrospray source (ES). The charged droplets are seeded with the neutral ablated material, which results in the production of sample-specific ions that are analyzed by a mass spectrometer (MS). The sample is optionally cooled or kept frozen by a Peltier stage (PT) equipped with a heat sink (HS) and fans.

generation would be synchronized with the injection of ions into the ion mobility cell. Combining LAESI with ion mobility spectrometry raises the prospect of improving confidence in metabolite identification while maintaining fast sample analysis.

1.2. Metabolic Analysis

The flexibility that results from decoupling sampling from ion generation has far-reaching benefits in practical metabolomic investigations. Perhaps one of the most attractive features of LAESI MS is that the biomedical analysis is facilitated by the inherent water content of the sample. As shown in Figure 6.1, LAESI MS allows direct metabolic analysis because it eliminates the requirement to modify the biological or medical sample. For example, certain metabolite and xenobiotic molecules have been analyzed in water-containing samples, such as bodily fluids, within seconds without any sample preparation. Examples show that small metabolites (e.g., carnitine, phosphocholine, tetradecenoylcarnitine) and lipid molecules (e.g., glycerophosphocholines [PCs]; the lipid nomenclature accepted by the LIPID MAPS consortium [http://www.lipidmaps.org/] is used throughout this chapter) can be readily detected, and the excretion of drug molecules (e.g., the antihistamine fexofenadine) can be monitored directly from whole blood and urine (Nemes and Vertes, 2007). LAESI mass spectra often include multiply charged ions, a phenomenon typically observed in ESI. Deconvolution of the ion charge states reveals abundant proteins in human blood, including the serum albumin and the α and β chains of hemoglobin. Thus, the molecular mass range of LAESI MS analysis extends from small metabolites to macromolecules greater than 66 kDa.

Local analysis is another important feature of the LAESI MS method. The analysis area of the laser beam is typically approximately 200 μm in diameter and can be reduced to 50 μm without significant signal loss. The ablation dimensions can be carefully adjusted for various tissue and cell types to support in situ, ex vivo, and in vivo experiments. The latter can be observed in Figure 6.2, which shows the

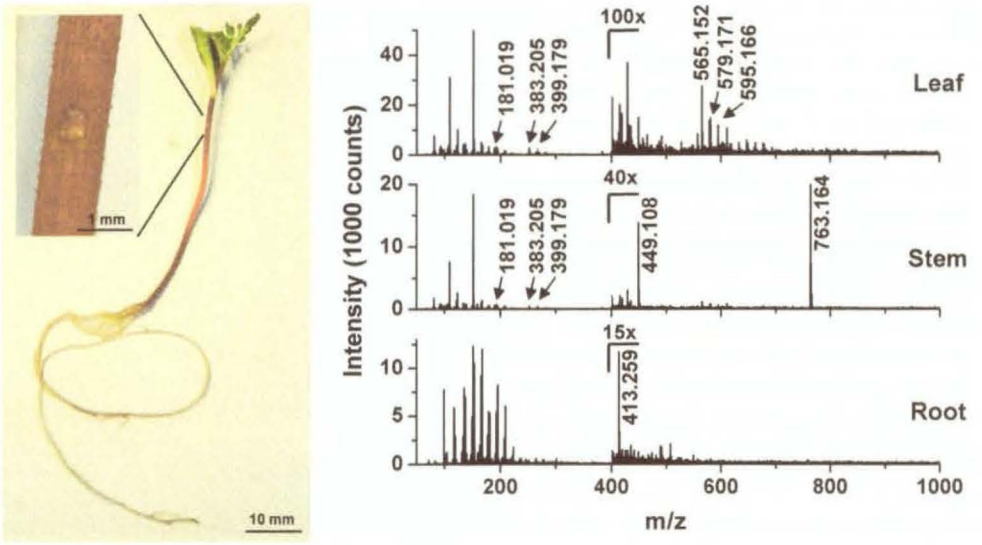

Figure 6.2. In vivo analysis of a French marigold seedling by LAESI MS. (*left panel*) In these profiling experiments, mass spectra, acquired on 350-μm-diameter areas of the leaf, stem and root, reveal that (*right panel*) the organs exhibit dramatically different primary and secondary metabolite composition. (Adapted with permission from Nemes, P., and Vertes, A. [2007]. Laser ablation electrospray ionization for atmospheric pressure, in vivo, and imaging mass spectrometry. *Anal. Chem.* 79, 8098–8106. Copyright 2007 American Chemical Society.)

metabolic profiling of the root, stem, and leaf regions of a live French marigold (*Tagetes patula*) seedling (Nemes and Vertes, 2007). The observed ions have been assigned to primary and secondary metabolites. With superficial damage to the live tissue, local analysis enables intensity profiles to be recorded across the various regions of the seedling. For example, the root primarily generates ions with m/z less than 250, the stem yields sodiated kaempferol 3-*O*-(2″,3″-di-*p*-coumaroyl)-glucoside or some of its structural isomers with an exceptionally high abundance, whereas the leaf produces various ions assigned to photosynthetic products.

Quantitative LAESI analysis facilitates metabolomic investigations. LAESI ion signal intensities from solutions of drug standards indicate that they follow a linear correlation with analyte concentrations over a four-decade dynamic range (Nemes et al., 2007). Metabolites in biological samples are present in a complex matrix of constituents, each of which can compete for the available charge within the electrosprayed droplets. In some cases, this results in narrower dynamic ranges or limits its quantitation to the determination of relative concentrations within the sample. Using deuterated analogues as internal standards, the absolute concentrations of small metabolites, including neurotransmitters and osmolytes (e.g., choline, carnitine, and betaine), have been identified in the electrical organ tissue of *Torpedo californica* (Sripadi et al., 2009). Other reports have established quantitation of lipids in the mouse brain, allowing the measurement of the absolute concentration of PC (34:1) with good analytical performance (Shrestha et al., 2010a). The analyte levels determined by LAESI MS have agreed with levels reported by traditional analytical methods. The ability to correlate ion signal intensities with native metabolite

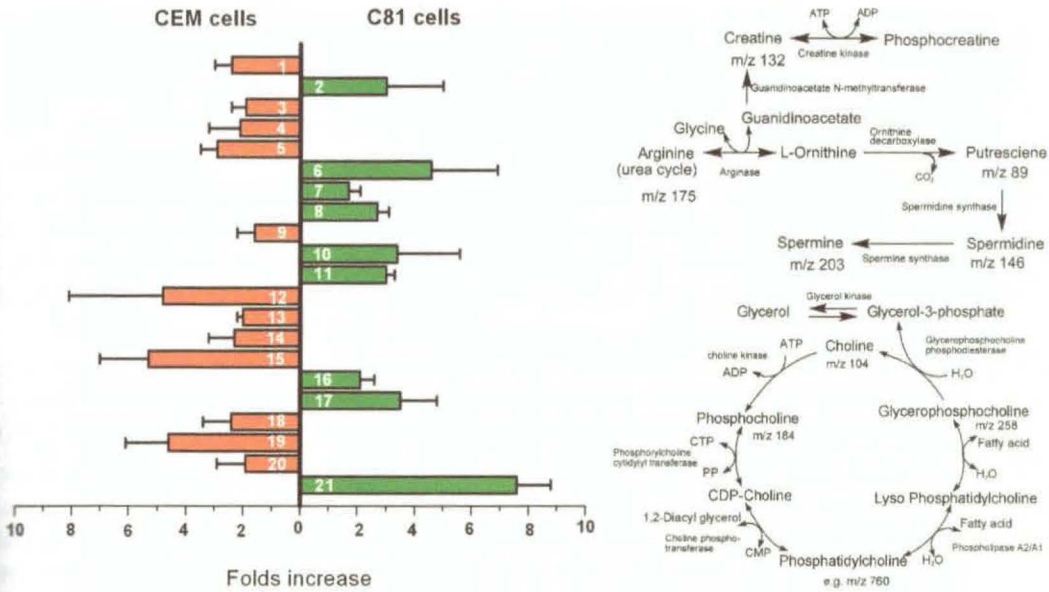

Figure 6.3. (*left panel*) Metabolic differences between control (CEM) and virally transformed T lymphocytes (C81) identified by LAESI MS. The most affected metabolites were (*1*) thioacetamide, (*2*) putrescine or pyrrolidine, (*3*) choline, (*4*) proline, (*5*) taurine, (*6*) creatine, (*7*) spermidine, (*8*) *p*-aminobenzoic acid, (*9*) iminoaspartic acid, (*10*) arginine, (*11*) dopamine, (*12*) phosphocholine, (*13*) carbamoyl-phosphate, (*14*) spermine, (*15*) methoxytyramine, (*16*) *N*-acetyl aspartic acid or *N*-formyl glutamic acid, (*17*) homovanillic acid, (*18*) glycerophosphocholine, (*19*) glutathione, (*20*) 8-hydroxyguanosine, and (*21*) adenosine monophosphate. These changes pointed to significant perturbations in the (*top right panel*) creatine and polyamine biosynthesis pathways and the (*bottom right panel*) lipid metabolism pathway. (Adapted from Sripadi, P., Shrestha, B., Easley, R. L., Carpio, L., Kehn-Hall, K., Chevalier, S., et al. [2010]. Direct detection of diverse metabolic changes in virally transformed and Tax-expressing cells by mass spectrometry. *PLoS ONE* 5, e12590.)

concentrations in tissues and cells is essential in the investigation of metabolic changes in an organism.

Human metabolic pathways and their changes can also be explored by LAESI MS. Virally transformed and Tax-expressing human cells were profiled more recently in high-throughput experiments (Sripadi et al., 2010). Figure 6.3 presents two of several biosynthetic pathways significantly altered by the viral transformation of host cells. Such measurements can be performed in a few minutes and require only small cell populations. With LAESI MS, several virus type–specific (HTLV1 vs. HTLV3), expression-specific (Tax1 vs. Tax3), and cell type–specific (T lymphocytes vs. kidney epithelial cells) changes were observed in the metabolite profiles. These features indicate that LAESI MS can be used in biomarker discovery and patient monitoring. More extensive investigations addressing how disease states alter cellular metabolic pathways can also enable new treatment strategies.

1.3. Metabolic Imaging

LAESI MS offers a means to achieve local analysis with spatial resolution to 50 μm. Under appropriate experimental conditions (e.g., temperature and humidity), the

structural and chemical integrity of biological tissues and cells is essentially retained around the ablated spot. Assuming proportionality between the LAESI MS signal and the metabolite concentration in the tissues, spatial variations in the chemical composition of these systems can be interrogated. The ion signal measured across the tissues allows the reconstruction of the corresponding molecular image. To perform LAESI mass spectrometry imaging (MSI), the sample is mounted onto a sample holder (Figure 6.1). Using two computer-controlled independent translation stages (*X* and *Y* directions), the sample is positioned at the focal point of the ablating mid-infrared light. The generated ions are simultaneously mass-analyzed, and the data are stored for each *X*-*Y* coordinate of the interrogated area. Detailed protocols of lateral imaging by LAESI MS and data evaluation can be found elsewhere (Nemes and Vertes, 2010a, 2010b). The molecular image of the sample for a particular species is reconstructed by representing the intensity of the related ion signal on a false color scale and correlating it with the coordinates for every pixel of the interrogated area.

Most plant tissues are structurally different from tissues found in animals and humans. In plants, the waxy cuticle and the rigid cell walls act as natural barriers for unwanted water loss in an MSI experiment. In contrast, when animal tissues are dissected, evaporative water loss is relatively rapid. Because water serves as the energy coupling medium in LAESI experiments, animal tissue sections are generally kept cold or frozen during analyses (Nemes et al., 2010; Shrestha et al., 2010a). A Peltier-cooling stage equipped with a heat sink and a fan, as shown in Figure 6.1, can maintain the appropriate conditions for several hours (Nemes and Vertes, 2010b) and ensure that the water content of tissues does not appreciably change during the LAESI MSI time frame.

Direct molecular imaging of thin animal tissue sections allows the appreciation of their metabolic organization at atmospheric conditions. In a more recent example, two-dimensional distributions of more than 200 distinctive ionic species were simultaneously measured from a 100-μm-thick coronal rat (*Rattus norvegicus*) brain section (Nemes et al., 2010). Among the monitored metabolites were neurotransmitter molecules, such as γ-aminobutyric acid (GABA) and choline; polyamines, such as spermidine and spermine; and metabolites essential for chemical energy transfer, including adenosine and adenosine monophosphate. Many lipid species were also detected, and several of them were identified as PCs and glycerophosphoethanolamines (PEs).

Using high mass-resolution time-of-flight MS in conjunction with LAESI, it becomes feasible to deconvolute the spatial distribution of metabolites that possess an identical nominal mass. Figure 6.4 presents the ion images of GABA and choline, which differ only by 38 mDa in their monoisotopic mass and have been found to populate the tissue section in a vastly different manner. Similar distributions can be obtained for any detected m/z.

The massive data sets generated in LAESI MSI can be evaluated via Pearson co-localization maps (Nemes et al., 2010). Figure 6.4 shows the tissue distribution of the unassigned lipid ion m/z 702.537 and the PC (37:6) and PE (40:6) on a false-color scale. The calculated Pearson co-localization map highlights the areas where these two ions are found together. Co-localization of metabolites can be exploited in the discovery of metabolic pathways active in certain tissue regions.

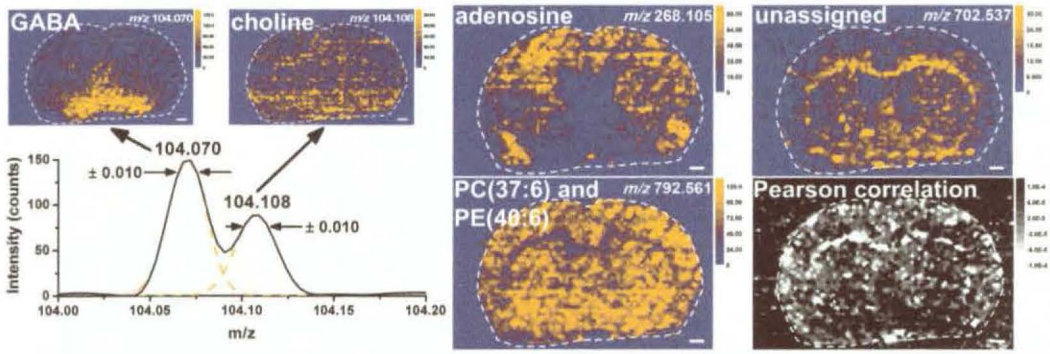

Figure 6.4. Metabolic imaging of a 100-μm-thick coronal section of a rat brain by LAESI MS. High mass-resolution differentiates among ions with identical nominal masses, including the GABA and choline ions, which exhibit dramatically different spatial distributions. Characteristic tissue localization is found for several metabolites and lipids, including adenosine, an unassigned lipid, and a combination of PC (37:6) and PE (40:6). The Pearson co-localization map between the unassigned lipid and combination of PC (37:6) and PE (40:6) distributions can help to explore metabolic relations in space. Scale bars denote 1 mm. (Adapted with permission from Nemes, P., Woods, A. S., and Vertes, A. [2010]. Simultaneous imaging of small metabolites and lipids in rat brain tissues at atmospheric pressure by laser ablation electrospray ionization mass spectrometry. *Analytical Chemistry* 82, 982–988. Copyright 2010 American Chemical Society.)

Various metabolites can also be imaged in plant tissues. Ion signals registered from plants by LAESI MS often correspond to primary and secondary metabolites, and many of them exhibit tissue-specific accumulation patterns. Leaves of the variegated zebra plant (*Aphelandra squarrosa*) can serve as an example. Some metabolites, such as kaempferol-(diacetylcoumaryl-rhamnoside), are found to be homogeneously distributed in the tissue, whereas others, including methoxykaempferol glucoronide and chlorophyll *a*, exclusively populate the yellow and green sectors, respectively (Nemes et al., 2008). In-depth investigations reveal that the distributions of certain secondary metabolites are linked to plant physiology. For example, in *Arabidopsis thaliana* leaves, LAESI MSI studies revealed that the outer lamina and the midvein synthesize 4-methylsulfinylbutylglucosinolate, indol-3-methylglucosinolate, and 8-methylsulfinyloctylglucosinolate in high concentrations (Nemes et al., 2009b). In agreement with these LAESI MSI results, independent studies found similar concentration profiles and suggested that they were consistent with a natural plant defense mechanism against herbivore attacks (Shroff et al., 2008).

1.4. Metabolic Depth Profiling and Three-Dimensional Imaging

If multiple laser pulses are delivered to the same spot on the tissue, each pulse removes a consecutive layer of cells. This enables the chemical composition of the underlying voxels (volumetric pixels) to be probed, producing a depth profile. The amount of material sampled by each laser pulse is governed by the applied laser fluence and the tensile strength of the tissue (Nemes et al., 2008; Nemes and Vertes, 2010b; Vertes et al., 2008). Mid-infrared laser ablation has been demonstrated with tissue removal rates between 30 μm/pulse and 80 μm/pulse (Nemes et al., 2008;

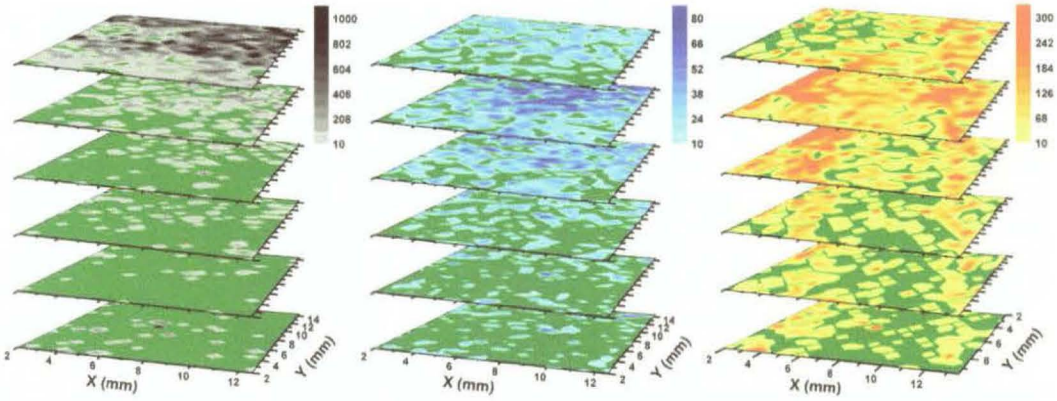

Figure 6.5. Three-dimensional metabolic imaging in leaf tissues by LAESI MS. The epidermal and palisade mesophyll regions of the *Spathiphyllum lynise* leaf tissue accumulate (*left panel*) cyanidin/kaempferol rhamnoside glucoside (m/z 595.2) and (*middle panel*) chlorophyll *a* (m/z 893.5) at high levels. (*right panel*) In the variegated leaves of *A. squarrosa*, acacetin (m/z 285.0) is abundant in the second and third layers of the tissue with a homogeneous distribution in the others. (Adapted with permission from Nemes, P., Barton, A. A., and Vertes, A. [2009]. Three-dimensional imaging of metabolites in tissues under ambient conditions by laser ablation electrospray ionization mass spectrometry. *Analytical Chemistry* 81, 6668–6675. Copyright 2009 American Chemical Society.)

Nemes et al., 2009a, 2009b). The results of single-cell analysis (Shrestha and Vertes, 2009; Shrestha et al., 2011) suggest that voxel dimensions can be tailored to reach subcellular levels.

Three-dimensional MSI is realized by the combination of lateral imaging with depth profiling (Nemes et al., 2009a). In practice, the tissue is positioned laterally while consecutive laser pulses explore the depth variations in its chemistry at each (X, Y) coordinate of the interrogated area. Detailed information on instrumentation and data analysis is available elsewhere (Nemes and Vertes, 2010b). In the acquired data sets, the intensity distribution for each m/z is mapped to the absolute position of the voxels, yielding the three-dimensional molecular images of the tissue for every detected m/z signal.

Results of three-dimensional LAESI MSI indicate that primary and secondary metabolites often populate plant tissues in a distinctive manner. For example, in the peace lily (*Spathiphyllum lynise*) leaflet, cyanidin/kaempferol rhamnoside glucoside was found in the top 40 μm (Nemes et al., 2009a). The corresponding three-dimensional distribution, seen in the *left panel* of Figure 6.5, reveals the elevated abundance of this species in the epidermal region of the tissue. In agreement with these results, independent studies reported higher concentrations of kaempferol glycosides in the upper epidermal layers, likely to serve in part as ultraviolet-screening pigments against the detrimental effects of solar radiation (Alenius et al., 1995). In contrast, the *middle panel* of Figure 6.5 shows that chlorophyll *a* is abundant in the palisade mesophyll layer of the leaf tissue from 40 to 80 μm, in agreement with the known localization of chloroplasts within plant tissues. A different distribution pattern was revealed in the three-dimensional MSI of *A. squarrosa* leaves (Nemes et al., 2009a). The *right panel* of Figure 6.5 presents the spatial distribution of

acacetin and indicates that this secondary metabolite follows the yellow sectors of the variegated tissue in the buried second and third layers but does not exhibit heterogeneity in the top and bottom epidermal layers.

The in-depth information gained by three-dimensional LAESI MSI enables metabolomic studies on a new level. For example, two-dimensional MSI shows that the distribution of kaempferol-(diacetylcoumaryl-rhamnoside) is homogeneous in the *A. squarrosa* leaf tissue (Nemes et al., 2008). However, the three-dimensional ion images indicate that this secondary metabolite accumulates only in the mesophyll layers (Nemes et al., 2009a), a piece of information that is lost in all lateral imaging experiments that fail to report on the absolute depth of analysis. The ability to interrogate biological systems in three dimensions has far-reaching implications in metabolomics and is likely to boost research in the life sciences.

2. Metabolic Analysis of Single Cells

Single-cell gene expression profiling using PCR has shown cell-to-cell variability even within the same cell types (Ståhlberg and Bengtsson, 2010). Classical analytical tools, such as laser capture microdissection coupled with microarrays, have shown heterogeneity for the gene expression among single neurons in a rat hippocampus (Kamme et al., 2003). However, standard metabolomic methods usually analyze numerous cells and provide information on the average metabolome, disregarding cell-to-cell heterogeneity. Direct single-cell metabolomics requires the analysis of chemically diverse metabolites in a small sample volume defined by the cell size. Ideally, analytical methods for direct single-cell metabolomic analysis require minimal sample preparation and exhibit high sensitivity and acute selectivity. Based on spectroscopy, chromatography, and MS techniques, a diverse array of analytical techniques have been used for studying a varying number of metabolites in single cells. Excellent reviews on this topic are available (Amantonico et al., 2010a; Schmid et al., 2010; Wang and Bodovitz, 2010). In this section, we briefly present the most common approaches used for the metabolic analysis of single cells with special emphasis on MS techniques.

One classical single-cell detection and sorting technique, flow cytometry, can be employed for the identification and separation of cells that exhibit a particular property, such as a disease state. In some cases, flow cytometry with antibody-based stains or genetically encoded fluorescence can be used to characterize a small number of biochemicals in single cells (Borland et al., 2008; Cohen et al., 2008).

Electrochemical detection of metabolites in single cells requires the use of microelectrochemical detectors (Ewing et al., 1992). The method has high sensitivity and results in label-free detection but is limited to a few easily oxidized biochemicals. In electrochemical measurements, a microelectrode can be positioned inside or near a single cell to detect easily oxidized biochemical species in the cell (Arbault et al., 1995; Ewing et al., 1992; Schroeder et al., 1992; Schulte and Schuhmann, 2007).

NMR is a well-established analytical technique for metabolic profiling of tissues and cell extracts (Beckonert et al., 2007) (see also Chapter 18). More than two decades ago, NMR spectroscopy allowed the analysis of metabolites in human erythrocyte cell populations, and NMR imaging was used to map the lipid content of a single *Xenopus laevis* egg (Aguayo et al., 1986; Brown et al., 1977). Osmolytes and

metabolites were studied more recently in single neurons isolated from the sea slug *Aplysia californica* employing NMR (Grant et al., 2000; Schoeniger et al., 1994). This spectroscopic technique can be used for the label-free, nondestructive, and in vivo interrogation of metabolites in these large cells, but it has insufficient sensitivity to work with single animal cells of more common sizes (Griffin, 2006; Kim et al., 2010). Developments in microstrip technology have enabled the NMR detection of 100 pmol of analyte and can provide enough sensitivity to study metabolites in small cell populations (Krojanski et al., 2008).

Fluorescence-based analytical methods in combination with microscopy have been particularly successful in studying the chemistry of single cells and subcellular structures (Muzzey and van Oudenaarden, 2009; Watson, 1987; Wu et al., 2008; Yip and Kurtz, 2002). These techniques are nondestructive, have impressive detection limits (down to the single molecule level), are capable of high-throughput analyses, and are quantitative in nature. Fluorescence-based metabolomics requires the introduction or presence of fluorophores inside a cell. For example, using coupled fluorescent proteins, the maltose levels in the cytosol of live yeast cells and the glucose concentrations in immortalized kidney cells were examined in single-cell fluorescence resonance energy transfer (FRET) experiments (Fehr et al., 2002, 2003). Some metabolites are natively fluorescent and can be directly monitored without chemical tagging. For example, the amount of carotenoids in yeast cells was calculated based on autofluorescence (An et al., 2000). Alternatively, genetic encoding for the production of the fluorophore (e.g., a green fluorescent protein [GFP]) is possible.

Capillary electrophoresis (CE) is another powerful technique and has been coupled to various detectors to characterize biochemical species in single cells (Arcibal et al., 2007). The direct analysis of single cells by CE is typically performed by inserting a microcapillary into the cell for sampling. The extracted material can be analyzed using electrochemical detection, laser-induced fluorescence, or MS (Cruz et al., 1997; Hofstadler et al., 1995; Hogan and Yeung, 1992; Kennedy et al., 1989; Lillard et al., 1996; Olefirowicz and Ewing, 1990; Sims et al., 1998; Yeung, 1999). The utility of CE combined with ESI MS for analyzing metabolites in single cells has been demonstrated on neurons of *A. californica* (Lapainis et al., 2009; Nemes et al., 2011).

The analysis of single cells using MS results in the simultaneous detection of multiple species. MS techniques operate without chemical labeling, can elucidate structural information, and, in many cases, have adequate sensitivity for single-cell experiments. With MS, multiple metabolites can be detected without selecting them before the analysis (Monroe et al., 2007). However, MS is a destructive technique and usually requires careful sample preparation. Metabolic profiling has been achieved for extremely large cells, the *X. laevis* oocytes, by GC-MS using in-liner silylation (Koek et al., 2009). Because of the high spatial resolution of the ion sputtering process, secondary ion mass spectrometry (SIMS) has also been used to analyze metabolites within a single cell and in subcellular structures (Chandra et al., 2000; Colliver et al., 1997; Ostrowski et al., 2004).

Matrix-assisted laser desorption ionization (MALDI) MS has been used to analyze neuropeptides in single neurons (Rubakhin et al., 2003) and peptides in mammalian cells such as single rat intermediate pituitary cells (Rubakhin et al.,

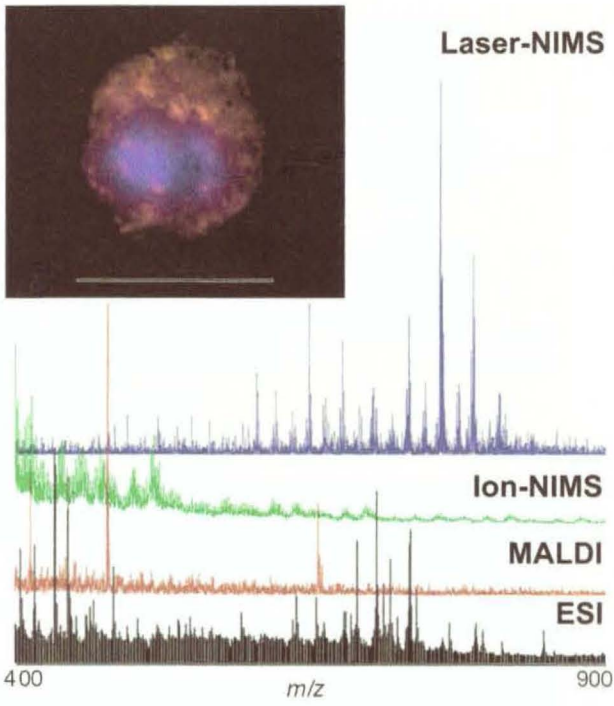

Figure 6.6. Comparison of mass spectra obtained by the analysis of 1, 100, 400, and 500 cancer cells by laser NIMS (*blue*), ion NIMS (*green*), MALDI (*red*), and nanoelectrospray ionization (*black*) demonstrates the superior sensitivity and metabolite coverage of laser NIMS. The *inset* shows a fluorescent image of the cell after laser NIMS analysis. The scale bar is 28 μm. (Adapted with permission from Northen, T. R., Yanes, O., Northen, M. T., Marrinucci, D., Uritboonthai, W., Apon, J., et al. [2007]. Clathrate nanostructures for mass spectrometry. *Nature* 449, 1033-U3. Copyright 2007 Nature Publishing Group.)

2006; Rubakhin and Sweedler, 2007). Nanostructure-initiator mass spectrometry (NIMS) with laser excitation has shown sensitivity at the single-cell level for analyzing eukaryotic microorganisms, yeast (*Saccharomyces cerevisiae*), and single human cells (Amantonico et al., 2009; Andrea et al., 2008; Northen et al., 2007). Figure 6.6 shows mass spectra obtained from 1, 100, 400, and 500 cancer cells (MDA-MB-231) by laser-NIMS, ion-NIMS, MALDI, and nanoelectrospray (Northen et al., 2007). These mass spectra seemed to indicate higher efficiency to ionize endogenous phospholipids and metabolites in laser NIMS experiments than by the competing methods. A similar MALDI-based technique has been employed to characterize metabolic heterogeneity within a cell population of unicellular eukaryotic alga, *Closterium acerosum* (Amantonico et al., 2010b). Direct laser desorption ionization MS has also been used to study strongly absorbing secondary metabolites in leaves, placenta, stamens, and styli in plants with single-cell resolution (Dirk et al., 2009). A more recently introduced matrix-free laser desorption ionization method based on silicon nanopost arrays (NAPA) has shown the ability to analyze the metabolites in single yeast cells and very small cell populations with a high coverage of the metabolome (Walker et al., 2011) in vacuum.

One direct method to analyze metabolites in single cells uses videomicroscopy in combination with MS (Mizuno et al., 2008); a schematic is shown in Figure 6.7. In this approach, contents of single cells are drawn into a nanospray tip under videomicroscopy, followed by the addition of an electrospray solution to the sampled cell material. At this point, the nanospray tip is converted into an electrospray emitter, and the sprayed cell content is analyzed by a mass spectrometer. This approach has enabled the detection of serotonin and histamine in granules of mast cells, known chemical mediators during allergy stimulation.

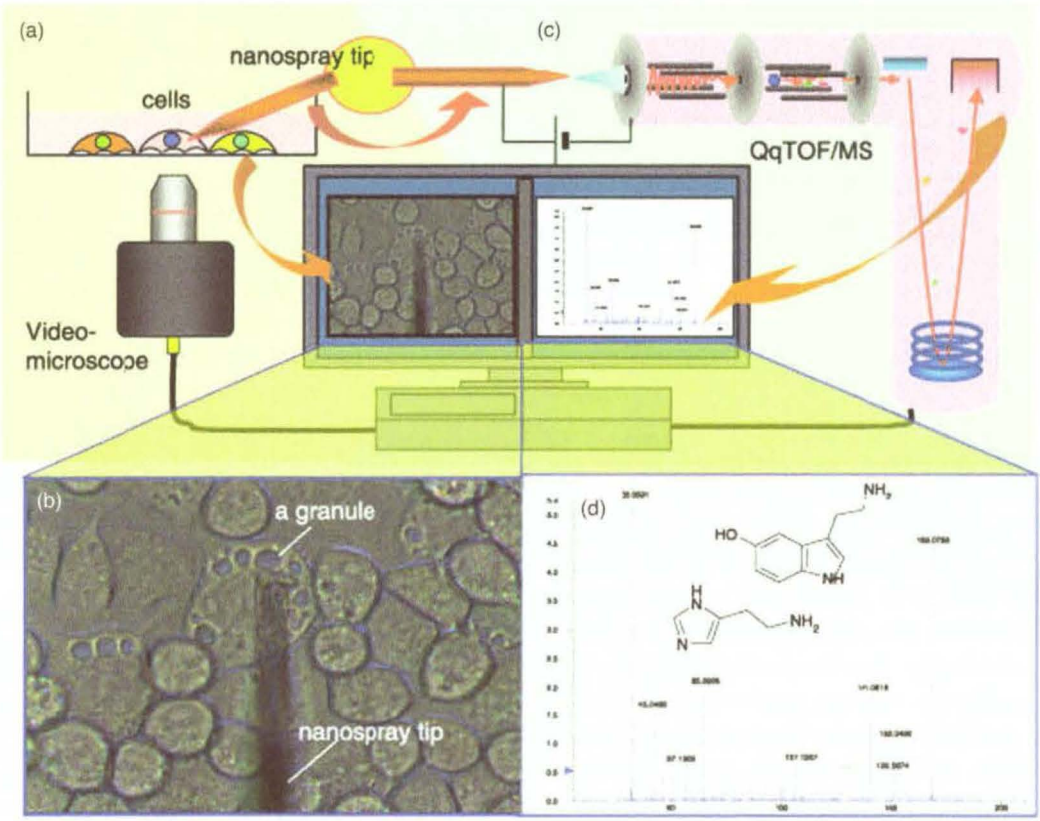

Figure 6.7. Analysis of a single cell by video MS. (a) The cells are observed by videomicroscopy. (b) Contents of a cell are extracted into a nanospray ionization tip. The ESI solvent is combined with the cell content in the tip and (c) the mixture is electrosprayed into the mass spectrometer to obtain a (d) mass spectrum. (Adapted with permission from Mizuno, H., Tsuyama, N., Harada, T., and Masujima, T. [2008]. Live single-cell video-mass spectrometry for cellular and subcellular molecular detection and cell classification. *Journal of Mass Spectrometry* 43, 1692–1700. Copyright 2008 John Wiley and Sons, Ltd.)

Single-cell metabolomics is a particularly promising application of LAESI MS. Several operating parameters allow fine-tuning of the amount of material sampled during mid-infrared ablation for such purposes. The pulse energy and focusing properties of the laser beam, the optical and mechanical properties of the sample, and the geometry of the LAESI setup are just a few underlying variables that can help tailor the analyzed area and volume to the physical dimensions of individual cells. The related instrumentation extends beyond the scope of this chapter, and the reader is referred elsewhere (Nemes and Vertes, 2010a, 2010b; Shrestha and Vertes, 2009, 2010). Among the available techniques for single-cell metabolomics by LAESI is the use of a sharpened optical fiber to deliver the laser pulse to ablate the selected cells. The selection of a single cell and the coupling of the laser pulse to the cell are assisted by two long-distance microscopes. Figure 6.8 shows such an arrangement that is used to analyze individual epidermal cells of *Allium cepa* (Shrestha and Vertes, 2009). Each cell yielded signals for more than seventy chemically different

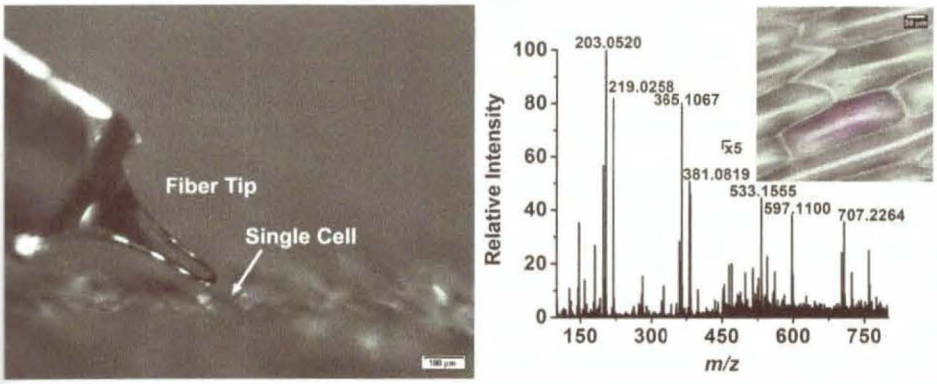

Figure 6.8. Single-cell metabolic analysis of *A. cepa* epidermal tissue by LAESI MS. (*left panel*) A chemically etched optical fiber is used to select and ablate the cells of interest. (*right panel*) MS analysis of the generated ions reveals phenotype-specific secondary metabolite composition, including cyanidin glucoside that is abundant in the purple cells of the tissue (*inset*). (Adapted with permission from Shrestha, B., and Vertes, A. [2009]. In situ metabolic profiling of single cells by laser ablation electrospray ionization mass spectrometry. *Anal. Chem.* 81, 8265–8271. Copyright 2009 American Chemical Society.)

ions. By targeting numerous cells, the metabolite content of neighboring cells can be analyzed. For example, it was found that purple cells contained significant levels of anthocyanidins, other flavonoids, and their glucosides, including cyanidin glucoside (Shrestha et al., 2011; Shrestha and Vertes, 2009). In agreement with these results, the latter is a known purple pigment and is contained in the cell vacuoles in purple plants.

Extending single-cell analysis to small cell populations offers new insight regarding cellular heterogeneity. In a more recent development, thirteen neighboring cells in the epidermal tissue of *A. cepa* were sampled for their metabolic composition (Shrestha et al., 2010b). The variance in these data can shed light on cellular heterogeneity on a metabolic level under native-like experimental conditions.

As discussed previously, the direct metabolomic analysis of single cells using MS is a quickly emerging field. We anticipate rapid growth in the development of new tools and methods for the ambient MS analysis of single cells and small cell populations.

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