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Ambient Mass Spectrometry in Imaging and Profiling of Single Cells: An Overview

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Abstract : It is becoming more and more clear that each cell, even those of the same type, has a unique identity. This sophistication and the diversity of cell types in tissue are what are pushing the necessity for spatially distributed omics at the single-cell (SC) level. Single-cell chemical assessment, which also provides considerable insight into biological, clinical, pharmacodynamic, pathological, and toxicity studies, is crucial to the investigation of cellular omics (genomics, metabolomics, etc.). Mass spectrometry (MS) as a tool to image and profile single cells and subcellular organelles facilitates novel technical expertise for biochemical and biomedical research, such as assessing the intracellular distribution of drugs and the biochemical diversity of cellular populations. It has been illustrated that ambient mass spectrometry (AMS) is a valuable tool for the rapid, straightforward, and simple analysis of cellular and sub-cellular constituents and metabolites in their native state. This short review examines the advances in ambient mass spectrometry (AMS) and ambient mass spectrometry imaging (AMSI) on single-cell analysis that have been authored in recent years. The discussion also touches on typical single-cell AMS assessments and implementations.

Key words: ambient ionization mass spectrometry, desorption electrospray, ambient MS, single cell MS, single cell analysis

Introduction

As the most fundamental structural and functional units in biology, cells are susceptible to a variety of influences that can change their metabolism, differentiation, and proliferation. This results in a significant degree of heterogeneity between individual cells, even those that are part of the same organism.[1-4] Even though two homologous cells have the same genetic makeup, their chemical make-up and concentration can vary.[5-7] Information regarding cellular individuality is frequently lacking because population cell analysis only produces averaged results.[8-10] Consequently, understanding cellular morphology and composition at the single-cell level can give precise information about specific cells in each microenvironment. The differential information provided by many distinct cells is essential for elucidating cell differences, and it is also crucial for the study of cell signaling, physiological pathologies of diseases, and the facilitation of the discovery of biomarkers that can enable the early diagnosis of serious diseases.[11-13] To detect and analyze the compositions and contents present within individual cells, however, can be very challenging due to the small sample volumes required and the complexity of chemicals encountered in these cellular environments.[14-17]

As a tool for biological and medical research, single-cell RNA sequencing is becoming increasingly significant. The most popular techniques for single-cell analysis include flow cytometry[18-21] and fluorescence microscopy.[22-24] The linear range of the fluorescent signal is limited, making it challenging to precisely quantify the cellular components, and different channels used to detect different dyes cannot be operated simultaneously due to the possibility of spectral interference. In response to cues from the environment and other external stimuli, cellular performance can vary greatly within a population. Additionally, depending on the physiological state of the corresponding cells, cellular responses to intra- or inter-specific information chemicals released by conspecifics or competing species can vary.[25] Rapid and high throughput methods are especially needed to address the dynamics of metabolite expression, which is causing significant concentration fluctuations throughout the growth of a culture or bloom.[26-27]

Mass spectrometry (MS) techniques have excellent

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potential for detecting many molecules without the need for labeling and can provide femtomolar sensitivity.[28-31] MS techniques are well suited for single-cell analysis due to additional benefits like low sample consumption, high throughput, and multiplexed detection, which enable quantitative, qualitative, multiplexed, and spatially resolved cellular level investigations.[30,32] AMS, has grown to become an effective tool in analytical science. This rise in popularity and observable applications is attributable to its ability to enable the rapid analysis of a variety of samples, usually in their natural state or with minimal sample preparation. In this article, we examine the advances in AMS and AMS-MSI related to single-cell analysis that have been authored in recent years.

Ambient Mass Spectrometry (AMS)

The application of mass spectrometry (MS) in analytical and bioanalytical research has grown significantly. This enormous success and breadth are largely due to MS's unrivaled capacities for detecting, recording, and characterizing molecules and atoms of various kinds, formulations, and dimensions.[33] High sensitivity, selectivity, and speed together have long been considered a key benefit of MS. In recent years, MS has also greatly expanded in terms of the kinds of molecules and compounds that it can handle, enabling it to contend with almost all types of biological molecules, inorganic and organic salts, organometallic complexes, supramolecular objects, and biological entities like viruses and bacteria, in addition to relatively small and thermally inert organic molecules.[34-40]

However, to handle the wide range of atoms, molecules, matrices, and mixtures, MS must promote effective ionization to produce diagnostic ions, preferably for every single component, which can be passed on to the high vacuum setting of MS in which they are identified and measured.[41] The transfer of the analyte compounds from their "natural" ambient setting, where target molecules are typically found frequently in condensed forms along with matrices and in complicated mixtures, into the clean but relatively "inhospitable" extreme vacuum MS environment presented significant challenges.[41] In MS analysis of mixtures, preseparation processes were frequently necessary as well, and thermally unsettling and less volatile materials could not be handled.[41]

Analytical chemistry has undergone an upheaval with the advent of ambient ionization mass spectrometry (AMS), which makes it possible to analyze samples swiftly and directly in their natural form. The analytical sector has been instrumental in advancing AMS since its debut, spurred on by the plethora of benefits it offers in addition to conventional MS.[42] The absence of chromatography permits rapid sample assessment; the versatility of ambient ionization sources allows them to be perfect for remote analysis when combined with portable devices; and time-consuming extraction and concentration procedures can be reduced or even removed. As a result, a growing variety of scientific disciplines are becoming aware of the potential uses of AMS, particularly in the fields of illness diagnosis, forensics, and environmental sciences.[43]

AMS techniques typically fall into three primary classes. Solid-liquid extraction techniques involve the extraction or desorption of molecules from the surface of a sample, generally achieving ionization by means of an electrospray ionization (ESI) mechanism.[44] Plasma desorption techniques achieve ionization using plasma with mechanisms akin to those in atmospheric pressure chemical ionization (APCI).[45] Finally, laser ablation techniques use infrared or ultraviolet lasers to ablate and desorb analytes from the sample surface.[46] Several well-known AMS methodologies fall under the umbrella of solid-liquid extraction methods, which entail the extraction or desorption of analytes from a specimen's surface followed by ionization. These methods commonly employ electrospray ionization (ESI).

Solid-Liquid Extraction Techniques

DESI

Desorption electrospray ionization (DESI, Figure 1) is still a highly prevalent spray-based technique for the spatially resolved assessment of complex specimens.[47,48] Small molecules in functioning systems that include resected[49] or unexcised tissues can be observed with the DESI-MSI technique.[50] Because it may operate in ambient conditions and requires minimum sample preparation beforehand, DESI-MSI has an advantage against alternative competing ionization techniques (such as MALDI, SIMS, etc.). The method also proved to be simpler to utilize than MALDI or SIMS because it only needs a cheap solvent spray and is not dependent on a vacuum environment for ionization. The DESI probe can quickly gather the molecular signatures of organs, tissues, and other materials.[49]

Nano-DESI

Using nano-spray desorption on both solid and liquid platforms, nano-DESI (Figure 2) is a technique utilized to

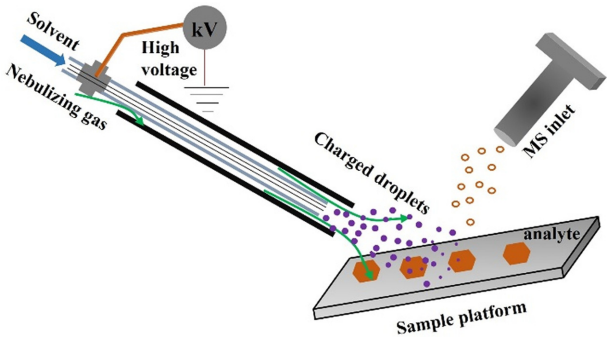

Figure 1. Schematic Illustration of DESI source

consistently gather and ionize analyte components.[51] The nano-DESI probe, which is frequently tilted, is made up of two integrated silica tubes. The analytical surface and two capillaries form a solvent bridge, which desorbs the analytes into it. Even though the desorbed analyte is sent to an MS inlet by the auxiliary capillary and produces ions by nanospray close to the point of entrance via self-aspiration or vacuum-assisted movement, the main capillary provides solvent to establish and maintain the bridge. Using nano-DESI, complex chemicals identified in biological samples have been sensitively located, examined, and visualized.[52-54] The dimension of the capillaries that constitute part of the probe, the solvent's flow rate, the properties of the surfaces, and the accuracy with which the space between the probe and the analyte can be adjusted all have an impact on how accurately measurements are made while using nano-DESI.

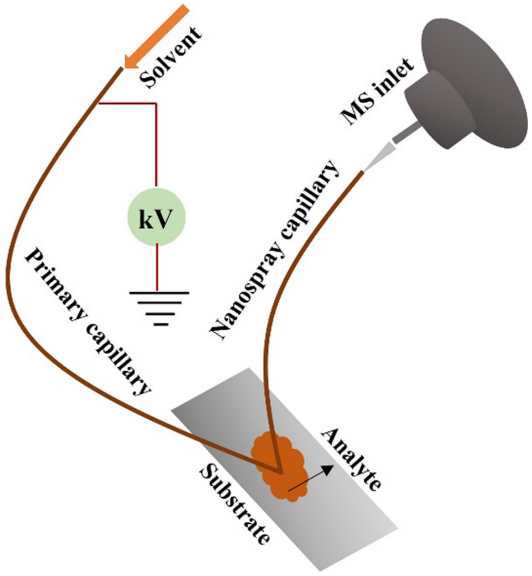

Figure 2. Schematic illustration of nano-DESI source

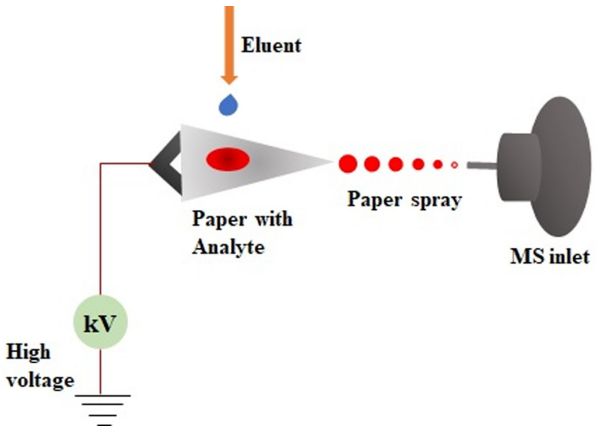

Figure 3. Schematic illustration of PSI source

PSI

In 2010, Wang et al. pioneered the simple, affordable methodology referred to as paper spray ionization (PSI, Figure 3).[55] In PSI, the sample is applied to a triangular-shaped strip of paper.[56] After the sample has dried, a spray solvent is applied to the substrate, and then a high voltage is given to the paper to cause the sample to electrospray from the triangle's tip. PSI has its foundation in ESI mechanisms, but with a key distinction from the methods. Instead of using a solid-liquid extraction technique, PSI produces ions right from the paper substrate that the sample is put on. Even though there are other compounds that can serve as a basis, including plant material[57] and polymers,[58] paper is the one that is most frequently utilized. Unlike many other ionization approaches, PSI enables quick access to a variety of sampling techniques, such as surface analysis and the direct evaluation of dried blood spots, including the separated compounds resulting from a TLC band.[55]

EESI

EESI-MS (Figure 4) separates the material being studied from the electric field and minimizes chemical regulator contamination, in contrast to conventional AMS techniques. Some of the essential elements of EESI include the following: Continuous and remote monitoring, in-vivo

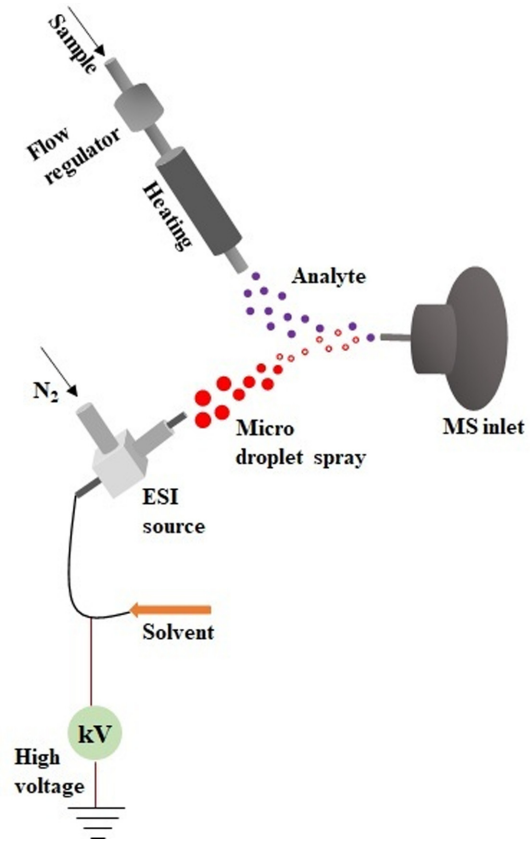

Figure 4. Schematic illustration of EESI source

research, the capacity to analyze polar and non-polar compounds, easier sample preparation techniques, the ability to monitor ion-molecule and ion-ion reactions, and the ability to analyze liquid, gas, and aerosol samples, among other capabilities, are just a few.[59] EESI-MS directly injects the bulk sample via the capillary at a predetermined rate of flow of extractive solutions driven by high voltage.[59]

The analytes were extracted from the specimen using the extractive solvent, and they were then transported into the bulk specimen via the changing direction of the electric field onto the adjacent MS for evaluation.[59] The following are some of the EESI-MS's unique features: There are several benefits, including (i) direct chemical evaluation within bulk specimens as opposed to on the surface; (ii) the solvent utilized is easily variable; (iii) a minimal amount of sample prior preparation; (iv) no sheath gas; (v) faster analysis time; (vi) minimal sample consumption; simple processes; and (vii) ease of integration with various kinds of MS.[59]

PESI

The ESI-based approach referred to as probe electrospray ionization (PESI) was originally developed by Hiraoka et al.[60] PESI (Figure 5) makes use of a grounded solid needle, typically constructed of titanium or stainless steel, to transfer a tiny amount of sample to the needle end. The use of a high voltage causes electrospray to form at the needle's tip after it has been manually or robotically oriented in the direction of the MS.

Laser Ablation

In laser ablation processes, analytes are removed from specimen surfaces using IR or UV lasers.[61] Following the development of MALDI, the ambient pressure alternative (AP-MALDI] was developed to facilitate MALDI-MS research by removing the need to place the sample in a vacuum.[62] Contrary to MALDI, several ambient pressure ionization techniques generate an approximately homogeneous ion mist under ambient conditions.[63] Nevertheless, with AP MALDI, additional distinct ambient pressure processes, such as the heating up of vibrationally energized ions and ion-molecule reactions, may take place over a longer period.[63]

LAESI

Considering lasers are capable of being optically focused to enable high-efficiency desorption at better spatial resolution and pulse frequencies beyond what is practical with solvent- and plasma-based desorption techniques, the application of lasers for accelerating sample desorption is particularly appealing. A stream of desorbed molecules is produced by LAESI,[64] and these molecules are then ionized by charged solvent molecules produced by electrospray. The key advantages of laser-based approaches, despite their less widespread application, are their ability to perform extremely focused sampling, which is especially advantageous for MS imaging applications, and their ability to dissociate the desorption and ionization procedures. The laser beam diameter, which is usually operated at 200 μm, determines the spatial resolution of LAESI.[65] However, samples must contain a high water content in order to efficiently encounter the infrared radiation and activate the target in LAESI. Pretreatment of the samples or the addition of a matrix are not necessary.[65,66]

IR-MALDESI

MALDESI was originally meant to be a method that integrated the stages of laser ablation and MALDI sample preparation with an ESI source for ionization. For laser absorption, samples needed to be co-crystallized with an organic matrix; afterwards, they had to be laser ablated

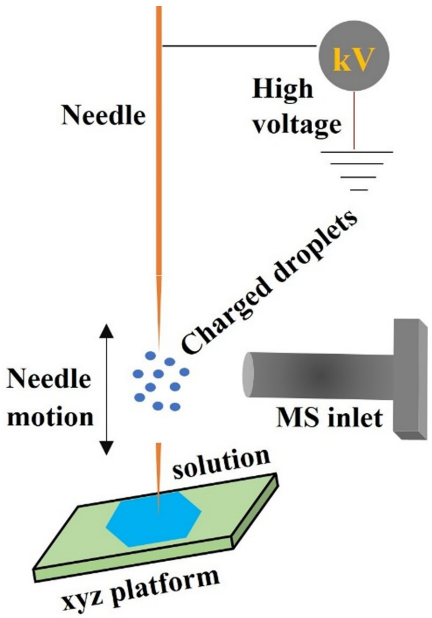

Figure 5. PESI Ionization source

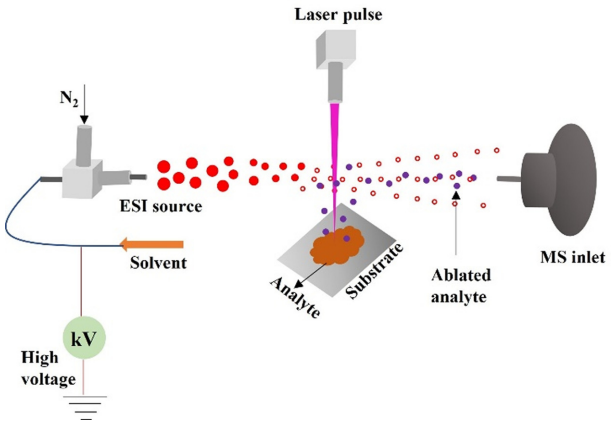

Figure 6. Schematic sketch of IR-MALDESI source

using a UV laser; next, the plume had to be ionized with an orthogonal ESI beam; and last, they had to be ionized.[67] The majority of MALDESI's updates have focused on technological developments for imaging applications, especially its spatial resolution.[68,69]

This ionization process was initially carried out using a UV laser, but more recently, an infrared (IR) laser (IR-MALDESI) has been utilized.[70] Prior to this, the spatial resolution of IR-MALDESI (Figure 6) was restricted to 150 μm, principally due to limitations on the IR laser point size. A multielement optical system allows the laser focal point to be reduced to a 50 μm spot size, enabling improved lateral resolution for tissue imaging applications.

Application of AMS in Single Cell Analysis

Table 1 presents pertinent information related to empirical studies on single cell analysis with AMS. Understanding the fundamental principles governing biological phenomena such as metabolism, stress, development, differentiation, procreation, relationships, mobility, apoptosis, aging, and development is greatly aided by the analysis of fluctuations in the chemical makeup or concentrations of metabolites in cells.[71] The sensitivity and accuracy, in addition to the instrument's capability for enrichment and the probes' capacity, are in great demand due to the continual increase in needs for identifying multiple metabolite species from just a single cell.[71] The study of single animal cells requires more sensitive techniques due to the negligible volume and lower intracellular substance content of animal cells, whereas PESI-MS-based single-cell examination only has relevance to plant cells. The design and development of functional probes to enhance trace elements from single cells and boost the degree of ionization and propagation efficiency of trace elements are the most practical and affordable ways to increase the sensitivity of PESI-MS.[71]

Functional probe electrospray ionization (FPESI) was developed by Zheng et al. to circumvent PESI's previously observed limitations.[71] A nitrogen gas heating system and a functional copper probe made of reduced graphene oxide (rGO-Cu probe) made up the FPESI system. The efficacy of the detection and the ion transport capability can both be improved by the nitrogen gas heating system. To enhance the analytes without losing electrical conductivity, the rGO-Cu probe created a significant specific surface area.[71] The FPESI system allowed for in situ direct sampling of PC12 single cells (neurotransmitters) and untargeted metabolomics evaluation of biological materials. It has the capability of directly sampling and detecting 12 metabolites in rat serum, 12 biosignatures in PC12 single cells, and 7 neurotransmitters. Duration, mobile phase, and additional expenses are drastically decreased when compared to conventional LC-MS.[71]

Although it has been suggested that depletion of healthy pollen grains brought on by physiological heat reactions is a major factor in temperature-induced spikelet sterility as part of global climate change, specific evidence at the pollen level has been lacking up to this point due to technological constraints. Wada et al. created the picoliter pressure-probe-electrospray-ionization (picoPPESI) for precisely determining the compounds in heat-treated individual mature pollen grains to get over constraints.[72] To determine the internal metabolites in whole plants growing in regulated high-temperature conditions, the picoPPESI system inserts the end of a finely tapered quartz capillary tip directly into the developing pollen grains. Pollen grains from the heat-tolerant cultivar N22 and the heat-sensitive cultivar Koshihikari were examined.[72] Despite the fact that each cultivar's in vitro pollen survival was unaffected by treatment, differences in phosphatidylinositol (PI)(34:3) and other 106 metabolites have been found in mature pollen. Irrespective of the intervention, a higher PI level was found in N22 pollen, but not in Koshihikari pollen.[72] On the other hand, both cultivars' pollen grains hardly exhibited any phosphoinositide. Our findings demonstrate that compounds in unaltered single pollen can be effectively identified by picoPPESI-MS analysis.[72] The active production of PI(34:3) ahead of germination is potentially tightly related to maintaining spikelet fertility despite high temperatures, as PI is a precursor of phosphoinositide that stimulates numerous signals for pollen germination and tube expansion.[72]

The exometabolome, which includes released metabolites, lipids, and peptides, is a component of the bacterial metabolome that is sometimes overlooked during research aimed at determining the chemical fingerprint of the cellular unit. Extracellular vesicles (EVs), a significant element of the bacterial exometabolome, are receiving greater importance in the microbiological and biomedical fields.[73] These expelled structures, created by cells in various realms of life, comprise an array of biological molecules involved in a wide range of cellular processes, making them a vital part of the bacterial extruded metabolome.[73] By demonstrating the creation, separation, verification, and assessment of bacterial EVs, Chamberlain et al. presented the utilization of PSI in bacterial exometabolomics for the first time.[73] As the study's model system, Oxalobacter formigenes (O. formigenes), a gram-negative resident of the human gut microbiome that holds a keen interest in the effects of its secreted metabolome on human well-being,[74-78] was selected.[73] The notion follows that O. formigenes generates and eliminates a secretagogue compound that, by promoting a net intestinal production of oxalate, a forerunner and risk factor for the development of stones, theoretically lowers its amount in circulation and renal excretion.[74,75,79-81] Investigating the EVs made by O. formigenes is potentially of considerable interest since a secreted bioactive substance of this kind could be anticipated to be encapsulated in and discharged via EV.[73] By comparing a panel of characteristics to an EV-free reference and observing representation from multiple kinds of biochemicals, such as lipids, metabolites, and

Table 1. Empirical studies on single cell analysis with AMS

Author	Domain	Sample	Ionization	Analyzer	Metabolite	AT
Chen et al.[110]	Biomedical	irinotecan-resistant (IRI-resistant) cells	nano-ESI	LTQ-Orbitrap	(LysoPC: lysophosphatidylcholine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PE-Cer: ceramide phosphoethanolamine; PG: phosphatidylglycerol; DG: diglyceride)	HPLC-MS, Piece BCA protein assay, MTT assay
Sun et al.[168]	Biomedical	irinotecan-resistant (IRI-resistant) cells	nano-ESI	LTQ-Orbitrap	phosphatidylcholines (PCs), monounsaturated fatty acids (MUFAs) to saturated fatty acids (SFAs), stearic acid/oleic acid	HPLC-MS, MTT assay, Western blot, Flow cytometry, & ROS
Li et al.[150]	Biomedical	MCF-7 cancer cells & WT lung cancer cells	DAESI	Qtrap-MS	Lipids	NA
Nguyen et al.[109]	Biomedical	Hela cells	nano-ESI	LTQ-Orbitrap	glycerophosphocholines (GPCs), PC(34:3), LPC(34:4), or PC(O-34:4)	LC-MS/MS
Huang et al .[169]	Plant biology	Allium cepa cells	CE-ESI	Orbitrap	malic acid, L-phenyalanine, cycloalliin, glucose, methylcysteine sulfoxide, and adenosine	NA
Chamberlain et al.[73]	Biological	extracellular vesicles	PSI	Orbitrap	phosphatidylethanolamines, phosphatidylglycerols, phosphatidic acid, phosphatidylinositol, phenylacetylglutamine (PAG) and violacein etc	NA
Zheng et al. [71]	Biomedical	PC-12 single cells	PESI	Orbitrap	biomarkers and metabolites	LC-MS
Zhang et al. [71]	Biomedical	MV4–11 and NB4 cancer cells	i-EESI	Orbitrap	Phospholipids such as PC(36:4), PC(36:2), PC(38:6) and PC(38:4) etc	NA
Wada et al.[72]	Plant biology	N22 & Koshihikari	picoPPESI	Orbitrap	Phosphatidylinositol (PI)	Microscopy
Liu et al.[169]	Plant biology	phytoplankton samples	LDI	Orbitrap FT-MS	glycosphingolipid and glycerophosphoserines	Microscopy

AT – alternate techniques

peptides, the investigators successfully managed to discern and putatively identify the features.[73] This work is innovative in three main respects: a) it demonstrates the ability to analyze EVs from any organism using PSI-MS; b) it applies PSI-MS to bacterial exometabolomics; and c) it validates and further explores EVs made by O. formigenes. Through the creation of a new scaffold for analyzing the chemical composition of EVs and the bacterial exometabolome, this innovative PSI-MS application will benefit the domain of metabolomics.[73]

In living organisms, the development of membrane structure, chemical energy retention, protein synthesis, cellular signaling, and other biological processes are all carried out by phospholipids.[82-86] The composition of phospholipids in tissues or cells can be indicative of certain metabolic processes. There is compelling evidence linking phospholipid metabolism dysregulation to a number of human disorders, including cancer,[87-89] diabetes,[86-90] Alzheimer's disease,[91-93] and cardiovascular diseases.[94,95] Chemical characterization of cancer cells at the single-cell level offers valuable information to advance our understanding of the fundamental mechanisms of disease and cell-to-cell variability.[96-100] Therefore, quick phospholipid determination in biofluid specimens is highly desirable in the field of life science. Internal extractive electrospray ionization (iEESI), an atmospheric ionization method combined with functional nanomaterials, has been developed recently for the targeted identification of trace analytes in complicated fluid samples, such as human urine,[101,102] and human blood.[103]

In a recent work, Zhang et al. created a system for the quick identification of phospholipids in microvolume biofluid specimens using the dispersed solid-phase microextraction (d-SPME) approach combined with iEESI.[104] Phospholipids based on Fe3O4@TiO2 nanocomposites were extracted and desorbed inside a glass capillary, and the solution containing the isolated phospholipids was then directly transferred to an electrospray emitter linked to it for MS analysis. With no centrifugation or filtration necessary, the magnetic Fe3O4@TiO2 nanocomposites enable quick separation of phospholipids from the sample substrate.[104] The analysis of a single sample was completed in 4 minutes. The phospholipids in blood plasma samples from 28 patients with pancreatic cancer and 23 control subjects, as well as 59 patients with ovarian cancer, were examined.

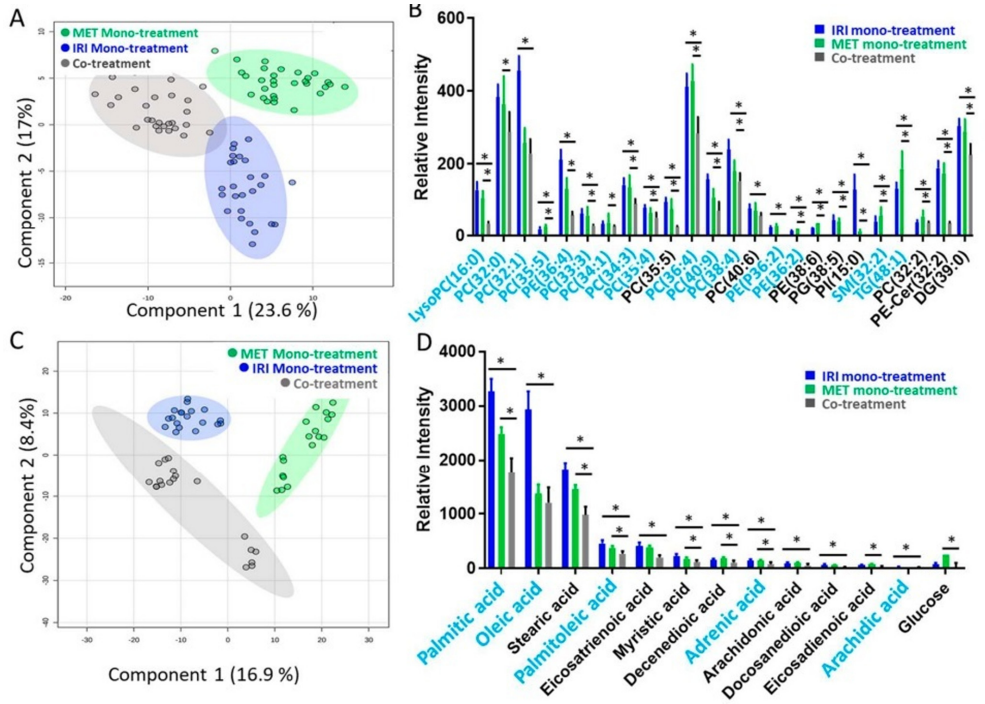

Figure 7. SCMS results of IRI-resistant cells from the co-treatment (0.56 μM IRI + 4.0 mM MET), IRI mono-treatment (22 μM IRI), and metformin mono-treatment (8.7 mM MET). (A) PLS-DA of positive ion mode results shows the metabolomics profiles were significantly changed by three different treatments ($p < 0.001$ from permutation test), and (B) representative lipids with significant abundance change (*$p < 0.05$ from both ANOVA and Tukey's test) among three treatment groups. (C) PLS-DA of negative ion mode results shows metabolomics profiles were significantly changed by three different treatments ($p = 0.004$ from permutation test), and (D) representative fatty acids with significantly different abundances (*$p < 0.05$ from both ANOVA and Tukey's test) among three treatment groups. Species labeled in blue font were identified using MS2 analyses using both single cells and cell lysates. (LysoPC: lysophosphatidylcholine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PE-Cer: ceramide phosphoethanolamine; PG: phosphatidylglycerol; DG: diglyceride). Reproduced with permission from Chen et al.110

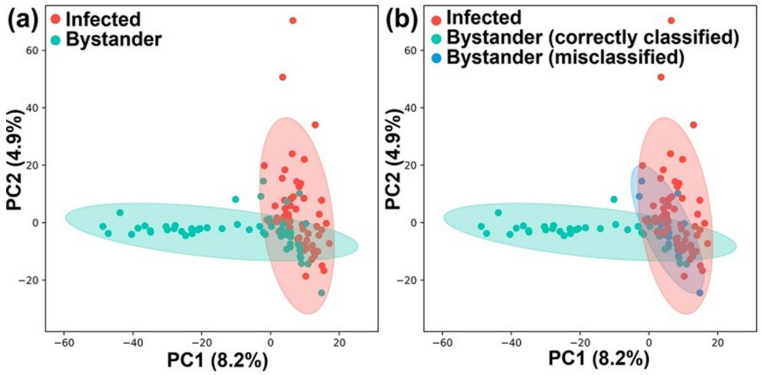

Figure 8. Impact of T. cruzi infection on the metabolome of bystander uninfected cells. (a) PCA of SCMS data highlighting metabolic overlap between T. cruzi infected cells and a subset of bystander cells. (b) PCA analysis of SCMS data as in panel a, colored based on random forest classifier prediction. Misclassified uninfected bystander cells have overall metabolomes similar to infected cells.Reproduced with permission from Ngyuen et al.109

The statistical analysis allowed for the confident separation of the cancer patients from healthy controls.[104] The determination of phospholipids in individual human cells (MV4-11 and NB4) demonstrates the sensitivity of single cell analysis. As a result, the findings showed that this method may rapidly differentiate cancer by detecting tiny amounts of phospholipids in small amounts of blood and cell specimens with high responsiveness and precision, minimal sample intake, and high throughput.[104]

Although single-cell transcriptomics[105] and proteomics[106] are being employed more often, single-cell metabolomics can examine cellular function in a way that may not be possible using other techniques. Smaller molecules called metabolites include amino acids, lipids, and carbohydrates.[107] Metabolites reveal the actions of related metabolic pathways and reflect the state of the cell. Single-cell metabolomics holds considerable promise for revealing the phenotypic differences between individual cells and, more especially, the variability among cells. By characterizing metabolites in individual cells, single-cell mass spectrometry [SCMS] can reveal cells' secret subpopulations. It is possible to examine and identify the cellular metabolites that are changed because of environmental perturbation using MS-based single-cell metabolomics.[108] Nguyen et al. examined the host cell variability that develops during infection with the Trypanosoma cruzi (T. cruzi) parasites that are the primary cause of Chagas disease (CD).[109] Only a small number of host cells in the chronic stage of CD are heavily infested with parasites, and symptoms may manifest elsewhere. During the T. cruzi infection, metabolomics data was collected from specific cells using SCMS.[109] The research demonstrated variation in the host cells' in vitro metabolic response to T. cruzi infection. According to the study findings (Figure 7), parasite-infected cells had a different metabolism from control cells.[109] Surprisingly, some nearby uninfected cells displayed metabolic effects as well. Increases in glycerophospholipids are among the specific metabolic alterations associated with infection. These findings offer a novel understanding of CD pathogenesis.[109] Additionally, the study is the first to use bioanalytical SCMS to evaluate mammalian-infectious substances, and it has the potential to have widespread applications in the investigation of infectious diseases.[109]

In a separate investigation, Chen et al. used SCMS to assess live, IRI-resistant cells under various treatment scenarios.[110] The chemotherapeutic drug irinotecan (IRI), a topoisomerase I inhibitor that inhibits DNA synthesis, is frequently used to treat metastatic colorectal cancer. Although it is a successful chemotherapeutic medication, drug resistance, both inherent and acquired, limits its clinical efficacy.[110] Metformin, an oral diabetes medication, has lately been linked to anticancer effects. This is probably because it specifically kills cancer stem cells (CSCs).[110] It is thought that it can restore therapeutic effects by sensitizing IRI-resistant cells. According to the study's findings (Figure 8), taking metformin was linked to a suppression of lipids and fatty acids, possibly because of inhibiting fatty acid synthase (FASN).[110] It's important to note that some species can only be identified from cells that are still alive.[110] Enzymatic activity assays also revealed that, as compared to IRI or metformin mono-treatments, the co-treatment exhibited the strongest FASN inhibition. Overall, the study shows that metformin can make IRI-resistant cells more sensitive, and this pharmacological effect is linked to metformin's capacity to suppress FASN.[110]

Mass Spectrometry Imaging (MSI)

A series of imaging techniques known as MSI (mass spectrometry imaging) do not require molecular labeling. It has been demonstrated that MSI is a useful technique for in-depth analysis of the spatial arrangement of molecular species.[111] It capitalizes on the receptivity and accuracy of MS, which makes it more effective compared to the capaci-

ties of spectroscopic techniques, in a study where the mass spectrometric evaluation is conducted totally from the sample rather than from isolates of the substance as conducted with conventional chromatographic techniques.[111]

MSI has been shown to be a highly efficient technique for in situ analysis of the spatial dispersion of biological constituents. Unlike other, more commonly employed biochemical imaging techniques, label-free screening is possible with MSI without the need for any prior understanding of the potential species of interest. Although MSI assessments are untargeted, sampling procedures can be customized for different categories of the pertinent molecules of concern.[112] By emphasizing key elements, MSI, an ex vivo molecular imaging approach, aids in a better understanding of the biochemical processes occurring in the tissue. Contrary to conventional imaging modalities, these techniques do not require anatomical view of the specimen. Molecular downregulation is a hallmark of many clinical diseases.[113-116] The MSI investigations of the biomarkers connected to these illnesses offer novel therapeutic and treatment options. Evaluation of tissue chemistry often yields more concrete details on the underlying cause when the morphological markers in conventional histopathology are unclear.[117] By not requiring prior knowledge of the target species, the MS-targeted methodology varies from traditional molecular imaging techniques such chemical discoloration, immunostaining, fluorescence, and radiolabeling.[117] With the help of this MSI characteristic, researchers can more thoroughly examine the chemical composition of tissues in order to understand the functioning of numerous biochemical pathways that are essential to biological processes and spot any discrepancies that might be connected to them.[117]

Another factor to keep in mind is that MSI investigates how metabolites are distributed across the sample in space as opposed to using isolated tissues, which is another way it differs significantly from standard MS-based approaches.[118] By anticipating the move away from laborious LC-MS technology to an effective and spatially defined in situ approach, MSI has opened new promise in the field of clinical MS. In fact, it has been shown that MSI is quite useful for determining the limits of malignancy by measuring the dissemination of biomarkers in tissue sections.[119-122] Pixel-by-pixel MS information was captured after the specimen's surface has been screened in 2D for imaging (Figure 9). As a result, a massive amount of MS data is collected across the specimen's surface, and any chemical entities that are found can have their ion signals merged with spatial data to create their ion images.[123] Because each ion signal can be spatially described, MSI may produce a large number of molecular images in a single scan. This makes it possible to continuously observe how various endogenous and alien species proliferate throughout the tissue samples, which can help with the identification of biomarkers, the diagnosis of diseases, the creation of novel therapies, and pharmaceutical research.[124]

Two variables are frequently given priority when evaluating the effectiveness and applicability of MSI for the analysis of diverse molecular species in tissues: (i) spatial resolution and (ii) m/z span. Both factors are impacted by the desorption process, which takes into account the sample preparation, mass detector layout, and probe size.[125] According to McDonnell and Heeren,[125] the various ionization techniques used in MSI are each uniquely characterized by complimentary advantages and disadvantages that mostly relate to the level of detail, mass precision, chemical sensitivity, specificity, and m/z range.[126] Sample ionization is important to transition the analyte from the condensed to the gas phase when analyzing gaseous ionic species in an MS.[126]

Ambient ionization techniques enable the rapid evaluation of complex materials in ambient (atmospheric) condi-

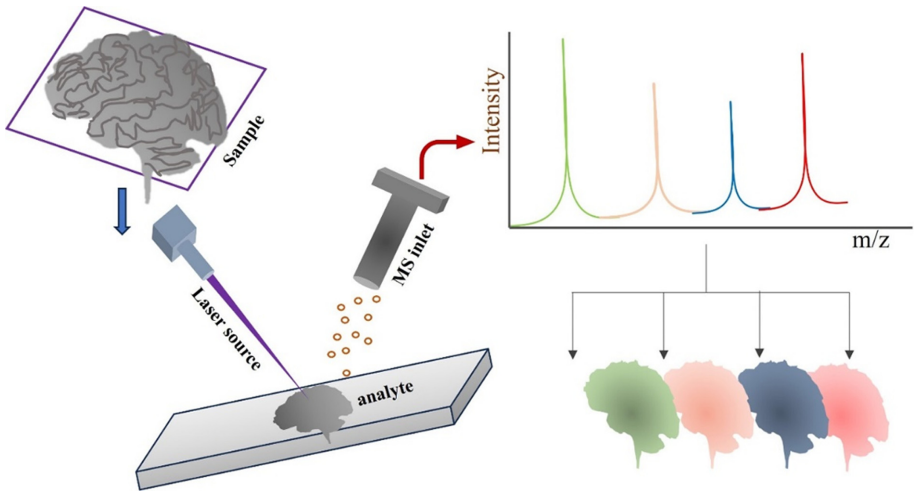

Figure 9. MSI workflow

Table 2. Empirical studies on single cell analysis with AMSI

Author	Domain	Sample	Ionization	Analyzer	Metabolites	AT
Xi et al.[70]	Biological	HeLa cells	IR-MALDESI	Orbitrap-MS	Phospholipids	NA
Pareek et al.[139]	Biological	HeLa cells	GCIB-SIMS	TOF	Purinosome	NA
Taylor et al.[153]	Plant biology	*Allium cepa* and *Fittonia argyroneura* cells	LAESI	Obritrap-MS	Catechol, furoic acid, phthalide, lysine Glycineamideribonucleotide	NA
Stopka et al.[155]	Plant biology	Allium cepa cells	f-LAESI	QTOF	malate, disaccharides, glutamate, and citrate	NA
Meng et al.[161]	Drugs and nanomaterials	HeLa cells	LA	ICP-MSI	NA	Bright field mircoscopy
Tamura et al.[173]	Biomedical	ccRCC tissue sample	DESI	QTOF	azelaic acid, palmitoleic acid, linoleic acid, oleic acid	H&E stain
Vijayalakshmi et al.[174]	Biomedical		DESI	Orbitrap-MS	small metabolites, fatty acids and lipids	H&E stain
Benussan et al.[175]	Biomedical	ADC & SCC tissue sample	DESI	Orbitrap-MS	asglycerophosphoglycerols (PG), glycerophosphoinositols (PI), glycerophosphoserines (PS), glycerophosphoethanolamines (PE), and fatty acids (FA)	H&E stain
Tian et al.[176]	Biomedical	liver tissue samples	DESI	QTOF	fatty acids (FA), phosphatidylinositols (PIs), phosphatidylserines (PSs), lysophosphatidylserines (LPSs), phosphatidic acids (PAs), lysophosphatidic acids (LPAs)	H&E stain, AP-MALDI, Immunostain
Li et al.[150]	Drug distribution	HeLa cells cultivated with proflavine & methylthioninium chloride	MLF-LDPI	TOF	NA	NA
Hieta et al.[177]	Plant biology	plant tissue samples	LAAPPI	micrOTOF	flavonol glycosides, fatty acids (FA), fatty acid esters, galactolipids, and glycosphingolipids,	NA

AT – alternate techniques; TOF – time of flight; QTOF – quadrupole time of flight

tions without the need for specialized sample pretreatment processes.[127-132] While analyzing the data, samples are processed in ambient ionization mass spectrometry. The localization of molecules in the specimen is not affected by sample preparation, and analysis can be greatly hastened.[127-130,133-135]

Venter et al. categorized ambient ionization techniques into three main categories based on the sample processing steps needed.[136] In liquid extraction procedures, analyte molecules are removed from the sample and acquired into a solvent prior to being ionized; in desorption procedures, free ions can be generated directly from substrates; and in liquid desorption procedures, larger fragments are generated and then ingested via an electrospray cloud followed by ionization.[136]

In addition to being easy to use, allowing materials to be evaluated in their natural environments, speeding analysis, and enabling the extraction solvent makeup to be customized to the experiment, liquid extraction methods offer several significant advantages. For instance, solvent makeup can be adjusted for estimate, online derivation via dynamic detection, or the efficient extraction of various analyte categories from the substance.[135-137] Additionally, the ability to perform quantitative MSI opens extraordinary opportunities for studying fluctuations in chemical concentrations in complex biological analytes by adding precisely chosen internal standards of known concentrations to the extraction solvent. Due to their special qualities, liquid extraction MSI technologies are particularly suitable for several tasks.

Although liquid extraction methods were the primary focus of nearly all the empirical investigations covered by this study, other ionization methods were used in fewer studies. This study offers a scientific and methodological review of the use of AMSI in single cell analysis. Table 2 presents pertinent information related to empirical studies on single cell analysis with AMSI.

Applications of AMSI

The lipid characteristics of isolated individual HeLa cells were investigated using IR-MALDESI-MSI.[138] The single HeLa cells contained 45 different lipid species that were putatively annotated, the majority of which were phospholipids. To further support the accuracy of the identifications, the theoretical carbon isotopic dispersion of individual lipid ions was measured and compared with the empirical value (Figure 10).[138] The relative abundances of four distinct lipids at m/z (369.3, 760.5, 782.5, and 808.6) seem to be in

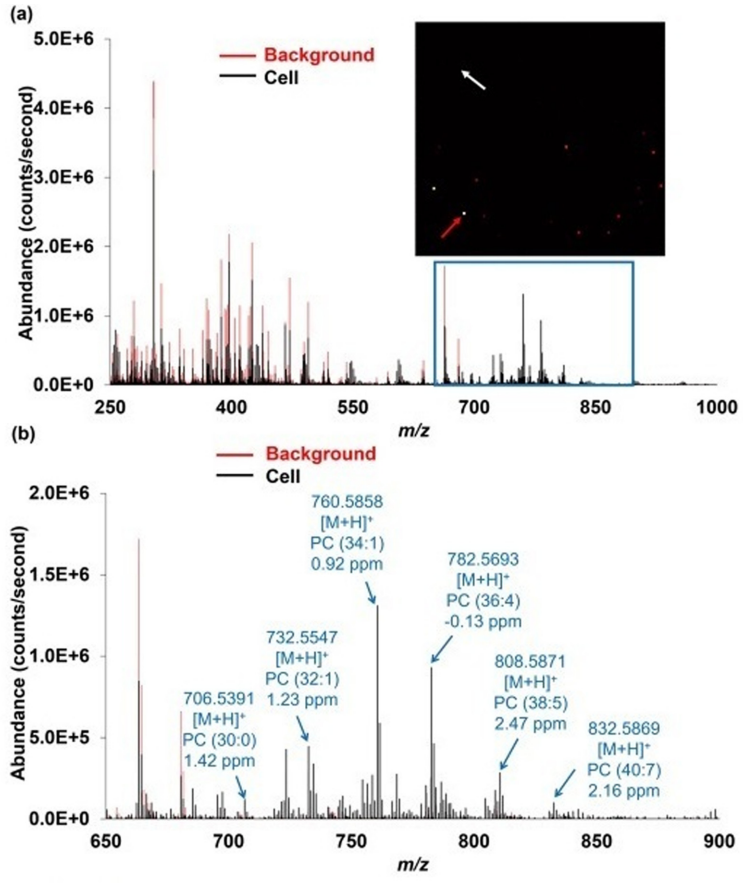

Figure 10. (a) Positive ion mode mass spectrum (m/z 250–1000) from a single cell (pointed by the red arrow) and a background pixel (pointed by the white arrow). (b) Expanded spectrum in the range of m/z 650–900 where most cell-specific ions were detected. Representative peaks are labeled and putatively annotated based on the accurate mass measurements. (Reproduced with permission from Xi et al.[70]

good agreement with one another. The frequency of detected lipid ions and their abundances seem to be positively correlated, indicating that lipids with increased abundances were observed in far more cells.[138] This might be connected to the analytical technique's limit of detection. Distributions of representative ion abundances reveal significant differences between individual cells, with lipid ion abundances sometimes deviating by more than two orders of magnitude. The evidence provided by the results can only be used to give a preliminary idea of lipidomic variation between cells; accuracy must be validated in follow-up work by using an internal standard for relative quantification to account for analytical variability.[138]

Although implementing spatially incorporated multiomics in single cells within tissues is at the cutting edge of biology, it has been made difficult by technological hurdles like a) detection limits that prevent imaging of low concentration biomolecules, b) the limited applicability of various MSI tools for cryogenic analysis of native cell states, c) sample preparation incompatibilities, and the challenge of maintaining dynamic metabolic gradients.[139]

The endogenous metabolism of individual cells in frozen tissue was imaged by Pareek *et al*.[139] Imaging using a secondary ion mass spectrometry - "SIMS" cryogenic water cluster ion beam (H2O)n>28K-GCIB-SIMS) with a resolution of 1 μm per cell was used in the study.[139] The development makes it possible for multi-modal mass spectrometry imaging (MSI) to detect proteins, metabolites, and lipids in individual cells within fully functioning liver zones and in different cell types in the native tissue state. The liver's functional units are mapped using a desorption electrospray ionization mass spectrometry imaging "DESI-MSI" workflow to create a map of metabolic zonation and variability.[139]

The DESI-MSI approach may identify tiny molecules in living systems, including tissues that have undergone resection[140] or have not undergone resection[141] (metabolites, lipids, medicines, etc.). DESI-MSI offers advantages compared to all competing ionization techniques (such as MALDI, SIMS, etc.) because it may function in ambient settings and requires little prior sample preparation. The procedure is also easy to employ because it simply requires an inexpensive solvent mist and doesn't require a vacuum setting for ionization, unlike MALDI or SIMS. From live organs, tissues, etc., the DESI probe can rapidly acquire molecular fingerprints.[141] The analyte segment was exposed to a stream of rapidly moving charged microdroplets in order to electrospray a solvent (such as water, methanol, acetonitrile, dimethylformamide, etc.) at an elevated voltage while employing nitrogen as the nebulizing gas.[142,143] The solvent droplet dampens the specimen surface as a consequence, dissolving the biochemical species that are present in the analyte (mainly metabolites and lipids). The ionization by electrospray procedure and the desolvation process both produce ionic species in the gas phase by the same mechanism, which involves solvent evaporation and Coulomb fission of the secondary microdroplets that contain the analyte species.[142] The bioimaging experiment collects mass spectra across the sample and scans the tissue surface in the x and y directions while bombarded by a spray of charged microdroplets. The two-dimensional image created from the pixel-to-pixel MS spectra can be employed to map the biochemical composition.[144] The study's results demonstrated that combined lipidomic and metabolomic profiling in individual cells can be used to categorize cells without using tissue dissociation or protein markers. Additionally, various metabolic signatures among cells expressing identical proteins are used to identify subsets of cell types. To fully understand the spatial diversity and its functional significance, this platform could be expanded to overlay spatial transcriptomics and proteomics, describing the proportion of enzymes involved in lipid metabolism. This would address the understudied roles of individual lipid species. This will offer a rare chance to research distinct protein-lipid-metabolite interactions and previously unidentified cellular subtypes.[139]

It is challenging to classify the lipidome in detail for single cells due to the rich complexity of lipids and the smaller sample sizes that can be obtained from an individual cell. One of the best procedures for the thorough identification and relative quantification of lipids at carbon-carbon double bond "C=C" location and sn-position levels is the Paterno-Büchi (PB) reaction coupled with MS.[145-148] The C=C positions of lipids in a single cell were observed to be identified using the MS method in conjunction with the PB reaction, but comparative quantification of isoforms has never been conducted, and its specific biological significance has not been proven.[149]

Li *et al*. (2021) developed a general method that permits high structural selectivity in the lipidomic evaluation of individual cells. Cell fixation is used to retain lipids inside the cell during batch treatments before single-cell assessment.[150] The C=C and sn-locations of lipids are determined by batch photochemical functionalization and individual-cell droplet treatment, respectively, and the fatty acyl-chain of lipids is confirmed by tandem MS analysis. The integration of electro-migration and droplet-assisted electrospray ionization, or "DAESI," allows for simple operation but high sample utilization, making single-cell MS analysis feasible. Four variants of breast cancer cells were successfully identified through quantification of lipid C=C or sn-location isomers in 160 cells.[150]

The main advantage of using laser ablation techniques to reduce spatial resolution is the achievement of highly focused sampling.[151] In LAESI, an infrared laser beam is employed to vaporize molecules from a sample's surface.[151] The ESI is used to ionize the samples in this plume, and the mass spectrometer is used to collect the ions for analysis. As a comparable alternative, ambient pressure photoionization (LAAPPI) uses an infrared (IR) laser beam to ablate

the analyte surface in addition to an ambient pressure photoionization source to ionize desorbed analytes.[152]

Taylor *et al*. used a special dual optical microscope/LAESI-source to carry out high-throughput optically informed single cell analysis and high spatial resolution MSI.[153] The metabolic information of every Allium cepa cell in a large sample set was evaluated using LAESI-MS in the first mode. To achieve this, 33 optically selected cell positions were manually entered for automated detection with an overall duty cycle of 575 ms per cell for every tissue sample. The second mode significantly improves the efficiency of LAESI-MS imaging by enabling the execution of 40 μm high spatial resolution MSI without oversampling.[153] The authors identified chemical species unique to the physical structures in *Fittonia argyroneura* plant leaves by imaging the leaves of the plant. Both sampling modes do not require extensive sample preparation, and native tissues can be sampled *in situ* while spatial metabolomics is being conducted. The potential of this molecular microscope for upcoming spatial metabolomics studies is demonstrated by targeted single cell imaging.[153]

Taylor *et al.* connected a LAESI microscope to a drift-tube ion mobility mass spectrometer – "DTIMS", allowing for the isolation of isobaric ions and the determination of the cross sections of ion collisions in addition to accurate mass measurements.[153] The mobiligram's DTCCS was calculated by the authors (Figure 11(d)), and they discovered that it was 217.26 Ao2, which allowed them to pinpoint the specific trisaccharide.[153] Based on comparisons to DTCCS values discovered in a metabolite registry on a DTIMS system, this value is most likely cellotriose (Zheng *et al*.2017).[154] The existence of cellotriose as a major compound in A. Cepa is anticipated because cellulose is an essential component of cell walls and the primary polysaccharide in onion skins.[153] These results underline how crucial ion mobility is for more accurate structural predictions.

In a related study, Stopka *et al*. presented a dual mode imaging system for increased, single-cell analysis guided by microscopy based on simultaneous brightfield and fluorescence imaging coupled with f-LAESI-MS.[155] The most progress in SC analysis has been made by the fiber-based LAESI-MS (f-LAESI-MS), which directs the laser to the surface of the analyte via an engraved optical fiber. In f-LAESI-MS single cell (SC) studies, microdissection for subcellular analysis and the detection of heterogeneous cell populations have both been applied.[156, 157] Frozen root nodules from soybean plants that have been infected with GFP-labeled rhizobia for 21 days are sectioned to show selective targeting of a particular cell type. Using brightfield and fluorescence images, infected cells are distinguished from uninfected cells by their fluorescence and differences in size and lack of fluorescence, respectively. As a result of comparing the mass spectra of the two cell types, numerous metabolites, including malate, disaccharides, glutamate,

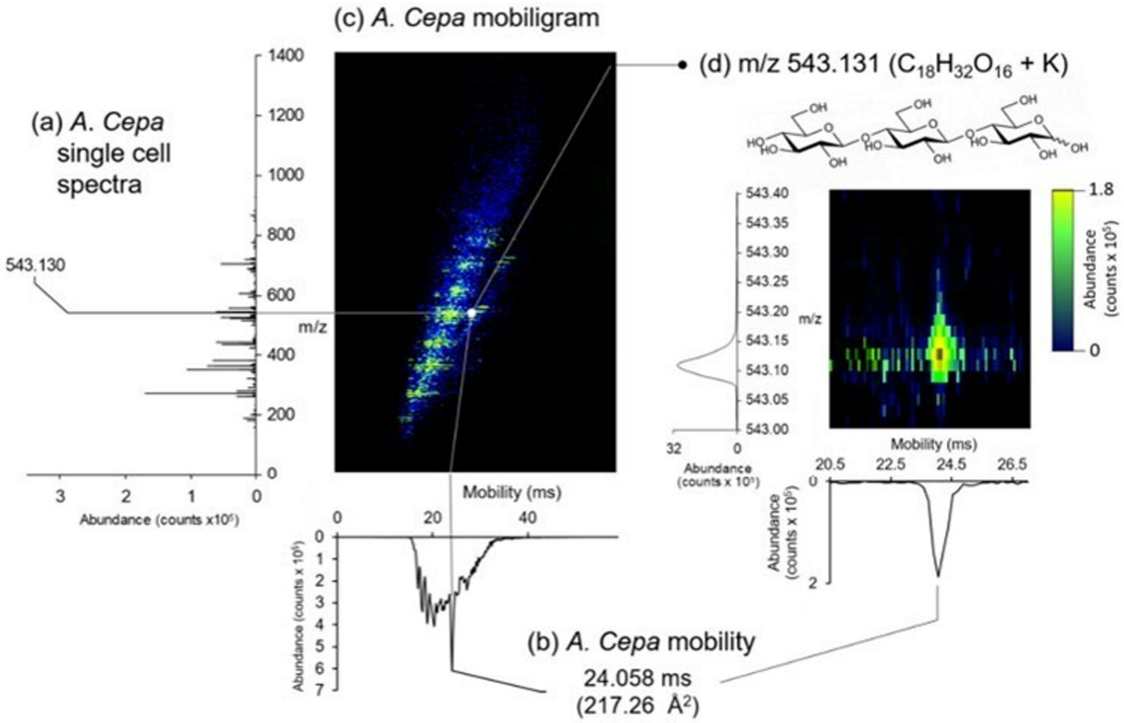

Figure 11. Example LAESI-DTIMS analysis of a single A. Cepa cell. (a) Spectrum with the saccharide peak labeled, (b) the corresponding mobility graph, and the (c) mobiligram showing detected species (green/yellow). (d) Spectrum and mobility graph of m/z 543.131 (C18H32O16 + K, Trisaccharide) with corresponding mobiligram. (Reproduced with permission from Meng et al.[161]

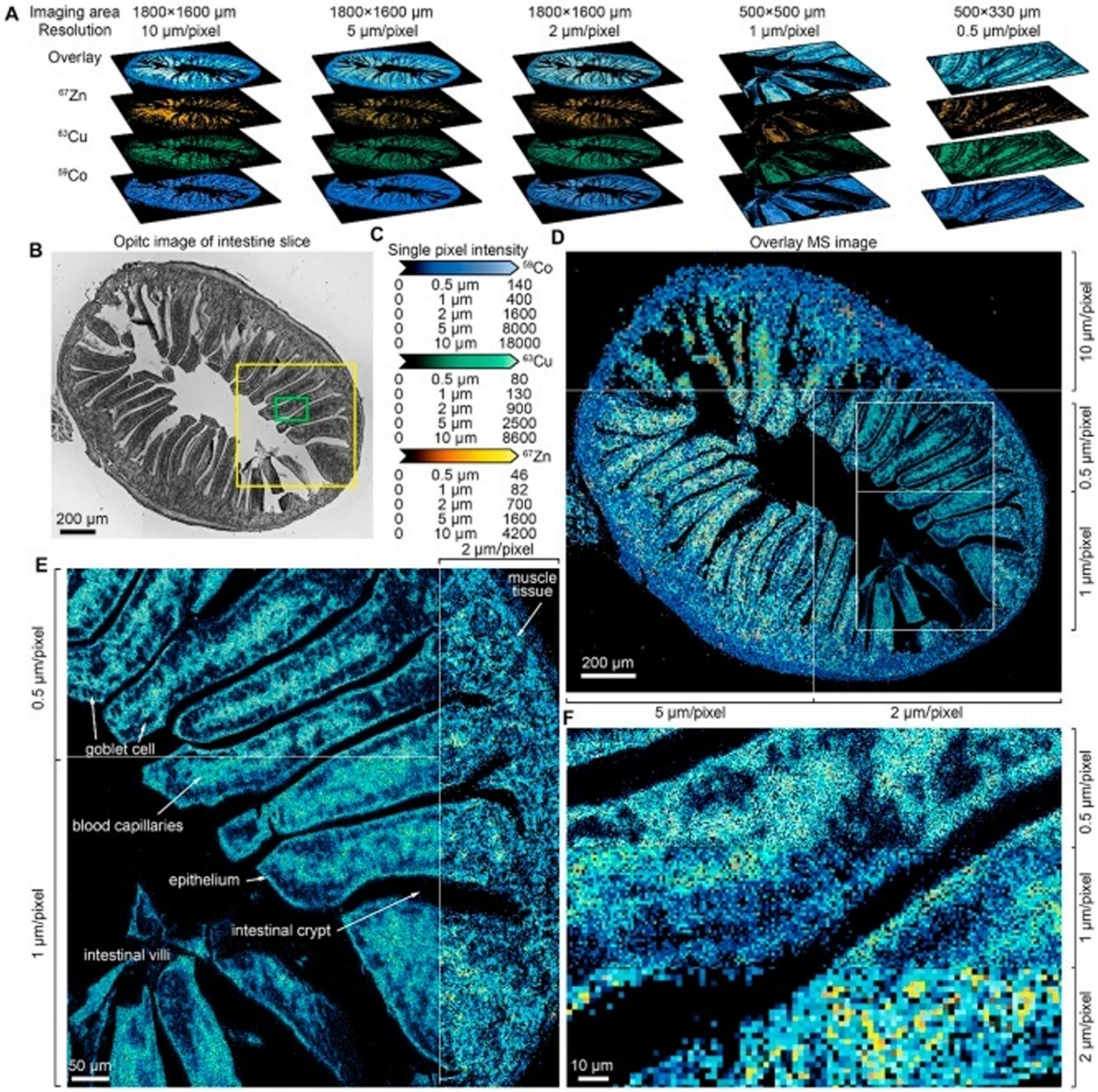

Figure 12. Example LAESI-DTIMS analysis of a single A. Cepa cell. (a) Spectrum with the saccharide peak labeled, (b) the corresponding mobility graph, and the (c) mobiligram showing detected species (green/yellow). (d) Spectrum and mobility graph of m/z 543.131 ($C_{18}H_{32}O_{16}$ + K, Trisaccharide) with corresponding mobiligram. (Reproduced with permission from Meng et al.[161]

and citrate, were discovered in each type of cell. The lipid region shows the most obvious difference, with membrane lipids in infected cells being significantly more abundant.[155] The lack of orthogonal separation in ambient analysis methods like LAESI-MS, however, opens the possibility of massive environmental noise and spectral sophistication.[158]

ICP-MS can detect and image *in situ* when used with a laser ablation (LA) system.[159,160] Meng *et al*. created a practical and affordable method that can increase the spatial resolution of LA-ICP-MS to about 400 nm (Figure 12).[161] For tiny volume ablation, a "three-way" structure ablation chamber has been develope.[161] For nanoscale sampling, laser desorption through a microlensed fiber, as used in our earlier research,[161] was used. Additionally, the method shows the potential of single-cell analysis. Knowing the intracellular pathways of drugs immobilized on nanocarriers presents a challenge for single-cell imaging. Carboplatin-based gold nanorods ("GNRs") were developed for single-cell MS imaging.[161] The authors were able to see how the drug was released from the GNRs carriers, entered the nucleus, and eventually caused apoptosis using the nanoscale LA-ICP-MSI technique based on the microlensed fiber.[161] Bright-field microscopy was used in the study to identify cellular apoptosis. High-resolution imag-

ing has several advantages, including the ability to detect nanomaterials, anticancer medications, and heavy metal pollution in lysosomes, cell nuclei, and cytoderm, respectively. This demonstrates the potential for studying chemical distribution at the organelle level.[161]

Conclusions

With the help of functional and mechanistic biology investigations, metabolomics is crucial for comprehending the physiological functions of metabolites. However, because metabolomics is still an emerging area, many discovered m/z values do not correspond to any items found in repositories. To achieve this, precisely identified metabolites are also necessary. Several databases ought to be queried to thoroughly analyze a dataset.[162] It is also possible that isotopic peaks, unanticipated adduct forms, spontaneous in-source degradation,[163] or unknowns are to blame for the absence of interpretation for detected m/z values.[164] Unknowns include real unknown metabolites, recognized compounds that are not yet included in databases, and epimetabolites.[164,165] Since metabolites' structural diversity is broad and they lack common building blocks like the amino acids or nucleotides present in proteins or the genetic code, respectively, the probability of unknowns is very high. Furthermore, there are no comprehensive lists of metabolites for every organism, making it impossible to fully identify an organism's metabolome.[166] A major difficulty that calls for informatics methods is moving past metabolite identification and ultimately determining the physiological functions of metabolites in metabolic pathways.

Due to its efficiency as a tool for comprehending the specific mechanisms underlying cellular and molecular patterns, single-cell analysis has gained increasing attention from researchers in a variety of biological domains. Chemical compounds can be evaluated and measured in single cells thanks to the development of sensitive and selective mass spectrometry techniques. MS techniques for single-cell analysis are now more varied than ever thanks to years of development. In this article, we have given an overview of these analysis techniques (DESI[167,170-172], IR-MALDESI, LAESI, DAESI, iEESI,nano ESI[168],CE-ESI[169] etc.) and a summary of the most recent developments in single-cell analysis and imaging research made possible by these various MS techniques. The studies presented here illustrate that the microsampling and imaging techniques presented are beneficial for probing the constituents of cells at different levels of chemical responsiveness and spatial resolution. Researchers interested in single-cell analysis will benefit from the differentiated advancement of these techniques because each one has benefits and drawbacks of its own. The need for MS systems with enhanced capabilities, particularly regarding detectability as well as time and spatial resolution, is further evidenced by the increasing technological demands associated with the study of cellular environments and single cells. MS will become more and more important in single-cell analysis as AMS technology develops and generates better spatial resolution and responsiveness; subsequently, this will advance biological research. With the help of this technology, it may be possible to identify various diseases early and gain important knowledge about cellular metabolism.

The discipline is advancing toward more sensitive approaches with higher throughput, quantitative capabilities, and less technical variability because of ongoing advances in MS-based single-cell metabolomics. Although single-cell approaches are still in their infancy, they commonly present results for species that are numerous with the hope that additional advancements may increase detection limits. The present challenges of single-cell metabolomics for biologically significant findings consist of a comparatively low throughput for techniques to distinguish between technical and biological fluctuations, a demand for higher sensitivity for metabolites with low abundance or low ionization productivity, and a need for advancements in databases to identify metabolites that are relevant and provide biological significance to the results.

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Conflict of Interest

The authors declare no conflict of interest.

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