Ambient mass spectrometry for *in vivo* local analysis and *in situ* molecular tissue imaging

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Recent technical innovations in mass spectrometry (MS) have extended the application of this powerful technique to direct chemical analysis at atmospheric pressure. These innovations have created an opportunity to appreciate the chemistry of biological systems in their native state, so tissues and single cells of plant, animal, or human origin can be interrogated *in situ* and *in vivo*.

Ambient MS also allows label-free detection of compounds and gives unique insights into temporal changes and tissue architecture in two and three dimensions. Compounds studied range from natural products (e.g., neurotransmitters, metabolites, organic acids, polyamines, sugars, lipids, and peptides) to xenobiotics (e.g., pharmaceuticals), dyes, polymers, explosives, and toxins.

This critical review covers analytical trends in ambient MS. Our discussions primarily touch on the mechanisms of sampling and the bioanalytical implications for *in situ* and *in vivo* experiments. We pay special attention to lateral imaging, depth profiling, and three-dimensional-MS imaging, all while working under atmospheric conditions. Our closing remarks highlight some of the present analytical challenges and developmental opportunities in this field.
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Keywords: Chemical imaging; Direct analysis; *In situ* analysis; *In vivo* analysis; Mass spectrometry; Metabolomics; Molecular imaging; Peptidomics; Single-cell analysis; Tissue analysis

Abbreviations: AP, Atmospheric pressure; DESI, Desorption electrospray ionization; ELDI, Electrospray-assisted laser-desorption ionization; fs, femtosecond; LAESI, Laser-ablation electrospray ionization; LDI, Laser-desorption ionization; MALDI, Matrix-assisted laser-desorption ionization; MS, Mass spectrometry; MSI, Mass-spectrometry imaging

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1. Introduction

Recent technological advances in mass spectrometry (MS) have lent fresh momentum to biochemical investigations under native conditions. Spurred by the 2004 introduction of ambient MS [1], a diversity of new MS techniques has emerged with many demonstrating impressive success in examining biological systems with molecular insight. This field is rapidly traversing from bulk analysis to probing ever-finer details of organizations and has already partially surpassed the single-cell level. The present review is to identify the major bioanalytical trends in ambient MS and also to draw attention to contemporary research opportunities.

Although MS is only one of many analytical techniques applied in biochemistry, it enjoys particular recognition when measuring chemically complex systems (e.g., tissues and cells) for fundamental and practical reasons:

(1) sampling minute amounts of material is compatible with most biological systems;
(2) label-free detection facilitates the identification of diverse compounds in their native state; and,
(3) quantitation over a broad dynamic range can address biologically relevant concentration levels.

These aspects brought certain vacuum-based and atmospheric pressure (AP) ion sources to the forefront of MS bioanalysis, and propelled research in the ''omics'' sciences and health-regulatory applications [2–5].

The advent of ambient MS translated sampling and ion generation from vacuum to AP while eliminating or

 0165-9936/$ - see front matter Published by Elsevier Ltd. doi:10.1016/j.trac.2011.11.006

Table 1. Major analytical terms and concepts in ambient MS and their implications for bioanalysis

Attribute of	Concept	Typical ambient MS meaning	Bioanalytical implication
Environment	Ambient	Of atmospheric pressure and room temperature	Ensures experimental conditions for direct analysis
	Native	Of physical and chemical characteristics inherent to specimen	Prerequisite for *in vivo* analysis
Analysis	Direct analysis	Sampling and ion generation with negligible physical and chemical modification to sample	Allows *in situ* analysis, and when in native sample environment, *in vivo* analysis
	Sampling	Removal of sample material	Determines the source of measured compounds
	Direct-contact	Part of the ion source is in physical contact with sample	Inherent for certain sampling technologies
	Contactless	Lacking physical contact with sample	Allows remote sampling and minimizes chemical cross-contamination
	In situ analysis	Direct analysis in the native environment of the specimen	Takes a snapshot of sample chemistry
	In vivo analysis	Preserving organism viability post analysis	Maintains organism alive during and after analysis
	Ex vivo analysis	Analysis in an artificial environment	Facilitates direct analysis under non-native conditions
	In vitro analysis	Separating components of an organism for analysis	Enables more in-depth analysis
Physical dimensions	Macroscopic	Of or above mm-scale dimensions	Enables long-range spatial profiling of chemicals
	Microscopic	Of μm-scale dimensions	Yields local analysis, and, when combined with rastering, chemical imaging
	Single-cell	Of dimensions comparable with mean cell size	Probes whole-cell chemistry
	Subcellular	Of dimensions below mean cell size	Investigates sub-cellular chemical heterogeneity
Compound	Endogenous	Of natural origin to specimen	Produced by organism
	Exogenous	Of artificial origin to specimen	Introduced into sample from outside

minimizing sample preparation [1]. An ambient ion source is characterized by direct sampling, minimal or no sample preparation, and typically high analytical throughput, so we need to keep in mind that, although ESI operates at AP, it is not an ambient ion source because it requires dissolution of the sample.

Table 1 clarifies other frequently used analytical terms and concepts in ambient MS and their implications for local analysis and tissue imaging. A brief literature survey indicates that there are at least 25 MS methodologies with ambient-probe characteristics [6–9] and this number is rapidly growing. These methods have mechanistic differences in their sampling and/or ion-generation method, which, in turn, manifest themselves in technique-specific sets of applications. Further details are available in the literature [6–9].

The current review is different from earlier efforts in that it aims to use the framework of ambient MS to gauge the trends in applications that have demonstrated *in vivo* local analysis and *in situ* chemical imaging on biological samples. With a primary focus on review publications and limitation in space, some excellent articles had to be excluded in preparing this manuscript, so we apologize to those omitted and welcome direct communications with the authors.

2. Ambient MS practiced *in vivo*

Rapid analysis is a unifying theme among ambient MS techniques with some demonstrated *in vivo* applications. For live specimens, the bioanalytical applicability of a given method can be assessed based on several factors, including analysis time, sampling dimensions, and mode of contact with the sample. Particular applications may require that we take into account additional aspects, including the class of detectable compounds or the presence of ionizing radiation. Some of the relevant techniques are listed in Table 2.

With the ability to utilize minute amounts of sample, the definition of *in vivo* analysis has to be revisited. In the case of a multicellular organism, sacrificing a few cells in the analysis may not significantly perturb its physiological processes to cause altered function or death. Thus these techniques can be viewed as operating *in vivo*. Conversely, even classic examples of *in vivo* analysis (e.g., immunostaining or X-ray imaging) can cause death if applied at excessive doses.

Stemming from the large differences in the sampling methods [6,7,10,11], the corresponding time requirements span several orders of magnitude. Most Q-switched laser-based ion sources couple ultraviolet (UV) or infrared (IR) light to the sample within a few

Table 2. Ambient MS methods with demonstrated capabilities for *in vivo* chemical analysis or *in situ* molecular imaging of biological systems

Scope of analysis	Ambient MS (acronym)	Samples	Endogenous compounds	Probe dimensions*		*In vivo?*	Potential application	References.
				Diameter	Depth			
Macro	DART	Bacterium, animal	Volatiles, fatty acids, esters, hydrocarbons	n/a (bulk)		Yes	Microscopic sampling	[16]
	EESI, SESI	Human, animal, bacterium	Saccharides, amino acids, polyamines, amines	n/a (bulk)		Yes	Biomarker discovery	[21–23,25]
	REIMS	Human, animal	Lipids, small acids	n/a, profiling		N/a	*In vivo* surgery	[19]
	LDI	Human, animal	Lipids, small acids	n/a, profiling		N/a	*In vivo* surgery	[28]
	LTP	Human	Explosives, drugs	250 μm on paper		Yes	Tissue MSI	[15]
	Paper spray	Human, animal	Hormones, lipids	<1 mm**	1 mm**	N/a	Chemical separation	[34,37]
	LMJ-SSP	Animal	Metabolites, lipids	<1 mm		N/a	Chemical separation	[38]
Micro	DESI	Animal, plant, microalga, human	Metabolites, acids, lipids, sugars	40–250 μm	20 μm for reconstructive 3D-MSI	Yes	Unicellular, biofilms	[1,34]
	SDAPCI	Animal	N/a	250 μm		N/a	High-resolution MSI	[50]
	AP MALDI	Plant	Acids, lipids	40–400 μm		N/a	*In vivo*	[13]
	PESI	Animal, plant	Lipids, carbohydrates, amino acids, phytochemicals	60 μm		Yes	Unicellular	[26,51]
	LA FAPA	Animal	N/a	50–250 μm	40 μm	n/a	Biological, *in situ* 3D-MSI	[17]
Single-cell	LAESI	Human, animal, plant	Metabolites, acids, neurotransmitters, lipids, carbohydrates, proteins, phytochemicals	50–250 μm	40 μm for *in situ* 3D MSI	Yes	*In vivo* surgery, subcellular, biofilms	[43,68,73,74]
Sub-cellular	AP (fs)-LDI	Plant	Primary metabolites	10 μm		N/a	High-resolution MSI	[75]
	Video-	Animal, plant	Amino acids, neurotransmitters, acids, terpenes	2 μm (1 pL)		N/a	Sub-cellular analysis	[76]

* Cited voxels are cylindrical with a circular base.
** Application for local analysis was supported by needle aspiration biopsy; Not applicable (n/a) indicates data not available.

nanoseconds {e.g., in electrospray-assisted laser-desorption ionization (ELDI) [12]}, but, in the case of mid-IR excitation, material ejection can extend to ~1 ms {e.g., AP IR-matrix-assisted LDI (IR-MALDI) [13] and laser-ablation (LA) electrospray ionization (LAESI) [14]}. By comparison, low-temperature plasma (LTP) MS measured warfare agents 1 m away from the sample with ~5 s analysis time involving sample evaporation and diffusion to the ionization source [15]. In most organisms, these time frames are deemed sufficiently fast to take a chemical snapshot of biological states, so ambient MS emerges as a valuable tool in the *in situ* study of sensitive specimens or dynamic processes.

Nearly all ambient MS ion sources can operate *in situ*, albeit not all are amenable to perform *in vivo*. Differences in the sampling mechanisms inherently impose limitations on live specimens [e.g., laser-based analysis of samples transparent in the ultraviolet (UV) region benefits from external matrices for enhanced light coupling but at the cost of potentially interfering with natural state and viability]. Similar limitations for *in vivo* studies may be alleviated by exploiting optically active endogenous compounds of high tissue concentration (e.g., primary and secondary metabolites), and we anticipate particular success in this respect with ambient MS via AP UV-MALDI and ELDI sampling. Once chemical-sample modification is eliminated, the ability to maintain system viability post analysis is primarily dictated by the relationship between the size of the organism and the physical dimension of the sampling volume, and is further hindered by the presence of ionizing radiation. Ambient probe dimensions range from several millimeters to a few micrometers in two- and three-dimensional space, and are surveyed in Table 2.

Desorption-ionization techniques minimize microscopic-scale damage to samples and are well-suited for live specimens. For example, gentle sampling characterizes direct analysis in real time (DART) that measured cuticular compounds in live fruit flies (*Drosophila melanogaster*); the specimen was immobilized to a capillary by gentle vacuum (Fig. 1A) and surface molecules were desorbed [16]. A survey of mass spectral features revealed hydrocarbons of longer chain length in the male flies than in their female counterparts (compare left and right panels). Similarly, an LTP source lifted cocaine molecules directly from a human finger, at an operational temperature of 30°C that prevented adverse effects to the subject (Fig. 1B) [15]. When finely tuned, dielectric discharge barrier desorption ionization (DBDI), plasma-assisted desorption ionization (PADI) and laser absorption with flowing AP afterglow (LA-FAPA) [17] may offer similar possibilities for *in vivo* studies [11,18]. *In vivo* applications by desorption techniques, including DART and LA-FAPA, benefit from short analysis times to minimize sample heating and/or drying due to high desorption gas temperatures (~250–500°C). More recently, charged droplets produced during electrosurgery combined with rapid evaporative ionization-MS (REIMS) allowed the detection of lipid and metabolic species directly from mammalian tissues *ex vivo* [19]. This technique seems well suited to the operating theater and promises eventually to aid surgical applications by *in vivo* screening of biomarkers in patients.

Alternative *in vivo* approaches utilize ESI to generate ions. For example, fast electrosprayed droplets facilitated spatially localized liquid extraction in desorption electrospray ionization (DESI)-MS to measure directly excreted antihistamine on skin [20]. In a spatially decoupled manner post sampling, electrosprayed droplets were also utilized to ionize nicotine from human breath and explosives from skin in extractive ESI (EESI)-MS [21,22]. The same approach allowed profiling of various human organs and monitoring of *Escherichia coli* on spinach [23]. Furthermore, volatile compounds of breath and organic acids released from human skin were fused with electrosprayed droplets in *in vivo* secondary ESI (SESI) [24,25]. In another direct-sampling set-up, carbohydrates, amino acids, and phytochemicals were collected with a sharp ESI emitter from live tulip bulbs in probe ESI (PESI)-MS [26].

Although laser sampling is locally destructive, the damage level is finely controllable to enable *in vivo* investigations. As shown in Fig. 1C, various organs of a live French marigold (*Tagetes patula*) seedling were chemically profiled with ~300 μm ablation spots in LAESI-MS, with viability of the organism demonstrated for several weeks [14]. Advanced light delivery can improve sampling to 50 μm areas [27], further minimizing the damage and extending the applicability of mid-IR ablation to smaller specimens. Another important aspect of LA sampling is its speed. The cells sampled by this method function in their normal environment until the ~1-ms LA event, so the analysis is likely to reflect the physiological state of the organism. Likewise, UV and far-IR regions of the spectrum provide a potential for LDI-MS for *in vivo* experiments and surgical applications [28].

2.1. Water-containing samples

An increasing number of ambient methods are beginning to utilize natural chromophores in biological samples for efficient laser sampling. The main tissue components with high absorption coefficients are proteins, DNA, and melanin in the UV region (180–400 nm wavelength), melanin and hemoglobin in the visible rage (400–780 nm wavelength), and water, hemoglobin, collagen, and proteins in the near- to far-IR regions (0.78–15 μm) of the spectrum [29]. At the appropriate absorption wavelength and volumetric energy density, microscale LA offers an efficient way to probe the chemistry of nearly all biological samples.

In LA sampling, water is the native energy-coupling matrix for a subset of techniques so as to give way to *in situ* and *in vivo* interrogations. Water is practically

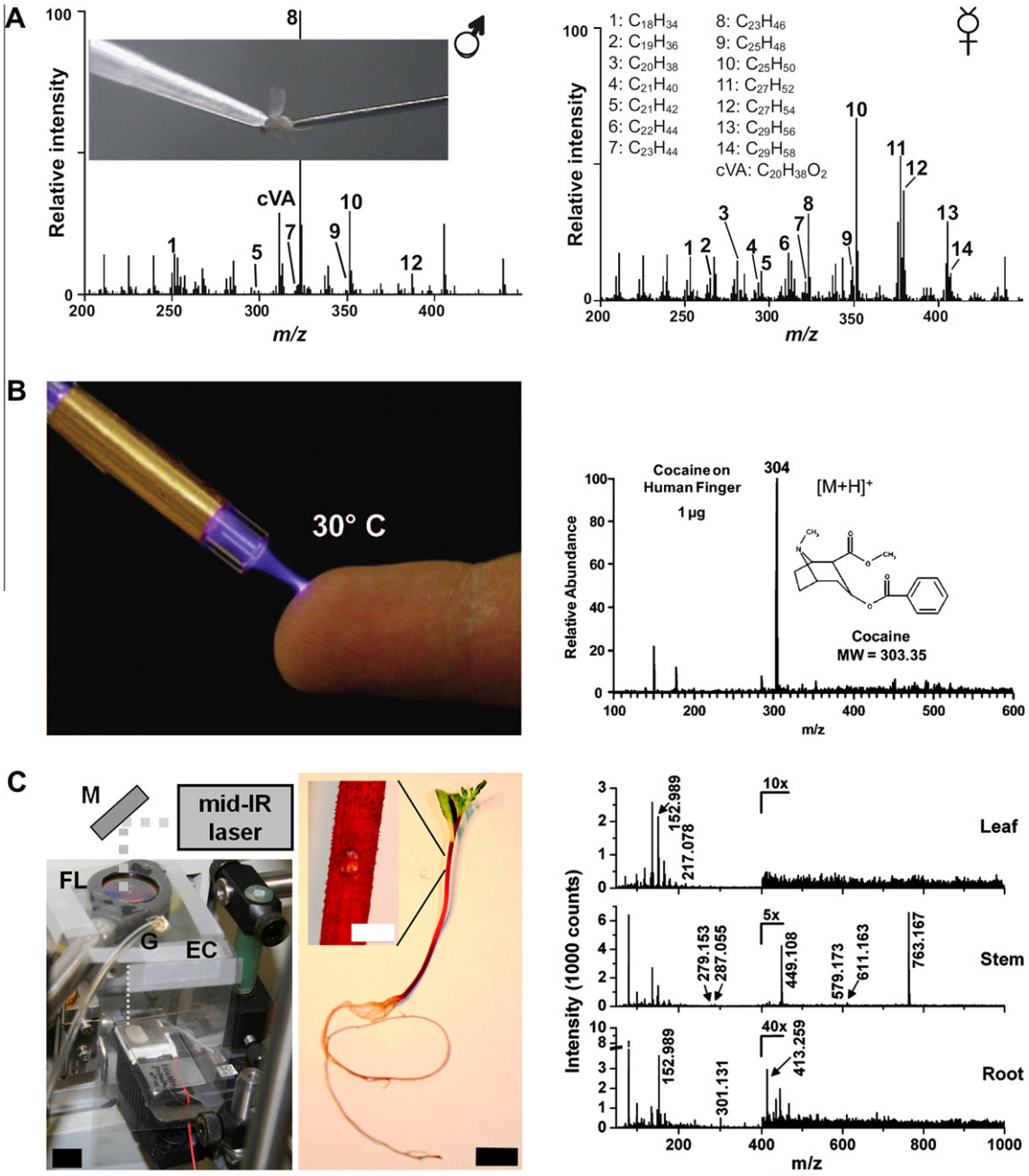

Figure 1. *In vivo* local analyses by ambient MS. (A) DART-MS identifies long-chain hydrocarbons on the cuticle of live male *D. melanogaster* flies. (B) LTP harmlessly desorbs cocaine molecules from a human finger. (C) LAESI-MS measures characteristic metabolite profiles for plant organs in a *T. patula* seedling with minimal damage to the organism. Key: M, mirror; GL, focusing lens; G, gas supply; EC, environmental enclosure. Scales in black = 10 mm. Scale in white = 1 mm. (Images were adapted from [14–16] with permission).

transparent in the near-UV region and exhibits several local absorption maxima in the mid-IR range (i.e. 1–10 µm). Energy coupling is most efficient at ~3 µm wavelength due to the symmetric and asymmetric stretching vibration modes of the OH bond [29]. Not only does this sampling method bypass chemical sample preparation but, working in the IR range, it also reduces or eliminates photochemical degradation of the biomolecules [82]. For comparison, many biomolecules exhibit absorption features in the UV range and may undergo degradation in UV-desorption sampling for ambient MS in the absence of a UV-absorbing matrix. Both AP-IR-MALDI and LAESI were among the earliest ambient ion sources to utilize 2.94-µm-wavelength light for the *in vivo* sampling of biological specimens for MS [13,14]. The features described above make mid-IR LA an attractive approach for the detection of intact biomolecules in live specimens by ambient MS.

Water-dependent direct analysis is not without its own analytical challenges. Mid-IR ablation is sensitive to evaporative loss, condensation, and inhomogeneous distribution of water in the sample, as well as spatial changes in mechanical characteristics, including tensile strength. Some of the former challenges may be mitigated by placing the sample into a microenvironment for fine control of humidity or composition of ambient air and/or to cool or to freeze tissues over an extended period of time (see Fig. 1c). For practical purposes, lateral water distribution and tissue tensile strength do not appreciably change in most biological samples, making mid-IR-based ablation a very robust technique. An exception is transitioning between water-rich soft and dry-bone tissues, which is expected to lead to biased sampling; water-rich soft tissue ablates more efficiently. Remedy may be provided by tuning the fluence and/or the wavelength of the laser beam *in situ* for regions of interest or retroactively correcting signal intensity for changes in sampled voxels. Similar experimental considerations can improve sampling reproducibility to ensure robustness in mid-IR-LA-MS.

These analytical benefits drove the emergence of an increasing number of mid-IR-based sampling techniques. For example, IR-LADESI [30] and IR-MALDESI [31] have recently coupled mid-IR-LA with ESI, and IR-LAMICI [32] coupled mid-IR-LA with metastable-induced chemical ionization (CI) for the direct analysis of small molecules, peptides and proteins from complex samples. Future developments are expected to lower the limits of detection (LODs) by improving the efficiency of capturing ablated particles {e.g., by air amplifiers [33]}, maximizing the yield of ion generation post-ablation {e.g., via reactive electrosprays [34]}, and enhancing collection of the ions produced for MS detection {e.g., by ion funnels [35]}. An important trend for *in vivo* biological applications is to generate ablation events with ever-decreasing dimensions.

3. Targeting varying levels of spatial organizations

3.1. From bulk analysis to spatial profiling

Ambient MS analyses exhibit a broad range of sampling dimensions, allowing different levels of biological organization to be targeted in the organism-organ-tissue-cell-organelle realm. Relatively large sampling areas characterize a number of techniques (see Table 2). These methods can help to reveal the characteristic chemical composition of selected areas locally or with low spatial resolution. An example is EESI-MS that measured metabolites *in vivo* in human breath or from large skin surfaces [23]. Furthermore, various bodily regions of fruit flies were sampled together in DART-MS (Fig. 1A) [16], and their chemistry was integrated for a total area of $\sim 5\ cm^2$ in LTP-MS [15]. To increase the spatial resolution of analysis with the latter approach, 250-μm × 250-μm sampling areas were interrogated on artificial samples [36].

Current progress tailors sampling dimensions to application needs, and *vice versa*. Biological samples, including bodily fluids and tissues, are generally present or can be harvested only in limited amounts, calling for sampling on a refined scale. For example, paper-spray-MS has succeeded in reducing the material requirement to less than 1 μL of blood or tissue by needle-aspiration biopsy [37]. Tissue components can also be locally extracted *in situ* into a single droplet by a liquid-micro-junction (LMJ) interface, and high-pressure liquid chromatography (HPLC)-MS can identify drug metabolites with confidence [38].

In translational projects (bench to bedside), local tissue analysis during surgical procedures is being pursued to provide direct information for supporting medical decisions. An example is low spatial-resolution chemical profiling of biomarkers that are indicative of cancerous tissues. REIMS and LDI-MS have recently been demonstrated *ex vivo* to aid surgeons in the operating theater [19].

Reduction in probe dimensions not only promotes the analysis of small specimens but also enables molecular imaging. If sampling is sufficiently localized, analyses can be performed in a repetitive manner, rastering across the sample to create an image reflecting the molecular organization of the specimen.

3.2. From local analysis to lateral chemical imaging

With a variety of emerging AP ion sources, molecular imaging is also feasible in the ambient environment. In vacuum, MS imaging (MSI) can follow two modalities:

(1) microprobe sampling integrates the chemistry for a small sample area; and,
(2) microscope analysis simultaneously reports chemical constituents in a spatially resolved manner for a selected target surface [39,40].

Molecular collisions and the sub-μm mean free path at AP have limited ambient MSI to microprobe-type imaging (Fig. 2A). In this arrangement, the sample is mounted on a sample holder and is laterally positioned by computer-controlled independent translation stages (X and Y directions), while the ions generated are simultaneously analyzed and the mass spectra are stored for each coordinate pair (pixel) of the interrogated area. Finally, the molecular image of the sample is reconstructed by correlating the intensity or area of the ion signal for a given m/z with the absolute coordinates of analysis for every pixel of the analyzed surface.

With the commercial availability of the necessary equipment, ambient MSI has been carried out in rapidly growing numbers, albeit with variations in analytical performance. The success of an imaging application can be partly rated based on the spatial resolution achieved

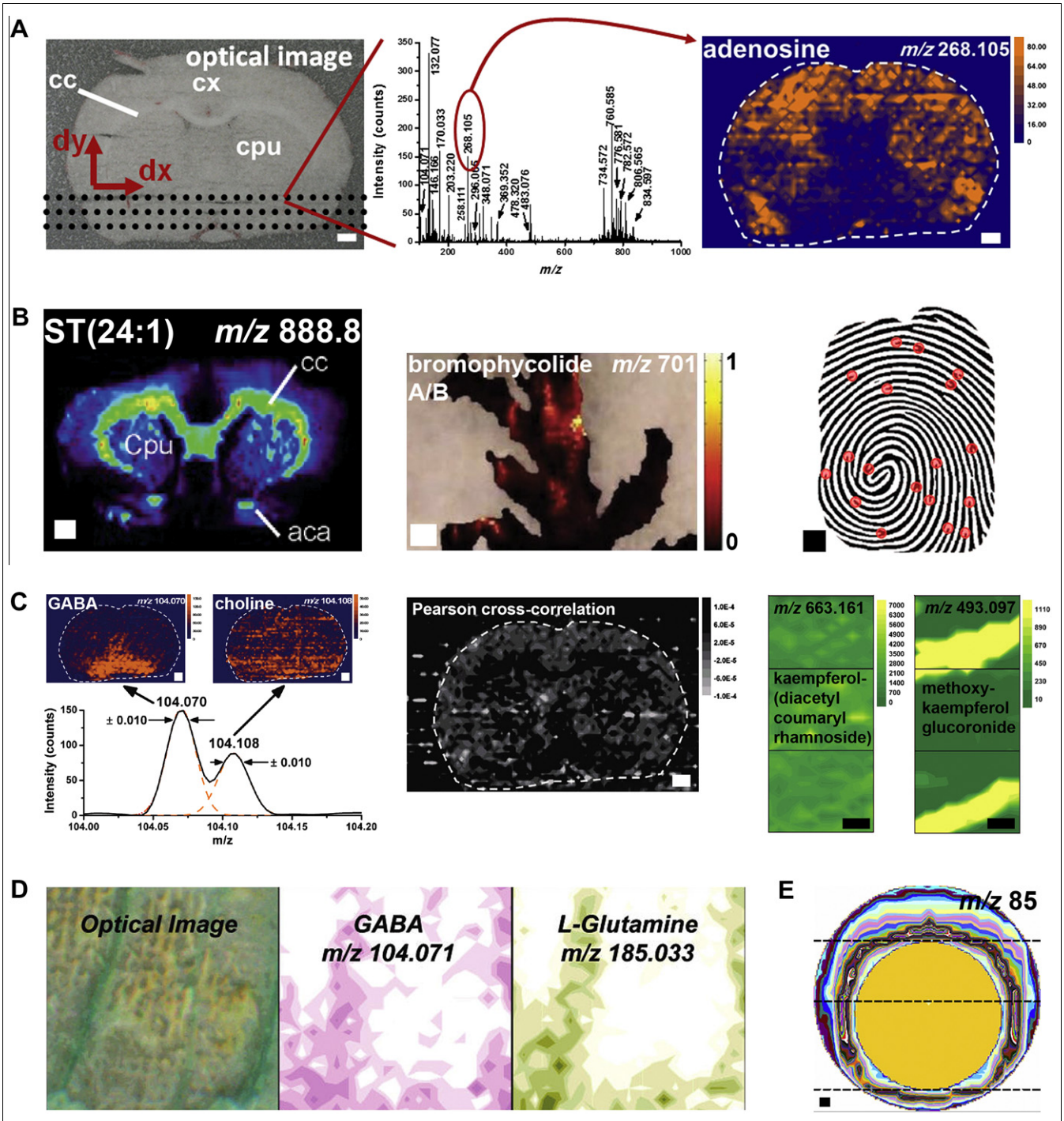

Figure 2. Lateral imaging by ambient MS. (A) Microprobe-mode MSI records the chemistry of each pixel on the surface, and ion images correlate the signal intensity with the absolute coordinate of analyses. (B) DESI: (left) sulfatide (24:1) (ST) accumulation in the corpus callosum (cc) of *R. norvegicus* brain; (middle) bromophycolide A/B in pale patches in *C. serratus*; (right) latent cocaine fingerprint and identified minutia (red dots). (C) LAESI: (left) spatial distributions of isobars GABA and choline distinguished in *R. norvegicus* brain (left and right insets, respectively); (middle) in darker pixels, Pearson cross-correlation analysis showed co-localization of cholesterol and PC(O-33:3) and/or PE(O-36:3) plasmalogens in the cc; (right) secondary metabolites imaged in *A. squarrosa* leaf. (D) AP IR-MALDI: GABA and glutamine imaged in veins of *L. candidum* flower petal. (E) SDAPCI: imaging of melamine fragment (*m/z* 85) revealed heterogeneous contamination in cooked egg. All scales = 1 mm. (Images were adapted from [45,46,50,56,79–81] with permission).

and the analytical limit of detection. At present, the customary performance for lateral imaging is in the ~40–250-μm range for biological specimens and is detailed for various ionization platforms in Table 2. DESI and LAESI have achieved considerable success in imaging animal and plant tissues with 250-μm resolution.

For example, DESI-MSI case studies in Fig. 2B revealed the accumulation of specific lipids in rat (*Rattus norvegicus*) brain, natural products in the tropical seaweed *Callophycus serratus*, and drugs of abuse in human fingerprints [34,41]. These results have been extended to drug metabolism in whole-body sections, and exciting applications have gained a foothold in biomarker studies and chemical diagnostics; profiling and imaging revealed differences in biochemical compositions between cancerous and healthy regions of tissue [7,11,34]. New technical developments, including electrode-assisted approaches, promise to empower DESI with 25-μm resolution [42].

LAESI-MSI is well suited to the study of water-containing samples [43,44]. As shown in Fig. 2C, high mass-resolution LAESI-MSI measured different distributions for the isobars GABA and choline in a thin *R. norvegicus* brain-tissue section. In stark contrast, adenosine was detected close to the cerebral cortex of the tissue (Fig. 2A). Pearson colocalization maps [45] have been introduced as a way to facilitate the evaluation of metabolic connections in LAESI studies. For example, two-dimensional (2D) colocalization was striking for cholesterol and phosphocholine (PC) (O-33:3) and/or phosphoethanolamine (PE) (O-36:3) in the corpus callosum (Fig. 2C). Plant tissues are also well suited to LAESI-MSI because the waxy coatings of the cuticles prevent rapid sample drying during the analysis. In the leaves of the Zebra plant (*Aphelandra squarrosa*), numerous secondary metabolites were detected with different distribution characteristics [46] (Fig. 2C).

Other laser-probe techniques also demonstrated abilities for *in situ* MSI. The molecular organization of plant tissues was the focus of AP UV-MSI and IR-MALDI-MSI experiments (Table 2). Fig. 2D presents fluid transport monitored through the vasculature; glutamine and GABA distributions paralleled the optical image of toluidine blue O-stained veins in a white lily (*Lilium candidum*) flower petal. Proof-of-principle experiments confirmed that AP IR-MALDI can obtain a spatial resolution of 40 μm [13], nearing the dimensions of an average plant cell. With low primary-ion yields obtained at AP [47], LA and desorption techniques can benefit from incorporating secondary ionization. For example, ESI of the ablation plume increased ion yields by up to two orders of magnitude in LAESI-MSI experiments compared to AP IR-MALDI [47]. In addition, chemical interferences can be minimized during ionization by combining laser-based sampling with *in situ* chemical separation. As an example, LMJ captured the chemical constituents of ablated particles in a droplet and employed HPLC-ESI-MS for analysis with ~70-μm sampling resolution on mock samples [48]. HPLC also offered a compound-specific piece of information to aid chemical identification. Higher signal-to-noise ratios and an ability to differentiate structural isomers are among the anticipated advantages of similar developments in laser-based ambient MS and MSI.

Data-dependent acquisition is a significant area of developmental opportunity in ambient MS. Despite advantages in higher signal-to-noise ratio and chemical specificity, so far relatively few pertinent examples have surfaced in the literature. For example, MSI in tandem operational mode achieved higher chemical specificity for DESI [49] and surface desorption AP CI-MS (SDAPCI-MS) [50]. As shown in Fig. 2E, imaging of a melamine fragment with 250-μm resolution in a cooked egg indicated contamination in the egg white rather than the yolk. Notably, melamine levels were slightly elevated in the central portion of the egg, which corresponded to the umbilical cord of the developing embryo. With recent instrumental advances routinely enabling data-driven acquisition in most MS systems, virtually all ion sources of ambient MS can anticipate analytical benefits from related applications.

Physical contact with the substrate is an alternative approach for AP interrogations. In this case, the spatial resolution is primarily determined by the size of the sampling object. For example, a fine needle was used to sample a thin mouse brain section in PESI with ~60 μm resolution [51]. To improve lateral resolution, we expect efforts to reduce the size of the probe. Using smaller droplets in LMJ-MS, micro-scale extraction promises high spatial-resolution chemical imaging, albeit with lower sample throughput due to the need for solvent delivery.

We expect that, in the years ahead, numerous ambient MS techniques will yield biochemical images with increasingly finer details. These developments are driven by the goals of aiding biological investigations and enhancing chemical diagnostics [36,52,53]. Preliminary results on mock samples have already indicated the feasibility of ~20-μm resolution with IR-LA metastable-induced CI (IR-LAMICI) [32] and ~8 μm with AP UV/IR-MALDI-MS [13,54–56]. Smaller spot sizes, potentially only defined by the light diffraction limit, are achievable for UV-based desorption techniques {e.g., ELDI [12], LA coupled to a flowing AP afterglow (LA-FAPA) [17] and microprobe LDI-MS [57]}. Near-field sampling can further improve spatial resolution. As demonstrated by recent results on artificial surfaces, coupling scanning near-field optical microscopy (SNOM) to MS promises chemical imaging with <1-μm resolution [58].

Novel approaches are breaking away from tissue surfaces and opting to sample submerged surfaces and potentially sub-surface layers. Intriguing developments have shown some success in profiling mock samples {e.g., scanning MS (SMS) probes [59], LMJ scanning sampling probe (LMJ-SSP), scanning capillary microscopy MS (SCM-MS) [60], and nanospray-DESI [61]}. These techniques offer ways to gain insight into sub-surface chemistry [62] and to study dynamic biochemical processes while performing under native-like

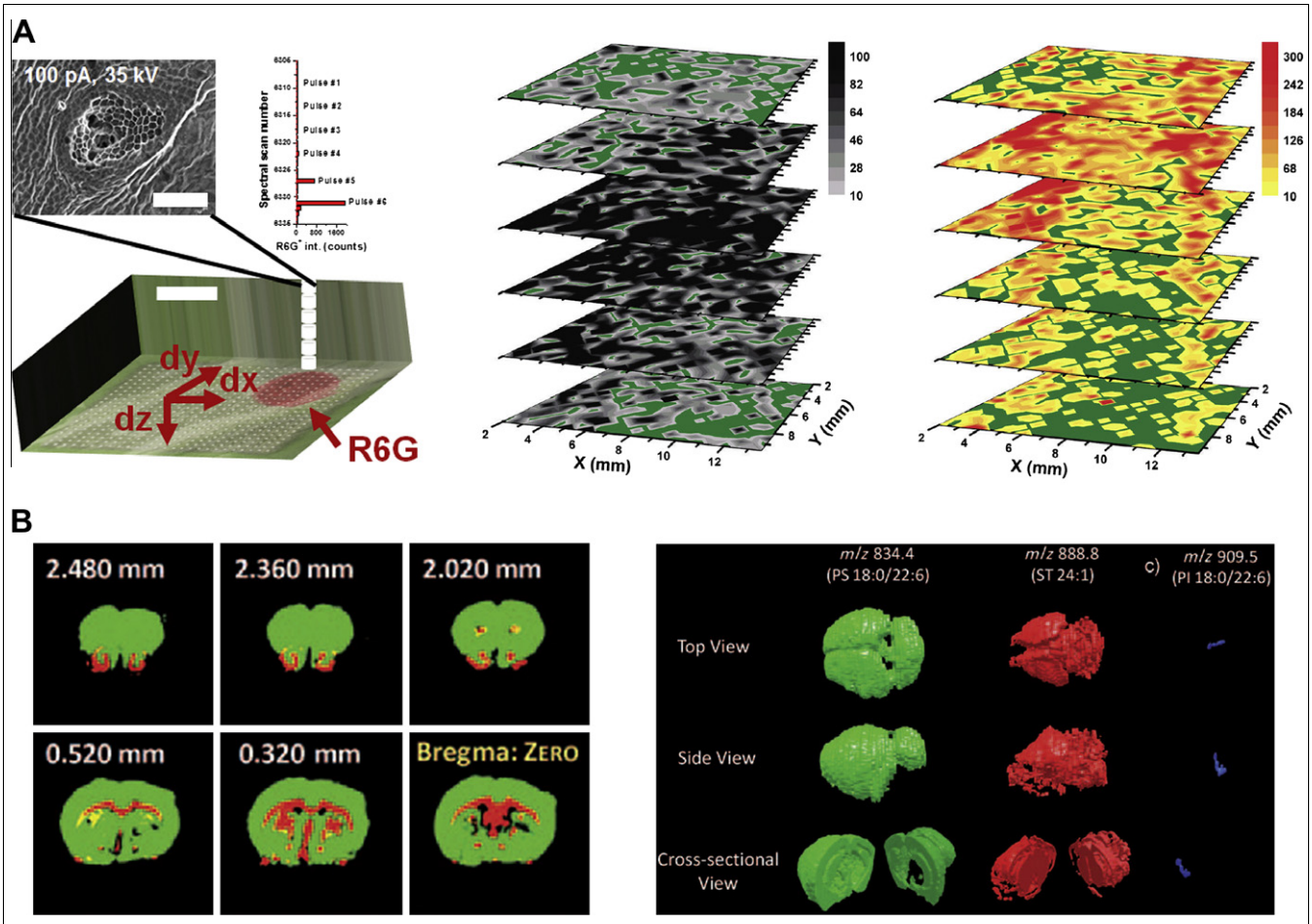

Figure 3. Ambient MSI in three dimensions. (A) LAESI-MS: *in situ* 3D imaging combined (left) depth profiling (dz) and lateral imaging (dx, dy). Rhodamine 6G (R6G) dye on abaxial leaf surface was detected by the sixth laser pulse (see $R6G^+$ signal), showing ~40 μm depth resolution. Scale bar = 200 μm. In *A. squarrosa* leaves, (middle) kaempferol-(diacetylcoumarylrhamnoside) populated the mesophyll layers (third and fourth from the top) with uniform lateral distributions; (right) acacetin accumulated in the yellow sectors of the second and third layers with homogeneous distribution in the others. (B) 3D-image reconstruction by DESI: (left) phosphatidylserine, PS, 18:0/22:6 (green) and sulfatide, ST, 24:1 (red) laterally imaged in serial coronal sections of *M. musculus* brain; (right) these lipids and phosphatidylinositol, PI, 18:0/22:6 (blue) were rendered into a 3D model. (Images were reprinted from [65,70] with permission.)

experimental conditions, including the presence of biological fluids and media for nutrition supply.

3.3. Volume sampling and depth profiling

The sampling methods described in the previous sections exhibit large differences in their ability to access subsurface regions. For example, based on SEM imaging of LTP-exposed paper, this method is thought to sample the surface layers to less than ~1 μm in depth [63]. Laser-based methods can interrogate the target to a depth determined by the light-penetration depth at the given wavelength. At low laser intensities and high absorption coefficients, UV laser pulses tend to remove less material (<1–2 μm/pulse) [64], whereas mid-IR ablation of hydrated tissues exhibits a removal rate of 30–40 μm/pulse [65]. This situation changes dramatically for ultrashort (~100 fs) laser pulses. Due to the highly non-linear nature of the laser-sample interaction at the intensities created (10^{13} W/cm^2), material removal rates for these lasers are less wavelength dependent.

There are diverse approaches to sub-surface sampling. For example, a sharp needle is brought in direct contact with the tissue (i.e. porcine retina) in PESI-MS to probe its chemistry *in situ* to ~20 μm depth. Alternatively, ablation by a focused laser beam allows remote sampling, reducing the potential of voxel-to-voxel contamination [9,66]. As shown in Fig. 3A, an individual laser pulse can remove *A. squarrosa* epidermal cells with ~35 μm average depth [65], corresponding to ~60 pL–20 nL voxel volumes, depending on focusing conditions.

Repeated sampling of underlying voxels enables chemical depth profiling. With limited depth resolution, LAESI-MS has been used to probe cross-sectional heterogeneity for a number of endogenous metabolites in plants, including chlorophyll *a*, kaempferol-(diacetyl coumarylrhamnoside) and acacetin (Fig. 3A) [46,66]. In

LA-FAPA [17] and IR-LA-MICI [32], alternative methods of ion generation are applied in combination with LA to achieve chemical depth profiling with resolution similar to LAESI. In biological applications, depth resolution depends on an intricate balance between the tissue tensile strength, water content, and laser fluence and stability.

Scanning microprobe microscopy techniques can offer impressive depth-profiling resolutions when combined with laser desorption or ablation events. Atomic force microscopy (AFM) tips [57] and near-field effects [58] confined material removal to voxels with ~1 μm diameter and ~0.6 μm depth in mock samples. Reduced voxel dimensions mean smaller amounts of sample and fewer detected constituents in the mass spectra, which may impose limitations for biological samples.

Efforts are under way to improve sample collection and ionization methods following ablation [33,35]. If successful in combination with nanoscale ablation, they can enhance the analytical figures of merits and expand the detectable range of chemical classes.

3.4. Three-dimensional chemical imaging

Ambient conditions are crucial for performing *in vivo* or *in situ* studies by MS. Three-dimensional (3D) MSI is a recent development under AP conditions. Similar to microprobe 3D imaging by SIMS and MALDI in vacuum [66,67], 3D-MSI has two basic operational modes at AP with demonstrated volumetric imaging capabilities: 3D volume reconstruction and the combination of depth profiling and lateral imaging. Differences between these approaches are illustrated in Fig. 3. At this point, only three ambient MS techniques have demonstrated the potential for 3D-MSI: DESI, LAESI and LA-FAPA.

In LAESI 3D-MSI, lateral chemical imaging is combined with depth profiling. The experimental protocol is available elsewhere [68]. As shown in Fig. 3A, the depth profile of the tissue is recorded voxel by voxel for each position across a raster, allowing the reconstruction of the 3D molecular image from the corresponding mass spectra. In plant tissues, 3D-MSI has revealed numerous distribution types for endogenous primary and secondary metabolites. Representative examples are presented for kaempferol-(diacetylcoumarylrhamnoside) and acacetin in Fig. 3A. Careful adjustment of the laser fluence and accurate *X*-*Y*-*Z* translation stages are required for robust LAESI 3D-MSI imaging.

The second approach, 3D volume reconstruction, is used in combination with DESI. Also applied in vacuum MALDI-MSI [69], this technique is based on the lateral imaging of serial sections of the tissue and the 2D images generated are rendered into a 3D image through computation [66,70]. Under ambient conditions, DESI-MSI has demonstrated impressive 3D-MSI results [70]. Fig. 3B shows representative examples for the lipids phosphatidylserine, sulfatide and phosphatidylinositol in mouse brain (*Mus musculus*). Importantly, the depth resolution here is determined by the 10–200-μm thickness of the sections produced by a cryogenic microtome. This makes 3D volume reconstruction highly reproducible, but it can be demanding on analytical throughput.

As laser-based ambient MS becomes available on other platforms, we expect 3D imaging to gain momentum. For example, because LA-FAPA has demonstrated 2D imaging with 50–250-μm resolution on a tissue sample and depth profiling in 40 μm steps on a tablet [17], we can anticipate a combination to yield *in situ* 3D-MSI. Among the laser-based ambient MS techniques, LAESI, LA-FAPA and IR-LAMICI will probably offer dual-operation modes for *in situ* 3D imaging (i.e. combination of depth profiling and lateral imaging and 3D volume reconstruction). Also, some platforms [e.g., DESI (Prosolia, Inc., Indianapolis, IN, USA) and LAESI (Protea Biosciences, Morgantown, WV, USA)] have already been commercialized for high-throughput imaging applications. The related software packages can aid data interpretation by producing colocalization maps and performing multivariate analyses.

Automated data processing with high-throughput 3D-MSI will help to explore physiological and biochemical details of biological specimens on a new level. Distributions of chemical species can only be revealed partially or obscured completely in 2D-MSI, which integrates for the analyzed depth within the tissue. For example, 3D-MSI identified a heterogeneous depth profile for kaempferol-(diacetylcoumarylrhamnoside) in an *A. squarrosa* leaf (Fig. 3A), whereas this pattern was reduced to a uniform distribution in the corresponding 2D-MSI (Fig. 2C). These results underline the importance of 3D-MSI and confirm that it can facilitate exploring the biological organization of specimens.

3.5. Single-cell and sub-cellular analysis and imaging

There are considerable research efforts under way to reduce sampling dimensions to or below the size of individual cells [71]. In the ambient setting, until now only a few techniques have achieved sufficiently small probe volumes to achieve this on biological samples. The important characteristics of these techniques are listed in Table 2.

Contactless sampling via LA opened up the possibility to probe single cells by LAESI and femtosecond (fs)-LDI. As illustrated in Fig. 4A, in the LAESI approach, an etched optical fiber is employed to ablate individual epidermal cells of *Allium cepa* [27,72,73]; the experimental protocol is available elsewhere [74]. With a 30–50-μm-diameter probe, adjacent cells were imaged one by one and the registered data revealed heterogeneous distributions for a number of metabolites. Some secondary metabolites (e.g., quercetin and cyanidin) were more abundant in the pigmented cells, whereas certain primary metabolite levels were slightly elevated in the non-pigmented cells [73] (Fig. 4A).

Single-cell imaging resolution was enhanced using optically non-linear fs-laser excitation in the near-IR

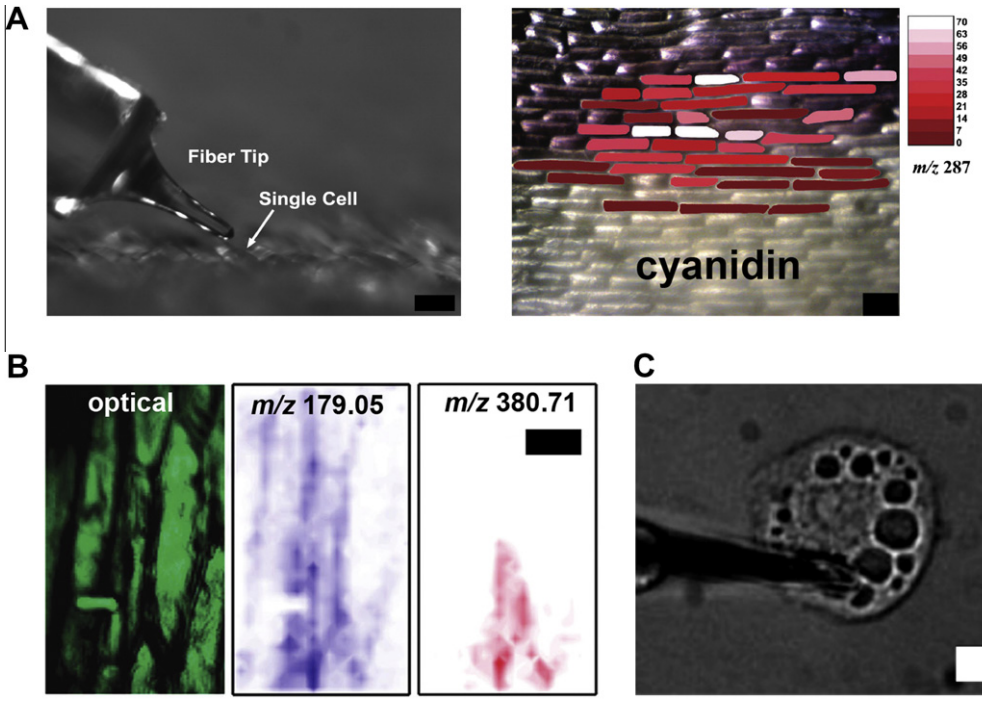

Figure 4. Ambient single-cell and sub-cellular sampling and chemical imaging. (A) LAESI-MSI: (left) an etched optical fiber ablated a single cell of *A. cepa* with 50 μm sampling, and (right) revealed heterogeneous cyanidin content among adjacent cells of a cell population. Scales = 100 μm. (B) Femtosecond near-IR LDI imaged (left) sucrose and a dye in *A. cepa* epidermis with 15 μm resolution. Scale = 100 μm. (C) A nanospray tip aspirated a granule of a selected mast cell, and electrosprayed serotonin was measured in video MS. Scale = 10 μm. (Images were adapted from [72,73,75,76] with permission.)

region of the spectrum for LDI. By focusing laser light of 800-nm wavelength with a microscope objective, *A. cepa* epidermis (Fig. 4B) was imaged with 10–15-μm lateral resolution in AP near-IR (fs)-LDI [75]. Sucrose was detected with higher ion counts in the cell walls, whereas an artificial dye was located in the intracellular compartment of the plant cells (Fig. 4B).

Direct-contact sampling takes a different approach to sample collection and has already demonstrated sub-cellular analysis. Video MS utilized an electrospray emitter with 2-μm inner diameter to aspirate ~1 pL of selected granules in rat mast cells, and the material collected was subsequently electrosprayed. In these experiments, histamine and serotonin were directly measured [76]. Alternatively, we expect the combination of microdissection and LAESI to create an opportunity to sample cells directly at sub-cellular scales.

These examples demonstrate the trend to sample progressively smaller volumes in biological organisms with ever-decreasing probe dimensions, improving yields of ion generation and MS collection.

4. Current limitations and future prospects

Chemical identification in the direct analysis of biological systems relies on a few principles in ambient MS that have worked well in many applications. Assignment protocols typically involve accurate mass measurements and searches against species-specific MS and tandem-MS databases. Positive matches are elucidated, based on isotope distribution and molecular fragmentation behavior in tandem-MS experiments, and putative assignments are ultimately confirmed against chemical standards. Due to the high mass accuracy and mass resolution offered by present-day MS systems, this protocol works well for a large number of compounds, but it is incapable of addressing chemical ambiguity in some cases.

A growing number of studies face the need for alternative approaches to further confidence in chemical assignments. Ambient MS typically seeks high-throughput analysis, operational convenience, and ability for *in situ* and *in vivo* analysis by doing away with sample-preparation and sample-separation methods. Sample separation is a necessity when structurally similar compounds are to be elucidated or chemical complexity poses the risk of matrix-suppression effects (e.g., in metabolomics where chemical diversity is pronounced with optical isomers serving different biological roles).

Future developments in the field are likely to reinstate chemical separation in one form or another. Chromatography and electrophoresis can efficiently remove interferences and add orthogonal dimensions for

identification [71]. HPLC coupled with LMJ-SSP and *in situ* electrophoresis in paper-spray-MS are promising initiatives to reduce the chemical complexity of samples in a direct-analysis setting [34,38]. An attractive alternative approach is ion-mobility separation because it is compatible with most ambient ion sources.

Research efforts should also focus on improving the analytical figures of merits. Mechanistic differences in ambient sampling and ion generation appear to have translated into niches of applications, mostly in metabolomics and lipidomics. Most ambient ion sources exhibit a strong bias toward small compounds and enable a diversity of applications targeting biochemicals of naturally high concentrations (metabolites, lipids, and peptides). In comparison, some endogenous compounds are present in a broad dynamic range, so biological specimens necessitate improvements in limits of detection and quantitation. In addition, enhancements in analytical performance will also be a prerequisite to probing biological organizations with increasing granularity.

In situ/*in vivo* applications are expected to move out of the laboratory, and reach the clinical theater and find applications on site. These methods have already shown an ability to identify biomarkers, chemical contaminants, and biological agents rapidly in a variety of samples [77,78]. When combined with field-portable mass spectrometers, ambient MS will probably propel biochemical research and regulatory applications in food, drug, and environmental safety to new levels.

Acknowledgements

The views and conclusions expressed herein are solely those of the authors and should not be construed to represent the Food and Drug Administration, US National Science Foundation (NSF), US Department of Energy (DOE), or The George Washington University (GWU). The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either actual or implied endorsement of such products by the Department of Health and Human Services. AV acknowledges financial support from the NSF (Grant 0719232), the DOE (Grant DEFG02-01ER15129), and the GWU Signature Program. PN thanks Dinesh V. Patwardhan (FDA), Benita J. Dair (FDA), Kenneth S. Phillips (FDA), and Martin K. McDermott (FDA) for their assistance during the preparation of this publication.

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