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REVIEW ARTICLE

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Biological mass spectrometry enables spatiotemporal 'omics: From tissues to cells to organelles

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Abstract

Biological processes unfold across broad spatial and temporal dimensions, and measurement of the underlying molecular world is essential to their understanding. Interdisciplinary efforts advanced mass spectrometry (MS) into a tour de force for assessing virtually all levels of the molecular architecture, some in exquisite detection sensitivity and scalability in space-time. In this review, we offer vignettes of milestones in technology innovations that ushered sample collection and processing, chemical separation, ionization, and 'omics analyses to progressively finer resolutions in the realms of tissue biopsies and limited cell populations, single cells, and subcellular organelles. Also highlighted are methodologies that empowered the acquisition and analysis of multidimensional MS data sets to reveal proteomes, peptidomes, and metabolomes in ever-deepening coverage in these limited and dynamic specimens. In pursuit of richer knowledge of biological processes, we discuss efforts pioneering the integration of orthogonal approaches from molecular and functional studies, both within and beyond MS. With established and emerging community-wide efforts ensuring scientific rigor and reproducibility, spatiotemporal MS emerged as an exciting and powerful resource to study biological systems in space-time.

KEYWORDS

imaging, mass spectrometry, metabolomics, peptidomics, proteomics

1 | INTRODUCTION

Interdisciplinary efforts sculpted mass spectrometry (MS) into an indispensable tool in the molecular study of life processes (Figure 1). Virtually all levels of the molecular architecture—from genes to transcripts to proteins to peptides to metabolites—can now be measured using this technology, some in exquisite detail. MS detection and quantification of proteomes, peptidomes, and metabolomes ('omes) expanded the analytical toolbox of biology across the taxa, helping to generate and test new hypotheses. Over the last decades, technological advances broke down classical limitations in detection limits (sensitivity), speed, and scalability to enable the analysis of limited amounts of materials. This review offers examples of innovations that extended MS-based 'omics to study the spatiotemporal organization of diverse biological systems at the realms of the organism, organ, tissue, cell, and organelle.

1.1 | MS meets research needs

Life processes respond to intrinsic and extrinsic events with complex and dynamic molecular changes across broad spatial and temporal dimensions, and measuring

Issue dedicated to Catherine Fenselau (Invited Contribution).

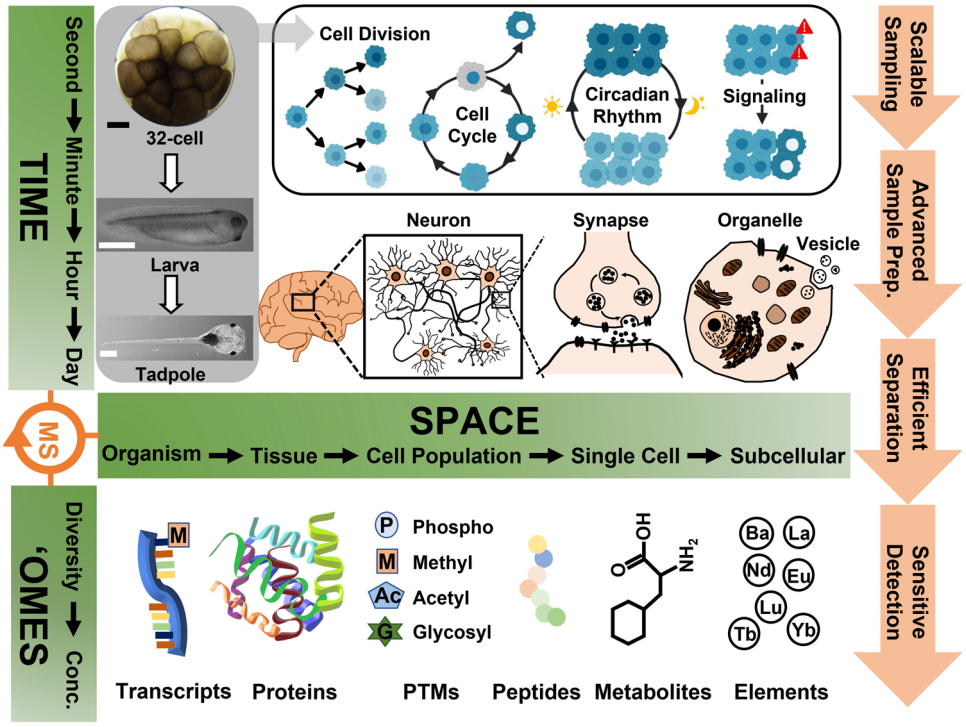

FIGURE 1 Understanding biological processes hinges on the analysis of molecular heterogeneity that they orchestrate over broad spatial and temporal dimensions. Recent technological innovations and the development of specialized methodologies in sample collection and preparation, chemical separation, and HRMS analysis and detection provide sufficient sensitivity and spatiotemporal resolution for studying molecular processes at the levels of transcriptomes, proteomes, peptidomes, and metabolomes ('omes) in ever-deepening information content. Information from these studies fuels basic and translational research forward. Representative examples are highlighted from whole organismal to subcellular studies. Adapted with permission from (Gnann et al., 2021; Lombard-Banek et al., 2021). [Color figure can be viewed at wileyonlinelibrary.com]

these perturbations is key to their understanding. Systems biology promotes holistic understanding, in part, by characterizing all types of molecules and their potential interactions from whole organismal to subcellular scales. Vast signal amplification via polymerase chain reaction (PCR) facilitated next-generation sequencing of minute amounts of transcripts and genes, thus leading single-cell sequencing to become quasi-routine. Subcellular RNA-seq, for example, recently uncovered transcriptomic differences between the cell body (soma) and dendrites in individual GABAergic and glutaminergic neurons during biological functions (Perez et al., 2021), raising the question of subcellular heterogeneity for functional molecules downstream of transcription. Without technologies capable of amplifying whole proteomes to metabolomes, information from sequencing is often used as a proxy for the proteome.

However, the relationship between messenger RNA (mRNA) expression, protein production, and metabolism is complex in dynamic systems (Y. S. Liu et al., 2016). Posttranscriptional and -translational regulation as well as protein turnover rates contribute to widely variable correlation between transcription and translation. This relationship is further complicated by processes occurring heterogeneously in space and time, as was recently found during cell culturing (Vogel & Marcotte, 2012), signal transduction between cells and neurons, differentiation of stem cells in developing organisms (Lombard-Banek, Reddy, et al., 2016, Peshkin et al., 2015), cancer invasion and metastasis (Saadatpour et al., 2015), and responses to drug treatments (Tian et al., 2004). Similarly, bioactive peptides and metabolites, which also carry out important physiological functions, are the result of interconnected molecular pathways, complicating their predictions from information upstream. Elucidating these molecular relationships over space-time is important for understanding states of health and disease, but this goal requires direct detection of proteins, peptides, and metabolites.

MS, especially high-resolution MS (HRMS), emerged as the technology of choice to bridge this knowledge gap. It integrates exceptional molecular specificity with a capability for quantification, typically without the need for functional probes. Readers interested in the fundamentals of MS and HRMS, ranging from ion generation to mass analysis, are referred to recent reviews (e.g., Feng et al., 2008). From tissues and cell cultures, hundreds to tens of thousands of biomolecules are routinely assessed in discovery studies, with near-complete coverage of proteomes (Geiger et al.,

2012; Hebert et al., 2014). For example, HRMS on two- and three-dimensional (2D and 3D, resp.) tissue cultures of human embryonic stem cells (hESCs) and induced pluripotent stem cells helped delineate molecular pathways critical for cell differentiation, cellular repair, and degeneration (Arnold et al., 2022; Cervenka et al., 2021; Sperber et al., 2015). Quantitative HRMS on proliferating neuronal stem cells revealed proteomic alterations between cellular and secreted proteomes in response to the microenvironment that would be complicated to predict from the transcriptome (Cervenka et al., 2021). HRMS quantified ~2800 proteins and their temporal expression profiles at progressive stages of differentiation, offering a glimpse into molecular mechanisms toward developing next-generation cell-based therapies such as cell grafting (Cervenka et al., 2021). Similarly, metabolic profiling of naïve and primed hESCs revealed alterations in epigenetic dynamics underpinning cell fate determination (Sperber et al., 2015).

The advent of highly sensitive HRMS propelled 'omics to dynamic and microscale biological systems. This group of technologies integrated existing approaches and invented new ones to accomplish trace-level sensitivity, sufficient to probe molecules at physiological concentrations. In doing so, they also allowed for scaling the spatial and temporal resolution of an analysis to the specific biological systems and questions at focus. Figure 1 conceptualizes broad spatiotemporal dimensions that contemporary HRMS is able to address. With sample collection capable of spatiotemporal scalability, high-sensitivity HRMS opened even more investigative possibilities. Rapid sampling enabled HRMS to assist studies on fast biochemical processes, including those underpinning cellular processes. For example, methods capable of fast sampling, such as capillary microaspiration with duty cycles on the order of seconds, can collect cellular materials even within a ~15–45 min cell cycle in cleavage-stage embryos, as shown from the South African clawed frog (*Xenopus laevis*) in Figure 1. Sampling over minutes to hours brought molecular studies to important physiological processes, including the maintenance of circadian cycles, feeding, and response to stress (see examples later). Additionally, scalability in physical dimensions raised the possibility of capturing spatial information on the distribution of molecules in specimens on the orders of millimeters to micrometers. MS imaging (MSI) played an important role by enabling the mapping of proteins, peptides, and metabolites across biological tissues, supporting functional studies and drug discovery. Profiling and imaging HRMS were recently adapted to subcellular structures. Chemical labeling enhanced spatial resolution to the domain of interacting molecules, thus vastly enhancing knowledge of the biochemistry underlying biology, such as signaling between neurons in the brain.

These advancements in HRMS support basic and translational studies with information on the spatiotemporal organization of the molecular composition in biological systems. Next-generation separations, enhanced mass analyzers, and intelligent data acquisition strategies facilitated molecular identifications and quantification from minute amounts of materials. Thousands of proteins and metabolites can now be measured, even from single cells. For targeted proteins, antibodies tagged with heavy elements extended HRMS to MSI at single-cell resolution. Novel mathematical algorithms helped processing complex primary data in depth. User-friendly software packages and community-wide efforts to freely share them aided adoptability across laboratories needing sensitive 'omics, raising an opportunity to support applications in biological and translational studies. Molecular information that emerges from these measurements holds answers to existing questions and help generate new hypotheses. Efforts in pursuit of sensitivity, scalability, and 'omic coverage progressively expand our technological toolbox, thus accelerating the scientific cycle of exploration and discovery. This review highlights technological and methodological milestones in the development of spatiotemporal HRMS 'omics.

2 | SENSITIVE HRMS FOR SPATIOTEMPORAL 'OMICS

Spatiotemporal HRMS builds on the general workflow of MS-based 'omics. The use of MS in systems biology was recently reviewed elsewhere (Feng et al., 2008). For spatiotemporal analysis, the interconnected steps of the analytical pipeline are presented in Figure 2. During sampling, specimens, such as tissues and cells, are collected and lysed to release their contents by a variety of methods and technologies, with each tailoring to specific benefits. The lysate is typically enriched for the biomolecules of interest, such as proteins or metabolites, then subjected to sample clean-up/purification to render compatibility for instrumental analysis.

The biomolecules are measured using a combination of technologies. The chemical complexity of the sample is spread out over time through separation in solution, e.g., by using liquid chromatography (LC) and capillary electrophoresis (CE), or the gas phase using, e.g., ion mobility spectrometry (IMS) or field asymmetric IMS (FAIMS). The biomolecules are next ionized, detected, identified, and quantified by HRMS. An array of fragmentation (tandem/multistage MS) and analyzer-detector systems provide complementary speed and

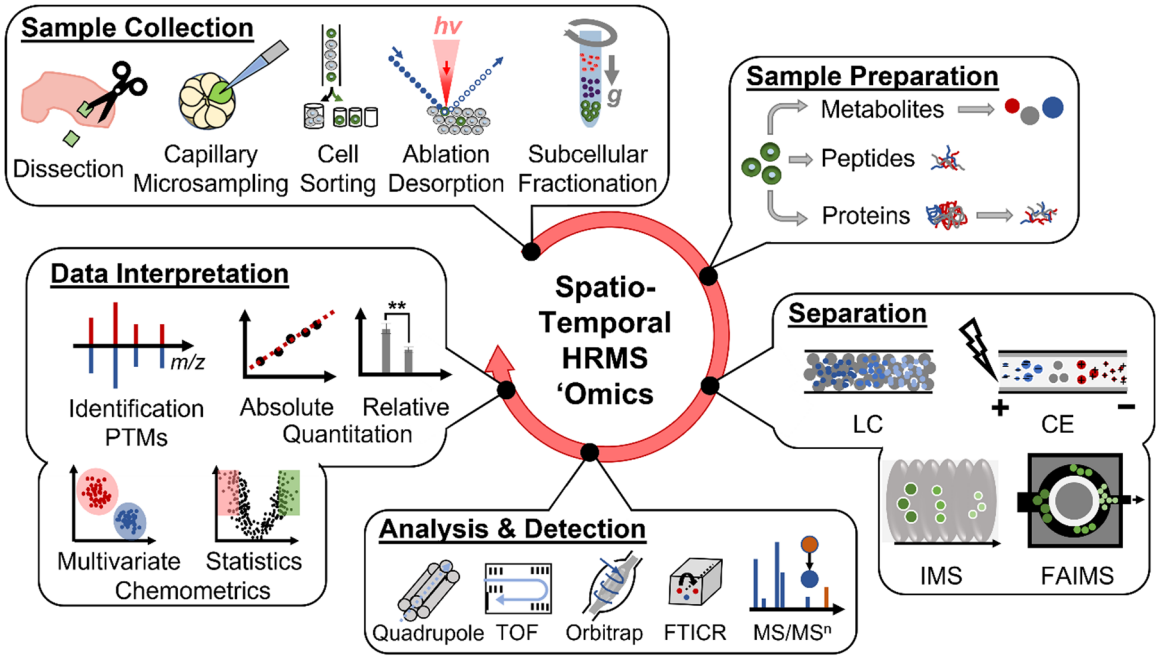

FIGURE 2 General experimental workflow and representative technologies enabling spatiotemporal MS. Scalable sample collection and processing allow for probing biological specimens across broad scales in space and time. Separation in the liquid and/or gas phase reduces spectral complexity. High-resolution mass analyzers, tandem/multistage fragmentation, and efficient detector systems support molecular identifications with high specificity, sensitivity, and a capacity for quantification. Development of advanced tools from bioinformatics and chemometrics facilitate the analysis and interpretation of MS data with spatiotemporal insights. Technological abbreviations are in the text. [Color figure can be viewed at wileyonlinelibrary.com]

spectral resolution, ranging from quadrupole to time-of-flight (TOF), orbitrap, and Fourier-transform ion cyclotron resonance (FTICR) instruments.

The primary data from these measurements are processed and analyzed, usually with specialized MS bioinformatics software packages supporting identification of proteins, peptides, metabolites, and their post-translational modifications as well as relative or absolute quantification. Statistical and multivariate models from chemometrics allow for systematic evaluation of the observed effects between different experimental conditions under study. The results are interpreted in relation to canonical knowledge and the biological context. In successful investigations, the specifics of each step of the workflow are matched to the goals of the study. Following sections focus on technologies that advanced spatiotemporal analysis in different biological specimens.

2.1 | Sample collection

The type of question and biological system at hand *de facto* determines the method of sampling. In tissues displaying heterogeneous biochemistry in space or time, such as the brain and kidney as well as tumors, local sampling with accuracy ensures contamination-free analysis from neighboring cell populations, thus aiding the interpretation of results. Likewise, sample collection with single-cell resolution unmasks differences between single cells (cell heterogeneity) within the same tissue, which are otherwise obscured due to signal averaging during cell pooling. As exemplified in Figure 3, various strategies were developed to collect and purify tissue biopsies, small populations of cells, single cells, or subcellular fractions of interest with different speeds and operational conditions.

2.1.1 | From biopsies to single cells

Manual dissection is simple and can have high accuracy. Surgical tools, ranging from sharp forceps to tungsten needles to eyebrow knives, are readily adaptable to excising distinct regions of tissues. With sufficient finesse, this approach also enables isolation of limited cell populations and individual cells from broad types of biological models. Dissection techniques, for example, enabled isolation of spatially distinct colon tumor biopsies from humans (Arnadottir et al., 2020), brain tissues from the mouse (X. H. Zhou et al., 2019), large single cells (Onjiko et al., 2015) and small cell populations (Baxi et al., 2018) from developing *X. laevis* embryos, neurons from *A. californica*

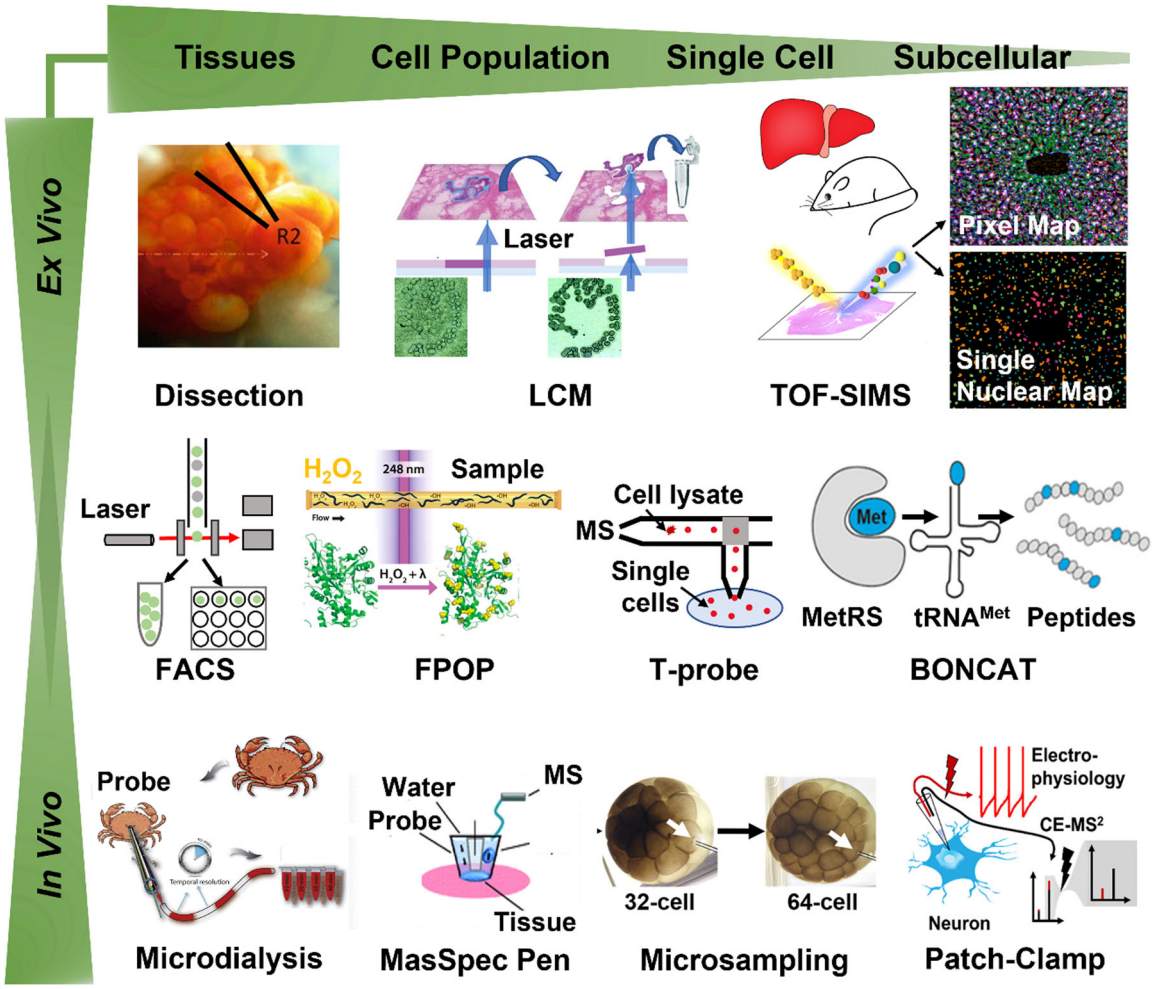

FIGURE 3 Representative strategies enabling scalable spatiotemporal sampling. Diverse technologies enabled HRMS with sufficient spatial and temporal resolving power to assess 'omics in broad types of biological systems and investigative contexts, ranging from ex vivo to in situ to in vivo analyses and from small populations of cells to single cells to subcellular organelles. Technological abbreviations are in the text. Adapted with permission from Azizian et al. (2021); Brown et al. (2018); Choi et al. (2022); Espino et al. (2019); Lombard-Banek, Moody, et al. (2019); Moroz et al. (2013); OuYang et al. (2015); Simone et al. (1998); von Eggeling et al. (2020); Yuan et al. (2021). [Color figure can be viewed at wileyonlinelibrary.com]

(Hanson et al., 2009), and distinct regions of nervous tissue from *Callinectes sapidus* (Hu, Helfenbein, et al., 2021). Needle-based biopsies, such as core needle (Satpathy et al., 2020) or fine needle (Piga et al., 2021) aspiration, were also used to collect small tissues or cells from humans. Labeling with dyes or fluorescent reporters enhances the accuracy of dissections under a microscope. Figure 3 demonstrates one such example, where identified cholinergic neurons from *A. californica* were dissected, permitting transcriptomics studies on aging (Moroz & Kohn, 2013).

Spatially resolved sampling reveals information on the physical location of the biomolecules. With narrow focusing and adjustable power (fluence), lasers naturally became a method of choice in this research field. As illustrated in Figure 3, laser capture microdissection (LCM) concentrates infrared or ultraviolet light to lift off small tissue areas to groups of cells to single cells (e.g., 3–5 μm in size), with a capability for resolutions down to micrometers (Simone et al., 1998). LCM-based HRMS facilitated biological studies on genes, proteins, and metabolites in broad contexts including cancer biopsies (Shen et al., 2021) and single-cell proteomics (Zhu, Dou, et al., 2018). The technology was recently reviewed elsewhere (von Eggeling & Hoffmann, 2020). Because the dimensions of laser ablation are scalable, LCM can be employed to access cellular and subcellular structures. Representative examples include analysis of growth cones of a retinal ganglion cell (Zivraj et al., 2010), soma and dendrites of single neurons (Perez et al., 2021), and invadosomes coordinating motility of normal and cancer cells (Ezzoukhry et al., 2018). Dissection by LCM enables accurate isolation of tissue areas that is scalable to even

smaller regions. For example, LCM with LC-MS profiled lipids between the cell bodies and synapses of *Drosophila* neurons (Hebbar et al., 2014). Likewise, HRMS of the optic tectum and telencephalon regions in the zebrafish brain identified over 400 proteoforms with heterogeneous distribution, including many with important activities for visual and motor functions and neuropeptide signaling (Lubeckyj & Sun, 2022). These approaches provide an effective snapshot of the 'omic state of the specimen at a given time.

For smaller cell populations or dispersed cells, alternative strategies enhance the fidelity of sampling. Flow cytometry-based techniques, such as fluorescence-activated cell sorting (FACS), were employed in several studies to purify heterogeneous cell populations (Chappell et al., 2016; L. Chen et al., 2020; Sergent-Tanguy et al., 2003). As illustrated in Figure 3, FACS enables accurate and high-throughput purification of cell populations to single cells based on fluorescent markers. Although specialized cytometers are able to sort particles ranging from 0.2 to 150 μm in diameter, commercial FACS instruments usually have a low-pass limit of ~20 μm cell diameter (Davey & Kell, 1996). Required expertise, expense, and technical limitations for larger cells culminated into alternative methods. In magnetic-activated cell sorting, for example, antibody-conjugated magnetic beads of several nanometers in diameter can be used to recognize surface markers before pulling down specific types of cells and subcellular organelles (Miltenyi et al., 1990).

Microfluidics renders added benefits in sample collection with minimal manual intervention. Cell sorting in microfluidic chips employs orthogonal mechanisms for isolation, ranging from acoustic waves to radiation forces to hydrodynamic flow to microfiltration and cellular immobilization. These and other emerging technologies were recently reviewed elsewhere (Y. Y. Zhang et al., 2021). It follows that these approaches can be extended to collecting single cells. However, due to mixing during processing, information on the spatial origin of each single cell is lost, which may be important in the context of biology.

2.1.2 | Subcellular organization

wFor targeted subcellular analysis, distinct organelles may be isolated prior to HRMS detection. Sequential lysis of cellular compartments and density-based centrifugation are well-established for cells and cell types from multiple biological models. Organelles such as nuclei, mitochondria, peroxisomes, lysosomes, and the Golgi apparatus can be separated based on their size, shapes, and surface charges (Tharkeshwar et al., 2018). Similar centrifugation-based strategies allowed for the isolation of exosomes from myeloid-derived suppressor cells involved in cancer tumor signaling (Burke et al., 2014) and from *Bacillus subtilis* at different developmental stages (Y. Kim et al., 2016). Organelles with similar densities, however, may pose challenges for high-purity isolation.

Biochemical approaches and bioinformatics provide alternatives for subcellular analyses. Subcellular labeling HRMS was the topic of a previous review (Y. H. Lee et al., 2010). Localization of organelle proteins by isotope tagging (LOPIT) and hyperLOPIT precluded the need for high-purity organelle purification by taking a multipronged approach, in which organelles were partially separated into multiple fractions using multistep density centrifugation for multiplexed quantification via LC-HRMS, followed by deep machine learning to extract information on the subcellular localization of detected proteins (Geladaki et al., 2019). The HRMS analysis of organelle fractionation is improved in MS-compatible buffers by reducing chemical interferences during detection. For example, radio-immunoprecipitation assay (RIPA) buffer with ethylene glycol and phase transfer surfactants aided isolation of cytoplasmic, nuclear, and organelle fractions from small populations of cells without the need for traditional proteomic cleanup steps (Masuda et al., 2020).

Integration of orthogonal methods of isolation enhances capacity, and so molecular specificity and accuracy. In fluorescence-activated organelle sorting (FAOS), subcellular targets are fluorescently labeled, for example, using antibodies for two-step purification via ultracentrifugation and FACS. This strategy enabled purification of synaptosomes without neuronal and glial contaminants, identifying novel proteins released in glutaminergic synapses (Biesemann et al., 2014). Magnetic isolation using differentially coated superparamagnetic iron oxide nanoparticles (SPIONs) helped purify endosomes during the study of Niemann-Pick disease, a lysosomal storage disorder, by pulling down endosomes enriched with dimercaptosuccinic acid-coated SPIONs and plasma membranes adhering to aminolipid-coated SPIONs (Tharkeshwar et al., 2017).

MSI techniques such as micro- and nano-probe HRMS with bioinformatics emerged for precision organelle analysis, precluding the need for physically isolating microscopic organelles. By tightly focusing beams of projectiles, from ions to photons (recall Figure 2), spatial 'omics recently opened the door to subcellular 'omics, as was the focus of a recent review (Seydel, 2021). High-definition multiplex ion beam imaging (HD-MIBI) secondary ion MS (SIMS) offered a 30-nm resolution, sufficient to localize cisplatin enrichment in nuclear

speckles with exclusion from chromatin regions, providing new information on subcellular microenvironments for drug development (Rovira-Clave et al., 2021). Spatial single nuclear metabolomics (SEAM) integrated TOF-SIMS with novel computational data analysis modules (Figure 3) to identify and localize single nuclei with variable metabolic patterns in healthy and fibrotic liver tissue (Yuan et al., 2021). In the 3D OrbiSIMS, the coupling of TOF-SIMS with an ultrahigh-resolution orbitrap empowered high-speed 3D molecular imaging, providing information on the subcellular localization of lipids and neurotransmitters in the hippocampus and single macrophages (Passarelli et al., 2017). Similarly, using image-guided matrix-assisted laser desorption ionization (MALDI) coupled to an ultrahigh-resolution mass analyzer, neuropeptides and lipids were identified from 0.5 to 2 μm vesicles in *A. californica* to reveal distinct metabolic subpopulations within these organelles (Castro et al., 2021). These and other advancements enabling subcellular and single-organelle spatial 'omics are expected to continue informing us of molecular profiles among cells and within their components.

In situ sampling can take a molecular snapshot of the tissues or cells under innate conditions with minimal external perturbations during collection. As illustrated in Figure 3, fast photochemical oxidation of proteins (FPOP) utilizes laser-induced photolysis of hydrogen peroxide to irreversibly label proteins in their native state within a cell, thus enabling studies on protein structure and folding and protein-protein interactions (Espino & Jones, 2019). Pioneering a new approach in epitope mapping, oxidative labeling was successfully used to map antibody-binding regions of the protein serine protease thrombin (Jones et al., 2011). Such detailed information on molecular identity, structure, and interactions is essential to develop monoclonal antibody-based therapeutics for targeted proteins.

2.1.3 | In situ/vivo

Chemical labeling enables the isolation of molecular partners in interaction. A plethora of labeling techniques allow for tracing biomolecules in subcellular compartments, and some in vivo. Bioorthogonal reactions, for example, use a wide array of click chemistry to anchor specific biomolecules without disturbing the intracellular environment. Such molecular handles were used to label proteins, lipids, glycans, nucleic acids, and enzymes, and for targeted delivery of small molecules, as was reviewed in (Bird et al., 2021). Bioorthogonal noncanonical amino acid tagging (BONCAT), Figure 3, enabled the measurement of newly synthesized proteins by incorporating biotin-tagged noncanonical amino acids in the nascent proteins with follow-up affinity purification or imaging by fluorescence microscopy (Landgraf et al., 2015). The approach recently allowed for mapping the proteome of tumor cells in ductal adenocarcinoma in the mouse (Azizian et al., 2021). BONCAT was recently used to find local translation of 42 proteins in the axoplasm during regeneration of the sciatic nerve following injury in rats (Di Paolo et al., 2021). Alternatively, proximity labeling using ascorbate peroxidase, APEX or APEX2 (Lam et al., 2015), or biotin ligase, BioID (Roux et al., 2012), localized enzymatic activity to map their proteomic environment in organelles, thus opening the door to proteomic studies on synaptic clefts of excitatory and inhibitory neurons (Loh et al., 2016). Isotope tracing adds a dynamic component to these studies via spatial fluxomics. Glutamine metabolism was monitored in mitochondria and the cytosol of cancer cells to elucidate metabolome remodeling (W. D. Lee et al., 2019). Alternatively, subcellular fractionation with activity-based protein profiling (ABPP) monitored proteomic changes in secretory pathways in the endoplasmic reticulum in response to cellular stress (Bechtel et al., 2020).

Sampling in vivo extends HRMS 'omics to physiological states. Figure 3 shows examples of in vivo probes capable of minimally invasive sampling. Microdialysis has a long history in the research of neuropeptides, neurotransmitters, and metabolites with ca. millimeter resolution in live biological models. This strategy was used to access molecular information on physiologically relevant timescales (e.g., from minutes to hours) in functional, behavioral, and clinical studies. Representative examples helped decipher the spatiotemporal dynamics of neuropeptides and their roles in feeding using crabs (Schmerberg & Li, 2013; Schmerberg et al., 2015), decision-making and drug response in rats (Zestos & Kennedy, 2017), and the tumor-promoting impact of radiation in glioblastoma using human xenografts in mice (Gupta et al., 2020), as well as clinically for cerebral metabolic analysis of human patients with aneurysmal subarachnoid hemorrhage among others (Helbok et al., 2017).

Real-time biopsies with rapid chemical analysis opened exciting new capabilities for health research (Figure 3). Liquid biopsies helped capture circulating cancer cells for diagnosis (Abouleila et al., 2019) or the assessment of effectiveness during therapy (Bensen et al., 2021). In the clinical setting, desorption electrospray ionization (DESI) and MasSpec Pen (Figure 3) gained recognition for its ability to downscale microextraction into <10 μl of water droplet on tissue regions of interest, even in morphologically complex samples (Brown et al., 2018; J. L. Zhang et al., 2017). With fast sample transfer and HRMS detection, the approach aided cancer

diagnoses of ovarian, lung, thyroid, and breast biopsies ex vivo, and recently also in vivo, thus empowering surgical applications with a new tool.

Microsampling confined to capillaries and nanochannels downscales sampling to the realm of cells and organelles. Whole cells, portions of a cell, or subcellular contents may be aspirated using precision-fabricated capillaries, usually with assistance from fine translation control and optical microscopy. The introduction of ambient ionization and ultra-sensitive HRMS ushered the extension of capillary microsampling to endogenous and exogenous metabolites in broad types of single cells, as was recently reviewed elsewhere (Y. Y. Yang et al., 2017; Zhang & Vertes, 2018; Zhu, Shao, et al., 2021). Early on, for single cells, live-video MS with direct electrospray ionization (ESI) detected metabolites from single adherent embryonic fibroblasts (mouse) and plant cells (Fujii et al., 2015; Tsuyama et al., 2008), T-probe (Figure 3) profiled metabolic perturbations in HeLa cells on anticancer treatment (R. M. Liu et al., 2018), and laser ablation electrospray ionization (LAESI) surveyed secondary metabolites in *Allium cepa* cells under guidance by optical microscopy (Taylor, Mattson, et al., 2021). The live cell analysis device (LCAD) exchanged capillaries for nanofabricated channels, where localized electroporation facilitated tyrosine phosphatase extraction from cells during proliferation and hydrogen peroxide treatment in human triple-negative breast cancer cells with protein detection enabled by self-assembled monolayers for matrix-assisted laser desorption/ionization (SAMDI) MS (Mukherjee et al., 2020).

To detect deeper into single-cell 'omes, microsampling can be combined with high-efficiency separations before in situ/vivo HRMS. By aspirating <5% of the total cell volume using a pulled borosilicate capillary, proteomic and metabolomic cell heterogeneity was found in embryos of *X. laevis* and zebrafish (Lombard-Banek, Moody, et al., 2019; Onjiko et al., 2017; Portero & Nemes, 2019; Saha-Shah et al., 2019). Capillary microsampling facilitated integration of HRMS 'omics with functional biology. Patch-seq has a history of leveraging this strategy for sequencing mRNAs from neurons after their electrophysiological characterization (Cadwell et al., 2016), as was recently reviewed elsewhere (Lipovsek et al., 2021). After eavesdropping on the electrical activity of single neurons using a patch pipette, neurotransmitters in the mouse (Aerts et al., 2014), the American cockroach (Neupert et al., 2018), and recently also proteins in the mouse (Choi et al., 2022) were measured in identified single neurons in the central nervous systems of these model organisms. Multi-step capillary microaspiration also enabled in vivo analysis of dynamic reorganization in the metabolome and proteome of single identified blastomeres dividing in the live *X. laevis* embryo, which successfully developed to a functioning tadpole with indistinguishable behavior from wild-type siblings in a background color preference assay (Lombard-Banek et al., 2021). Accessing such information on molecular interactions in space-time offers an important piece of the puzzle towards the holistic understanding of molecular mechanisms underlying biological functions and disease states.

2.2 | Sample preparation

Sample preparation can make or break studies requiring trace detection, and those addressing spatiotemporal processes are no exception. Specimens encompassing few cells, single cells, or subcellular organelles provide only micro- to picogram quantities of starting materials (Ctortecka & Mechtler, 2021; Zhang & Vertes, 2018). Processing such limited amounts poses nontrivial challenges in handling of miniscule volumes without solvent evaporation and with minimal-to-no losses for proteins, peptides, and metabolites due to surface adsorption (Z. C. Yang & Sun, 2021). Advances in small-scale sample preparation was the topic of several reviews (Hu, Li, et al., 2023; Kelly, 2020; Matula et al., 2020; Qin et al., 2022; Taylor, Lukowski, et al., 2021; Z. C. Yang & Sun, 2021). The following section highlights developments that ushered biological HRMS to spatiotemporal 'omics from small tissue biopsies down to the single-cell and subcellular level.

2.2.1 | Proteomics

HRMS provides critical information on proteomes during states of health and disease. Methods and technologies enabling bottom-up, middle-down, and top-down proteomics are able to identify and quantify broad types of proteins, assess their structure, gauge their interactions, and determine modifications during biological processes in space and time. Deep proteome coverage is an enabling feature of bottom-up proteomics, which is the most widely used of the three workflows. In this approach, proteins are enzymatically digested to peptides, which can be efficiently ionized and sequenced to pinpoint their source proteins using advanced bioinformatics. Thousands of different proteins can be identified using this approach, even from limited samples. Bottom-up proteomics was applied to study complex spatiotemporal processes at the organismal to subcellular levels in development (Baxi et al., 2021; L. W. Yang et al., 2020), neuroscience (Hobson et al.,

2022; Velasquez et al., 2019), and various disease states (Vo & Palsson, 2007; Waas & Kislinger, 2020). The information from these measurements provides the foundation for hypothesis-driven and functional studies.

HRMS-based proteomics executes multiple analytical steps with each capable of affecting sensitivity. Lysis, extraction, reduction, alkylation (of disulfide bonds), enzymatic digestion, and desalting are routine stages of sample processing requiring care for limited samples. Simplification of the workflow was recently found to improve performance. For single-cell measurements, for instance, MS-compatible detergents and mechanical lysis methods (i.e., freezing/thawing or laser pulses) and exclusion of reduction and alkylation eliminated the need for downstream clean-up, and reduced nonspecific adsorptive losses and dilution (Lombard-Banek, Moody, et al., 2019; Schoof et al., 2021). Select strategies enabling high-fidelity proteomics are presented in Figure 4 and discussed further.

"One-pot" strategies confined the analytical steps to a single location. For amount-limited samples, immobilization of molecular processing and reduction or elimination of sample transfers proved particularly beneficial. For example, gel-aided sample preparation (GASP) (Fischer & Kessler, 2015), suspension trapping filter (S-Trap) (HaileMariam et al., 2018), and filter-aided sample preparation (FASP) (Wisniewski et al., 2009) used gels and filter membranes, respectively, to reduce sample transfer steps and improve proteome coverage from hundreds of micrograms of protein. By miniaturizing and confining sample processing onto a multi-well plate, micro-FASP was able to identify ~3400 proteins from ~200 ng of yolk-free proteome from single blastomeres that were isolated from early-stage *X. laevis* embryos (Zhang, Dubiak, et al., 2020). Single-pot solid-phase-enhanced sample preparation (SP3) demonstrated similar capabilities for comparable protein amounts from 1000 HeLa cells and *Drosophila* embryos by immobilizing proteins and peptides on carboxylate-coated paramagnetic beads throughout sample preparation (Hughes et al., 2014).

Sample processing in situ provided an opportunity to streamline the analytical workflow. As illustrated in Figure 4, single-cell proteomics by MS (SCoPE-MS) confined sample processing to one pot, allowing for multiplexed protein quantification among single cells (Budnik et al., 2018). Capillary-based systems may serve as alternative substrates for single-cell sampling and processing. As shown in Figure 4, the integrated proteome analysis device (iPAD) directly supplied a

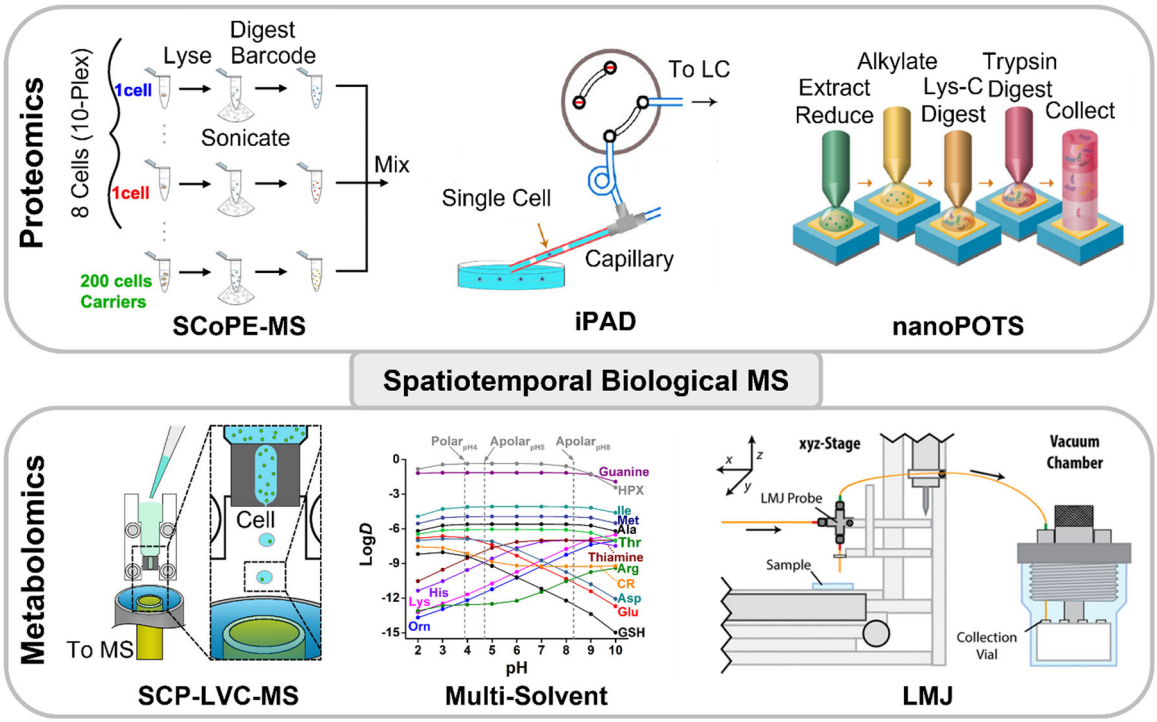

FIGURE 4 Scalable and sensitive sample processing for metabolites and proteins for spatiotemporal MS. One-pot sample processing (SCoPE), direct cell capture and processing (iPAD), and miniaturization of sample processing steps (nanoPOTS) deepen the detectable proteome by reducing analyte losses. Direct cell capture (SCP-LVC), multi-solvent processing, and in situ metabolite extraction (LMJ) after MALDI imaging improve coverage of the single-cell metabolome. Technological abbreviations are in the text. Adapted with permission from Budnik et al. (2018); Cahill et al. (2019); Comi et al. (2017); Kelly (2020); Onjiko et al. (2016); Shao, Wang, et al. (2018). [Color figure can be viewed at wileyonlinelibrary.com]

suspension of living cells (iPAD-100) for online cell lysis, protein digestion, and peptide desalting and trapping to a 10-port valve-based system on a commercial nanoLC, identifying ~600–650 proteins from a single injection of 100 cells (~10 ng protein) using nanoLC-HRMS (Q. Chen et al., 2015). In pursuit of cell heterogeneity, iPAD-1 (Figure 4) used an ultranarrow-bore analytical column to identify ~270 proteins from a single HeLa cell (220 pg proteome) in 1 h of analysis with nanoLC-HRMS (Shao et al., 2018). These and other simplified devices facilitate technology adaptation.

Microfabricated devices naturally give way to "one-pot" workflows. ProteoCHIP used polytetrafluoroethylene (PTFE)-based microfluidics to automate single-cell isolation toward small-volume handling, enabling the processing of up to 192 samples simultaneously for LC-MS (Ctortecka, Hartlmayr, et al., 2022). As shown in Figure 4, nanodroplet processing in one pot for trace samples (nanoPOTS) miniaturized the processing of 100 cells to a single mammalian cell onto nano-wells on microfabricated glass chips using nanoliters of reagents for deep proteomics (Zhu, Clair, et al., 2018; Zhu, Piehowsk, et al., 2018). Using LCM to dissect ~100 μm squares from mouse tissues, the approach helped uncover spatial differences between uterine cell types during pregnancy in preparation for blastocyst implantation (Piehowski et al., 2020). NanoPOTS also enabled proteomics on single sensory hair cells measuring ~0.6–1 pl in volume, facilitating the study of cell differentiation in chicken embryos (Y. Zhu et al., 2019). Nested nanoPOTS (N2) arrayed multiple wells to enhance measurement throughput for multiplexed quantification using nanoLC-HRMS (J. Woo et al., 2021). Recent efforts sought to further refine one-pot sample preparation strategies to make the setups cost-effective and allow scalable sample preparation, as was recently reviewed in Slavov (2022). Automated preparation in one pot for trace samples (autoPOTS), the latest upgrade of nanoPOTS, now allows automated processing of 1–500 cells using commercially available instrumentation (Liang et al., 2021).

Similar strategies also emerged for top-down proteomics. Detection of intact proteins provides insights into proteoforms and posttranslational modifications (Catherman et al., 2014). With fewer analytical steps than its bottom-up sibling, miniaturized formats for detergent removal and desalting (Nickerson et al., 2021; Yu et al., 2021) and "one-pot" sample preparation (Dagley et al., 2019; M. W. Zhou et al., 2020) extended top-down proteomics to limited sample amounts as low as hundreds to tens of nanograms. As mentioned earlier, analyte recovery can be enhanced by eliminating the routines of sample handling and desalting. Freeze-thaw lysis and nonionic surfactant-based extraction from ~200 brain cells enabled spatial top-down proteomics for ~400 proteoforms between the optic tectum and telencephalon regions of the zebrafish brain (Lubeckyj & Sun, 2022). Further advancements in technology raise exciting potentials to leverage proteoforms as biomarkers of diseases (Smith et al., 2021).

2.2.2 | Peptidomics

Endogenous peptides, such as neuropeptides, can provide additional insight into biological processes, particularly related to the nervous and neuroendocrine systems. These peptides are synthesized from larger proteins via enzymatic cleavage, and their biological activity is often distinct from the preprohormone or precursor-related peptides (Hook et al., 2018). Many neuropeptides contain similar sequences that may vary by only one or a few amino acids, but their biological functions could be identical or largely different (DeLaney et al., 2018). Furthermore, neuropeptides are thought to be the most diverse class of signaling molecules, are present in low abundances in vivo, and are prone to rapid degradation. As such, analyzing endogenous peptides in space and time presents unique challenges and requires a rapid, sensitive workflow with specificity capable of differentiating between peptide isoforms. MS emerged as the gold standard for profiling neuropeptides, as it is capable of rapidly detecting neuropeptides at sub-attomole levels and can identify peptides without prior knowledge (DeLaney, Phetsanthad, et al., 2022).

Peptidomics consists of peptide extraction, purification via molecular weight cutoff filtration, and desalting before MS analysis. A critical aspect of this process is deactivation of proteolytic enzymes to prevent peptide degradation and achieve highly sensitive peptidomics analyses, which can be accomplished by flash-freezing the tissue immediately after dissection, heat stabilization, or acidification. Acidification is particularly effective for deactivating proteases when the acid is in an organic solvent (e.g., acidified methanol or acidified acetonitrile), as while proteases are deactivated by the low pH, large proteins are precipitated from the solution. For example, acidified methanol was recently used to successfully extract neuropeptides from single mollusk neurons, enabling sequencing of peptides in the cytoplasm and nucleus of the neurons and identification of differences in peptide abundances based on subcellular location (L. W. Zhang, Khattar, Kemenes, et al., 2018). In MALDI-MS workflows, 2,5-dihydroxybenzoic acid (DHB) was found ideal for peptide extraction, as it served as both the extraction buffer and the matrix (Romanova et al., 2008; J. H. Wang et al., 2009). Though other stabilizing agents

such as EDTA and protease inhibitor cocktails can also be added to the extraction buffer to improve peptide stability (R. B. Chen et al., 2009; Kwok & Tobe, 2006; Onorato et al., 2019), MS compatibility is important to consider, particularly with limited samples, so as to reduce additional cleanup steps and sample losses.

Peptidomics from limited samples may also benefit from molecular enrichment to boost sensitivity. Affinity-enhanced magnetic beads offered one such approach to improve detection for low-abundance peptides (Schmerberg & Li, 2013; Vocat et al., 2020). The incorporation of magnetic beads coated with NPY02 antibodies during sample preparation led to femtomolar sensitivity and quantitation of neuropeptide Y in human plasma (Vocat et al., 2020). Considerations of sample recovery for the desalting step are similar as in bottom-up proteomics workflows. Additionally, solid-phase extraction (Bardsen et al., 2019) and on-plate droplet desalting for MALDI-MS (S. Wang et al., 2017; Yoon et al., 2018) improved the sensitivity of neuropeptide analyses. Solid-phase extraction was also brought online to seamlessly clean up neuropeptide samples before LC-MS (Axel et al., 2017).

Alternatively, MSI strategies are popular for direct measurement and spatial localization of bioactive peptides, particularly in limited samples. Sampling with a focused laser beam requires minimal sample preparation, yet yields a sufficient amount of analytes for ionization and detection. Development of validated protocols made sample preparation for MSI straightforward and robust (Chatterji & Pich, 2013; DeLaney, Phetsanthad, et al., 2022; Reyzer & Caprioli, 2013). They predominantly involve sectioning of frozen or embedded tissues or dissection of cells; however, careful steps need to be undertaken to maintain sample integrity and reproducibility within measurements. To remove interfering contaminants and reduce signal suppression, washing or desalting steps are common before freezing. By stretching the tissue, the spatial resolution of MSI was improved to enable localization of neuropeptides in individual neurons of *A. californica* (Zimmerman et al., 2011). Similarly, electroblotting through trypsin-containing membrane was found to enhance tryptic digestion directly in tissues to improve protein identification without compromising their spatial location (Andrews et al., 2021). Employing a secondary step of ionization on the expanding MALDI plume improved ion yields, boosting the sensitivity of spatial imaging of the tryptic peptides (McMillen et al., 2021).

2.2.3 | Metabolomics

MS-based metabolomics can be streamlined to deliver high reproducibility facilitating both absolute and relative quantification of metabolites. Common sample preparation protocols in metabolomics include quenching of enzymatic activity, analyte extraction, purification, and desalting, and/or chemical derivatization before separation. To deepen metabolite coverage, detection sensitivity can be improved by tailoring the chemical composition, pH, and temperature of the extraction solvent to the chemical properties of the compound classes of interest (e.g., amino acids, lipids, nucleotides). For example, a discovery study in *Escherichia coli* culture reported that polar solvents (e.g., water, ethanol) in combination with high temperature effectively extracted hydrophobic and hydrophilic metabolites as compared to less polar solvents (e.g., acetone) (Yanes et al., 2011). Several advances for cellular and subcellular sample preparation were developed, including optimized extraction solvent mixtures (Figure 4; Onjiko et al., 2016), on-column preconcentration or sample stacking (Kawai et al., 2019; Liao et al., 2020; J. X. Liu et al., 2014), and microfluidic devices (Wu et al., 2004). Recent technologies in droplet microfluidics were reviewed elsewhere (Matula et al., 2020).

Detection sensitivity benefits from sample cleanup. These strategies aim to reduce ion suppression while minimizing the risk of concentration dilution to help distinguish analyte signals from chemical interference. Recently, six targeted metabolites were detected in MCF-7 cells without lipid interference using simplified microextraction, which used the same pulled glass capillary to dispense a droplet of solvent onto the targeted cell for selective extraction and sampling by aspiration (X. C. Zhang et al., 2016). Alternatively, an on-probe derivatization step was reported to selectively enhance the signal of target metabolites, thus reducing ion suppression (Cao, Zhang, et al., 2020). Simplified extraction methods can also be employed to broaden the types of metabolites detectable in single cells and subcellular organelles, as was accomplished for polar and non-polar metabolites in single cells using analyte partitioning within a microcapillary (Hamilton et al., 2017). The single-step method successfully decreased ion suppression during lipidomics by MALDI-MS.

As with other 'omic areas, droplet-based microfluidic chips allow limited sample handling by confining single-cell workflows to low-volume droplets. These approaches were the focus of several comprehensive reviews (Feng et al., 2015; Gao et al., 2013; Joensson & Svahn, 2012; Y. F. Liu et al., 2019; Matula et al., 2020; Shang et al., 2017). A major advantage of microfluidic methods is the resulting high throughput that enables the rapid interrogation of hundreds of cells in a single experiment (Cahill et al., 2019; X. C. Zhang et al., 2016).

For example, by combining a single-cell printer (SCP) with liquid-vortex capture (LVC)-MS (Figure 4), a recent study demonstrated direct analysis of metabolites in single cells at a rate of ~30 cells/s (Cahill et al., 2019). LVC-MS has also been combined with LCM to enable matrix-free spatially resolved direct analysis of metabolites from 20 to 40 μm tissue sections (Cahill et al., 2016). An important aspect to consider when using microfluidics for cell isolation is the possible perturbation to the cell's native metabolome due to external factors, such as mechanical forces.

Techniques enabling in situ or minimally intrusive cellular or subcellular isolation can assist in these challenges. Recent methods using, for example, sharpened or dual-barrel capillaries, reproducibly targeted and aspirated picoliter-to-nanoliter sample volumes from neurons (Aerts et al., 2014; Choi et al., 2022), *Allium cepa* cells (Huang et al., 2021; Saha-Shah et al., 2016), and *X. laevis* embryonic cells (Lombard-Banek, Choi, et al., 2021; Onjiko et al., 2017; Saha-Shah et al., 2019). Likewise, a single-lysosome MS (SLMS) platform was recently reported which combines patch-clamp recording and induced nanoESI (InESI)-MS to enable electrophysiological profiling followed by direct metabolic analysis of individual enlarged lysosomes within ~80 s (Zhu, Li, et al., 2021). The same glass micropipette used as a recording electrode served as the electrospray emitter for subsequent ESI-MS analysis. Further advances aiming to extract and measure mitochondrial, cytosolic, and nuclear metabolites can open new avenues for investigating metabolic regulation within these subcellular compartments. Overall, the fast sampling afforded by these and other capillary probes help minimize metabolic changes and stress arising from endogenous metabolism, transitions in cell cycle, or metabolic degradation, thus providing a more precise view of the cell's metabolic state (Onjiko et al., 2017).

Integration of HRMS with light microscopy, fluorescent labeling, and MSI opened the possibility of high-throughput single-cell and -organelle metabolomics. SpaceM, an open-source method for in situ single-cell metabolomics, demonstrated the detection of ~100 metabolites from more than 1000 individual cells per hour to classify cell types with high accuracy (~91%) (Rappez et al., 2021). Microscopy-guided MALDI allowed for profiling over 30,000 individual rat cerebellar cells, revealing cellular heterogeneity (Neumann et al., 2019). Image-guided MALDI on a FTICR mass spectrometer enabled surveying lipid and peptide heterogeneity between 0.5- to 2-μm-diameter dense-core and lucent vesicles in *A. californica* cells (Castro et al., 2021). As shown in Figure 4, a liquid microjunction (LMJ) probe further enabled coupling optically guided MALDI with CE-HRMS to extend the metabolic coverage from single pancreatic islet cells (Comi et al., 2017).

Minimization or elimination of chemical matrix for the purpose of sampling or ionization facilitated higher measurement throughput and reduced chemical interferences. Under vacuum, nano laser probe-based LDI achieved a resolution of 300 nm and was able to capture the localization of anticancer drugs within organelles of a HeLa cell (Meng et al., 2020). Nanostructure-initiator MS (NIMS) (H. K. Woo et al., 2008) and silicon nanopost array (NAPA) laser desorption/ionization (Korte et al., 2019) used specialized substrates to promote desorption and ionization for high-resolution spatial imaging of tissues, cells, and organelles. SIMS has a demonstrated history of imaging small biomolecules with ~650 yoctomole sensitivity (Greving et al., 2011), with resolutions on the order of a few tens of nanometers, as was reviewed recently (Massonnet & Heeren, 2019). Early on, for example, the technology found changes in lipid domains in mating *Tetrahymena thermophila* (Kurczy et al., 2010). NAPA imaged triglycerides from tissues, which are otherwise difficult to ionize (Fincher et al., 2020). To facilitate chemical identifications, a TOF instrument equipped with dual C_{60}–SIMS and MALDI increased the impact area of molecular-ion projectiles (Lanni et al., 2014), while matrix-enhanced SIMS used a matrix as an energy sink, allowing for profiling metabolites and neuropeptides in tissues (Potocnik et al., 2017) and single cells with a high 6 s/cell measurement throughput (Do et al., 2017). These and other developments in SIMS and MALDI were the focus of recent reviews (Lanni et al., 2012; X. P. Zhu et al., 2022).

Ambient MS lifted spatial and temporal imaging to the ordinary environment. Live single-cell video-MS (Mizuno et al., 2008), DESI, and LAESI MS provided convenient detection of primary and secondary metabolites in scalable resolution from tissues to cells, as was reviewed (Nemes et al., 2010; Wiseman et al., 2006; L. W. Zhang & Khattar, Kemenes, et al., 2018). LAESI MS pioneered spatial interrogation of tissues from plants and mouse brain sections in 2D (Nemes et al., 2010, Stopka et al., 2019) and 3D (Nemes et al., 2009). Sequential tissue sectioning with ambient MSI on each section allowed DESI (Eberlin et al., 2010) and nanoDESI (Lanekoff et al., 2015) to reconstruct the 3D distribution of metabolites in the mouse brain. The ability to spatially resolve metabolic signatures from tissues to cells promises to drive new biological discoveries and applications, for example, in the study of cancer cell populations and their microenvironment. Moreover, the development of methods to measure subcellular metabolic signatures offers an avenue for new biological insights.

2.2.4 | Multi-'Omics

The integration of multiple 'omics information on the same sample provides a deeper and more complete picture of molecular processes in a biological system. Furthermore, measuring the levels of multiple types of biomolecules across space and time is necessary to better understand dynamic biological processes, such as development, which would not be captured by a single-time snapshot (Lopes et al., 2020). Protein, peptide, and metabolite levels also change as a result of intrinsic and extrinsic factors (i.e., conditions of sample collection), such that simultaneous collection of multiple biomolecules from the same samples is favorable to enhance the fidelity, robustness, and reproducibility of analyses, particularly on limited samples.

Several methods were developed to enable sample processing for multi-omics studies on the same sample. Representative examples range from simple molecular weight cutoff filtration for proteomics and peptidomics (Zougman et al., 2008) to multiphase partitioning via simultaneous metabolite, protein, lipid extraction (SIMPLEX) (Coman et al., 2016). A more recent tip-based extraction, simultaneous trapping (SiTrap), was developed to allow proteomics and metabolomics on tens of micrograms of proteins from the same cell or tissue (Zougman et al., 2020). With further miniaturization, this method could be advantageous for multi-'omic analysis of limited samples, as fewer transfer steps help to minimize sample handling and reduce analyte loss compared to traditional extractions using multiple phases. Although recent efforts introduced methods to collect samples from the same tissue or cell for dual proteomics and metabolomics (Lombard-Banek et al., 2021; Pace et al., 2021), techniques enabling simultaneous multi-'omics have yet to become routine.

2.3 | Separation

Chemical separation aids biological HRMS 'omics. Separation provides another layer of compound-dependent information, the time of separation, to aid molecular identifications. It also helps minimize or eliminate chemical interferences during ionization and detection, thus improving sensitivity and the confidence of molecular identifications. Further, by spreading the chemical complexity of the sample over time, separation enhances the efficiency of duty cycle utilization during tandem and multi-stage MS, which in turn deepens the detectable and quantifiable portion of the 'omes. This section highlights approaches, shown in Figure 5, that facilitate spatiotemporal studies on limited specimens, ranging from cell populations to single cells to organelles, using some form of separation.

2.3.1 | LC

As a result of extensive research in stationary phase chemistries and engineered systems capable of withstanding high pressures, automation, and commercialization, LC became the mainstream separation technology for HRMS. Virtually all types of proteins, peptides, metabolites, lipids, and drug molecules can be separated using high-performance LC, or HPLC (Aydogan et al., 2020; Broeckhoven & Desmet, 2021). Reversed-phase LC (RPLC) employing packed-bed stationary phases with C_{18} chemistries (Figure 5) is efficient in resolving peptides and hydrophobic metabolites including lipids, whereas C_4 or C_8 chemistries were found to work well for intact proteins (Catherman et al., 2014). The development of hydrophilic-interaction LC (HILIC) extended analyses to small and polar molecules, including neuropeptides, amino acids, and glycans (Aydogan et al., 2020; Mihailova et al., 2008).

Combining orthogonal mechanisms of separation enhances trace-sensitive analyses. Higher theoretical plate numbers resulting from the hyphenation of multiple dimensions of separation deepens the molecular depth of detection. The multidimensional protein identification technology (MudPIT) recognized these fundamental advantages for bottom-up proteomics early on by supplementing strong cation exchange (SCX) with reversed-phase chromatography (Schirmer et al., 2003). To reduce salt load from SCX, a modification of the approach used RPLC chemistries in both dimensions, first to fractionate at high pH (e.g., pH 8–10), then to separate each fraction at the conventional low pH (e.g., pH 2–3). High pH fractionation doubled the overall depth of the detectable phosphoproteome from mouse cell lines compared to SCX, identifying over 30,000 phosphopeptides (Batth et al., 2014). For limited cell populations, this approach recently yielded 70% improvement in the coverage of the measurable proteome, allowing for the molecular characterization of the developing neural ectoderm tissue during embryogenesis (Baxi et al., 2018). Fractionation using stage tips (H. Kim et al., 2019) or nano-liter volumes (Reubsaet et al., 2020) further aided in reducing sample loss for deeper molecular coverage.

Recent advances in HPLC extended HRMS to ever-decreasing amounts of materials (Figure 5). To reduce analytical requirements to few micrograms to nanograms of proteomes, new-generation columns enhanced the peak capacity of partition chromatography by packing

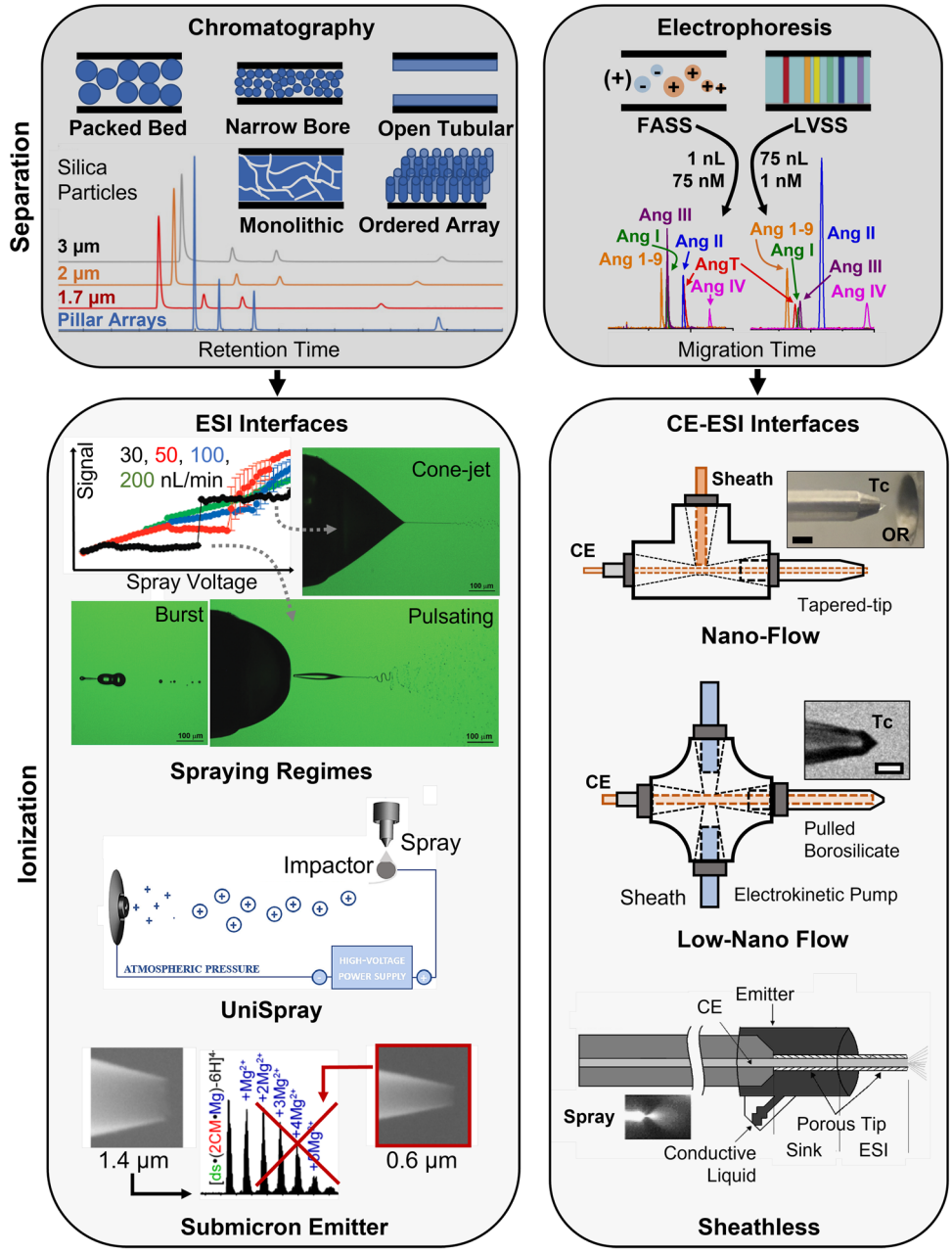

FIGURE 5 Advances in separation and ionization aided the chemical analysis of limited amounts of materials. Reducing the size of packing material and diameter of LC columns, newer generation columns minimize dispersion of molecules for high sensitivity during chromatography. Diverse stacking methods, including field-amplified sample stacking (FASS) and large-volume sample stacking (LVSS), boost sensitivity during CE. Specialized technologies and methods improve ion yields during electrospray ionization by controlling the spraying regime, charging of droplets, and reducing analyte dilution. Technological abbreviations are in the text. OR, orifice of mass spectrometer; Tc, Taylor cone. Adapted with permission from Bongaerts et al. (2020); Choi et al. (2017); DeLaney, Jia, et al. (2022); Desmet et al. (2020); Kenderdine et al. (2018); Lombard-Banek, Choi, et al. (2019); Lombard-Banek, Moody, et al. (2019); Marginean et al. (2007); Moini (2007); Nemes et al. (2007). [Color figure can be viewed at wileyonlinelibrary.com]

narrow-bore columns (inner diameters <50 µm) with small silica particles (<5 µm). These columns operate at nanoflow rates (<50 nl/min), thus concurrently reducing sample dilution and increasing ionization efficiency towards ultrasensitive detection. Recently, 362–874 protein groups became identifiable from a single HeLa cell (<150 pg) using flow rates of ~20 nl/min through an in-house-packed nanoLC column with 20 µm inner diameter (Cong et al., 2020). With 10–100-fold improvement in sensitivity than afforded narrow-bore packed columns (~15–30 µm inner diameter), these HPLC systems were recently coupled with microfluidic systems

capable of automating sample preparation, such as nanoPOTS (S. M. Williams et al., 2020), to address the challenge of protein loss on surfaces due to adsorption. Similarly, metabolomic coverage also benefited from reducing the flow rate from the analytical (0.5 ml/min) to the microflow (8 μl/min) regime, especially for sample-limited studies (Geller et al., 2022).

New-generation LC columns overcame challenges associated with packing narrow columns with robust performance. As illustrated in Figure 5, open tubular, monolithic, and micropillar array columns (μPAC) reduced band broadening and increased peak capacities using flow rates decreased to nanoliters to subnanoliters per minute (Desmet et al., 2020). The resulting enhancements in separation improved sensitivity and reproducibility for trace-level proteomics and peptidomics (Aydogan et al., 2020; Broeckhoven & Desmet, 2021; Yi et al., 2017) as well as metabolomics (Maes et al., 2014; Turiak et al., 2018; Zemenova et al., 2017). Capillary monolithic columns with 12 nl/min flow rate enabled identification of over 2300 proteins from 1 ng of HeLa digest (Gregus et al., 2020). Flows on the order of picoliters per minute through narrow open tubular columns with ~2 μm inner diameter recently elevated identifications to ~1000 proteins from 75 pg of *Shewanella oneidensis* tryptic digest, which was equivalent to half the amount obtained from a single HeLa cell (Xiang et al., 2020). Monolithic capillary columns were used to spatially profile the proteome and phosphoproteome of the plasma and the vacuolar membrane in *Oryza sativa* (Whiteman et al., 2008). Alternatively, fabricated monolithic capillaries were used for solid phase extraction and concentration enrichment of peptides released from spatially distinct regions of *A. californica* brain tissue followed by MALDI analysis (Iannacone et al., 2009).

Improvements in HPLC-ESI interfaces played an equally important role in advancing the sensitivity of HRMS analyses. In the conventional ESI interfaces, the LC eluent is fed through a metal emitter or pulled glass capillary, and sufficient potential difference is established between the spray tip and the mass spectrometer to form a stable electrospray. Electrosprays, however, can operate in different ways (Nemes et al., 2007), and transitions between these modalities can impact performance and the higher-order structure of molecules (Nemes et al., 2008). Figure 5 shows the impact the onset of different spraying regimes has on spray dynamics and MS ion signal when the ESI potential was adjusted. The relative ion signals, stability, and chemical composition of the electrospray are all impacted by alterations in the spraying regime. As illustrated, the cone-jet regime generates small droplets continuously, thus maximizing ionization efficiency (Nemes et al., 2007). The interface called UniSpray (Figure 5) placed an impactor rod of high voltage between the grounded capillary tip and inlet of the MS to facilitate electrospray ionization of the primary electrosprayed droplets, thus boosting S/N for neuropeptides (Bongaerts et al., 2020). Following the scaling laws of ESI, emitter tips with submicron-diameters (e.g., ~0.6 μm) were recently demonstrated to improve protein and oligonucleotide sensitivity by efficiently reducing their formation of cluster and adduct ions (Kenderdine et al., 2018).

2.3.2 | CE

While LC is a powerful separation method for complex biological samples, separation with CE can offer distinct advantages. Unlike HPLC, CE separates charged compounds in solution based on differences in their electrophoretic mobilities. With typical sample consumption on the order of a few nanoliters, this technology is a natural fit for volume- and mass-limited samples. Several methods allow for efficient preconcentration of analytes via in-column sample stacking (Osbourn et al., 2000). Field-amplified sample stacking (FASS) and exquisite separation power permit efficient differentiation of trace amounts of proteins, peptides, and metabolites, as was recently reviewed in multiple studies (DeLaney et al., 2019; Drouin & Ramautar, 2021; Lombard-Banek, Yu, et al., 2019; Zhao et al., 2012). With large-volume sample stacking (LVSS) using a dynamic pH junction, a 10-fold enhancement in sensitivity (Figure 5) enabled the detection of 5700 proteoforms in the *E. coli* proteome (McCool et al., 2018) and peptides from the renin-angiotensin system in the paraventricular nucleus of the mouse central nervous system (DeLaney, Jia, et al., 2022). An international study encompassing 13 different laboratories found CE robust and reproducible (Drouin et al., 2020), projecting this technology to complement chromatography-based HRMS for spatiotemporal 'omics.

Ultrasensitive measurements also benefited from the innovation of specialized CE ion sources for HRMS. The development of low sheath-flow and sheathless interfaces supported the coupling of CE with ESI-HRMS, reviewed elsewhere (Gomes & Yates, 2019; Lindenburg et al., 2015). Figure 5 highlights representative CE-ESI interfaces that enabled stable and sensitive CE-ESI operation using blunt-tip and tapered-tip metal as well as microfabricated borosilicate and fused silica emitters. These custom-built CE-ESI instruments extended 'omics measurements to attomole to zeptomole to yoctomole levels of metabolites and peptides from limited samples and broad types of biological models (Bonvin et al., 2012;

Choi et al., 2017; DeLaney, Jia, et al., 2022; Hirayama et al., 2018; Moini, 2007; Peuchen et al., 2017; Vermeire et al., 2020).

Microsampling and custom-built CE-HRMS platforms enabled spatiotemporal profiling of important biomolecules. Using micropunch sampling, the technology recently permitted characterization of nearly all the angiotensin peptides of the renin-angiotensin system in the subfornical organ and paraventricular nucleus of the mouse brain with a 30-zmol (~18,000 copies) limit of detection, raising a potential to facilitate future studies on thirst and water homeostasis and regulation of the neuroendocrine system to stress (DeLaney, Jia, et al., 2022; Lombard-Banek, Yu, et al., 2019). Further, microdissection and capillary microsampling extended ultrasensitive CE-HRMS to hundreds of different metabolites (Aerts et al., 2014; Onjiko et al., 2015) and up to ~2000 different proteins in single neurons in the central nervous system of the mouse (Choi et al., 2022) as well as blastomeres in *X. laevis* and zebrafish embryos (Lombard-Banek, Moody, et al., 2019, Lombard-Banek, et al., 2021; Lombard-Banek, Moody, et al., 2016). Results from these studies revealed previously unknown molecular differences between identified embryonic cells and reorganization of the single-cell proteome and metabolome during formation of cell lineages (Lombard-Banek, Choi, et al., 2019; Onjiko et al., 2017). Microdialysis combined with CE has enabled in vivo study of cerebral metabolism of peptide E in rodent brain (Behrens & Li, 2010).

Information from these studies supported the generation of hypotheses and led to biological discoveries. CE-HRMS metabolomics on dissected identified neurons revealed distinct metabolomes in *A. californica* neurons and their regional composition (Lapainis et al., 2009; Nemes et al., 2013). CE-HRMS played a central role in the discovery of noncanonical cell fate-altering molecules and mechanisms of embryonic patterning (Onjiko et al., 2015). Most recently, the technology enabled the characterization of ~700 proteins and ~150 metabolites from ~10 nl aspirates from single-identified blastomeres in cleavage-stage *X. laevis* embryos, enabling in vivo proteomics in live embryos (Lombard-Banek et al., 2021). Using top-down HRMS, the technology also enabled detection of ~400 different proteoforms in distinct regions of the zebrafish brain, containing ~250 cells (Lubeckyj & Sun, 2022). CE-HRMS aided temporal studies on the expression of N- glycans and N-glycosylated peptides during vertebrate embryonic development, revealing extensive reorganization of the N-glycome expression during neurulation (Qu et al., 2020).

Integration of CE with orthogonal HRMS technologies opened exciting new directions in basic research. CE-HRMS with CE modes (Xu et al., 2012) and LC fractionation (Z. C. Yang et al., 2019) deepened detection of proteomes. Coupling high pH fractionation followed by CE analysis helped identify 6500 proteins from 500 ng of protein from a human cell line. When coupled with ion mobility detection, optical isomers became distinguishable in single-cell bodies isolated from *A. californica* pleurin neurons (Mast et al., 2021). With MALDI-MS, CE enabled rapid fingerprinting of individual pancreatic islet cells with single-cell resolution, revealing metabolic differences between the different cell types (Comi et al., 2017).

Further automation and miniaturization through microfluidic chips also facilitated ultrasensitive measurements. Microfluidic CE systems expanded HRMS to ultrasensitive analysis by constraining critical steps of the analytical workflow, from sample lysis to separation, in a single platform with minimal sample transfer and, in turn, reducing sample loss. For example, an integrated microfluidic device enabled the analysis of α and β subunits of hemoglobin in 12 human erythrocytes per minute (Mellors et al., 2010). High throughput provided by this platform was used to analyze intracellular levels of metabolites in a large number of neuronal cells under different physiological conditions (X. T. Li et al., 2016). Such automated systems enabled high throughput 'omics on large populations of single cells, as was reviewed elsewhere (Abraham et al., 2019).

2.3.3 | IMS

Gas-phase ion separation provides speed and added resolution to aid molecular detection. With ion mobility separations taking less than 100 ms, ion mobility can be nested into most liquid-phase separations before HRMS detection. Most IMS techniques yield information to enable the calculation of collision cross section (CCS), which serves as compound-dependent information to aid molecular identifications. This approach enabled the elucidation of compound classes with large molecular diversity, including D- and L-amino acid-containing peptides (Fouque et al., 2017) and neuropeptides of the renin-angiotensin system in the paraventricular nucleus of the mouse central nervous system (DeLaney et al., 2022). IMS facilitated a broad range of investigations, ranging from proteins to small metabolites to lipids to glycans, as was reviewed elsewhere (Burnum-Johnson et al., 2019).

IMS techniques combined with other separation techniques, such as HPLC, can help distinguish isomers, isobars, and conformers. As a dual separation device and charge-state filter, FAIMS increased unique peptide identifications by ~30% and doubled the number of

quantifiable peptides over non-IMS from HEK293 cells due to a 10-fold enhancement in ion signal abundance and improved utilization of MS/MS duty cycles (Pfammatter et al., 2018). FAIMS was employed to discard singly charged ions to improve detection of peptides from single HeLa cells processed using nanoPOTS, providing over twofold improvement (Cong et al., 2020). Trapped IMS (TIMS) enabled regio-specific profiling of novel D-amino acid-containing peptides in the *A. californica* central nervous system (Mast et al., 2020). IMS also aids deepening molecular coverage from complex biological samples by providing an additional dimension of separation, the CCS, and reducing chemical noise and isobaric interferences. HPLC with TIMS-MS provided orthogonal separation to facilitate speciation for over ~1400 lipids and capture their spatial distribution in mouse brain tissues (X. Chen et al., 2022). HPLC with traveling wave IMS (TWIMS) was used to identify metabolic changes during colorectal cancer progression (M. D. Williams et al., 2015). Distinct metabolic profiles were captured to differentiate colorectal cancer from nonmalignant tissues, and across different stages of cancer progression.

IMS provides separation in cases when classical solution-based separations are restrictive or not feasible. Such is the case in most ambient ionization experiments, where LC- or CE-based separation would hinder analytical throughput and complicate sample processing. For example, capillary microsampling coupled with TWIMS-MS enabled neuropeptide measurements from cytoplasmic and nuclear components of single neurons from *Lymnaea stagnalis*, yielding previously unavailable data for functional biological experiments on their roles (L. W. Zhang, Khattar, Kemenes, et al., 2018). Similarly, dual-probe microsampling with drift-tube IMS-MS reported 73 lipids and 79 metabolites from live mammalian microglial cells (Domenick et al., 2020). Further, IMS boosts the reproducibility and confidence of identifications and quantification in MSI (Sans et al., 2018). LAESI with IMS imaged the distribution of isobaric primary and secondary metabolites, such as flavonoids, in plant species and lipids and metabolites in mouse brain sections (H. Li et al., 2015). MALDI with TIMS-MS enabled spatial mapping of isobaric and isomeric lipids in crustacean tissues (Fu et al., 2020). Recently, ambient ionization using liquid extraction surface analysis with TWIMS allowed imaging of intact proteins from mouse kidney tissue (Hale et al., 2020).

2.4 | Mass analysis and detection

Spatial HRMS 'omics profits from a still-expanding array of technological innovations. Development of specialized ion optics enabling efficient ion collection and transfer (e.g., ion funnel) as well as systems integrating new-generation mass analyzers, digitizers, and ion detectors (e.g., TOF, orbitrap, FTICR, SIMS) ushered in a new era of sensitivity (detection limit), speed, and spectral resolution for limited specimens. A subset of developments are shown in Figure 6. High-repetition lasers and fast electronics enabled record 50 pixels/s data acquisition rates in MALDI-TOF, sufficiently high to enable fast 2- and even 3D imaging. In a recent example, 3D-MALDI-TOF MS (Figure 6) revealed differences between the lipid architecture of metastatic and non-metastatic medulloblastoma tumors, yielding new information on disease progression (Paine et al., 2019). Customized nano-flow ESI (nanoESI) ion sources on TIMS-TOF MS (Figure 6) quadrupled the detectable ion signal, deepening proteome coverage to >3900 proteins from 1 ng of protein from single HeLa cells (Brunner et al., 2022).

Ultrahigh resolution from orbitrap and FTICR instruments supported molecular identifications and coverage of the 'omes. Late-generation orbitrap tribrid mass spectrometers increased the detectable proteome from a single HeLa cell by ~36% and ~20% at the peptide and protein level, respectively, compared to the earlier designs (Cong et al., 2020). Additionally, transferring identification based on FAIMS filtering (TIFF) permitted identification of ~1700 proteins from single HeLa cells using varying compensation voltages (J. Woo et al., 2022). A 3D SIMS instrument equipped with an orbitrap analyzer (Figure 6) permitted high-resolution spatial imaging while obtaining accurate mass-to-charge (m/z) information, revealing lipid dysregulation during macrophage differentiation (Suvannapruk et al., 2022). Microscopy-guided MALDI with FTICR MS (Figure 6) enabled profiling ca. 500 lipids from 30,000 cells from the rat cerebellum, finding previously unknown molecular cell heterogeneity in the tissue in record time (Neumann et al., 2019). In situ microsampling by fiber-optic LAESI, or f-LAESI (Figure 6), with 21 T FTICR MS tracked metabolite responses in single plant cells upon bacterial infection (Samarah et al., 2020). These and other advancements continue to expand the capabilities of spatial measurements down to the building block of life, the cell.

Spatiotemporal 'omics naturally benefited from advances in tandem MS with increasing throughput, sensitivity, and specificity. An intelligent DDA method monitored the elution order of peptides based on prior discovery measurements to target tandem MS events, resulting in 80% more protein identifications than the standard (Bailey et al., 2014). BoxCar DDA leveraged multiple narrow m/z windows to improve ion accumulation during MS scans, realizing an order of

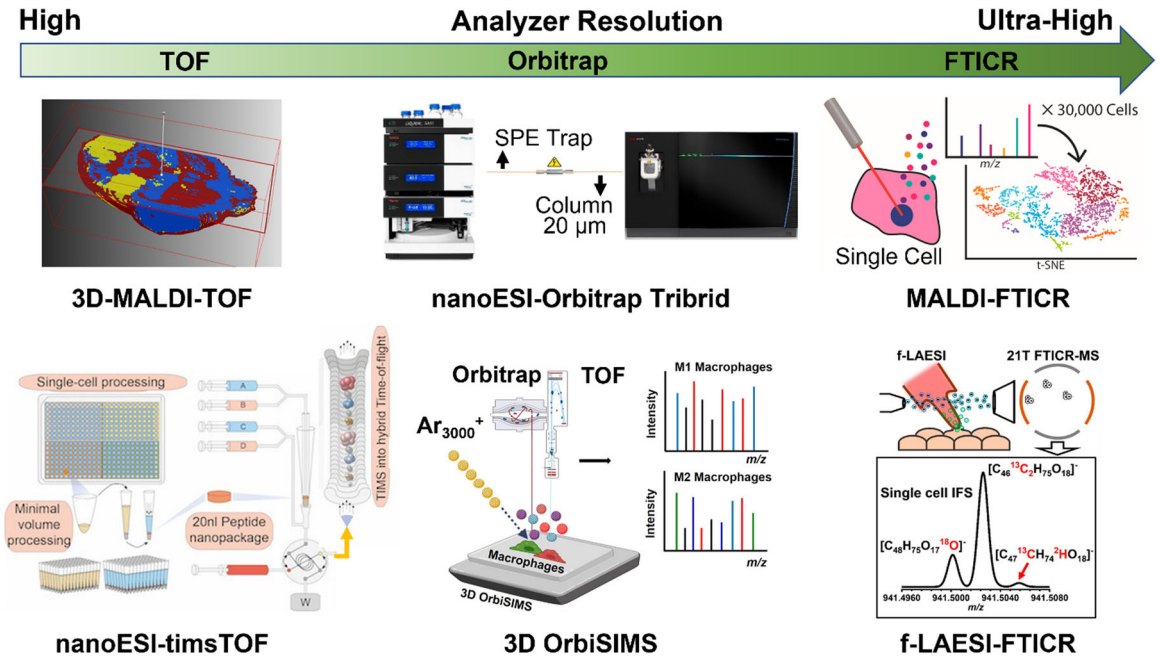

FIGURE 6 High- to ultrahigh resolution analyzers and instrumental configurations with enhanced ion transfer efficiency support spatiotemporal 'omics. TOF analyzers are capable of fast operation, enabling spatial interrogation of molecular distributions in tissues and cells using focused laser beams (MALDI) and in situ ablation/ionization (LAESI). Orbitrap and FTICR analyzer-detector systems provide ultrahigh resolution to distinguish isobaric species and their spatial and temporal distributions. Abbreviations are in the text. Adapted with permission from Brunner et al. (2022); Cong et al. (2020); Neumann et al. (2019); Paine et al. (2019); Samarah et al. (2020); Suvannapruk et al. (2022). [Color figure can be viewed at wileyonlinelibrary.com]

magnitude improvement in quantification (Meier et al., 2018). A DDA ladder employed a nested exclusion series of abundant peptide ions to reduce MS/MS redundancy, allowing identification of 35% more proteins than standard DDA and quantification of 415 proteins from ~5 ng of protein digest (approx. 10 neurons) from the mouse nervous system (Choi et al., 2021). In parallel, instruments with multiple fragmentation cells opened the door to using structurally diagnostic fragment ions to trigger sequential or multiplexed dissociation toward deeper sequence coverage. For example, electron transfer dissociation and higher-energy collisional dissociation (EThcD) discovered 4 different N-linked and 14 distinct *O*-linked glycosylated neuropeptides as well as 91 novel neuropeptides in identified crustacean neural tissues (Q. J. W. Cao, Yu, et al., 2020). The advent of data-independent acquisition (DIA) remedied limited tandem MS duty cycles during DDA, approximately doubling the number of neuropeptides in limited neural tissues (DeLaney & Li, 2019). This approach detected ~1500 proteins from single cells in the developing *X. laevis* embryo (Saha-Shah et al., 2019).

Spatial HRMS provides several strategies for quantification (recall Figure 2). Both label-free quantification (LFQ) and label-based quantification were adapted to limited specimens in the context of spatiotemporal biology. LFQ uses spectral counts, peak intensities, or peak areas to estimate the abundance of proteins, peptides, and metabolites without barcoding (Dupree et al., 2020; Lai et al., 2013). A reduction in sample processing steps aids analytics on trace amounts of samples, as was reviewed recently (Slavov, 2021). Chemical derivatization helps analytical throughput and reproducibility, and when performed online, enabled low-picomolar detection limits for peptides in microdialysates continuously collected from rat brains (Wilson et al., 2018). To enhance throughput, label-based quantification may integrate stable isotopes during sample preparation in vivo (metabolic labeling) or in vitro (chemical labeling) to enable multiplexing, as was reviewed elsewhere (Arul & Robinson, 2019).

Label-based quantification became quasi-routine for high throughput quantification across broad spatial and temporal processes, lately even for single cells. Reductive methylation (DeLaney et al., 2021) and DiLeu labeling (Sauer & Li, 2021) aided neuropeptidomics. The carrier approach pioneered the use of tandem mass tags (Thermo Fisher Scientific) in abundance to boost tandem MS on isobarically tagged protein digests from single cells (Slavov, 2022), while usage of a sacrificial protein helped reduce nonspecific protein losses during sample

preparation (Slavov, 2021). SCoPE-MS (recall Figure 4) is one such method, which distinguished cancer cell types at the single-cell level and characterized cell-to-cell heterogeneity during embryonic stem cell differentiation (Budnik et al., 2018). The new-generation SCoPE2 further downscaled volumes, automated sample preparation, increased throughput, and optimized MS data acquisition and analysis to quantify ~1000 proteins per TMT experiment with only 1 h of LC separation (Specht et al., 2021). Integration of carrier-based approaches with newer instrumentation further compounded the demonstrated improvements in proteome coverage and quantification from single cells. For example, FAIMS and real-time search in both MS/MS and synchronous precursor selection (SPS)-MS^3 methods on an orbitrap mass spectrometer attained higher proteome coverage and more accurate quantification in single acute myeloid leukemia cells by lengthening ion trap times, thus boosting sensitivity (Furtwängler et al., 2022). Combining narrow-window DIA acquisition with TMT tagging yielded high throughput multiplexing for trace samples (Ctortecka, Krssakova, et al., 2022). Additional improvements in efficient duty cycle utilization, sensitivity, and real-time search capabilities made possible by continued technological advances will further enhance performance of these carrier-based quantification methods for proteomics analysis of limited samples.

2.5 | Data analysis

Bioinformatics plays a critical and enabling role for spatiotemporal HRMS. This field of HRMS yields challenging data, not only in terms of complexity and size but also metadata to assess in relation to space and time. In Figure 7, representative examples are grouped based on their abilities to access these orthogonal pieces of information. General steps for data analysis include raw data processing (feature extraction or identification), data preprocessing, statistical analysis (quantitative information), and annotation and molecular network analysis (qualitative information). Data from imaging microscopy (optical to electron) and functional tests, such as electrophysiology and behavioral assays, complement the chemical data from MS 'omics with information on the biological phenotype. Analysis of such complex data poses several challenges such as handling large data sets, integrating orthogonal information obtained by combining different data acquisition techniques, accuracy of identification, and data processing speeds. Here we highlight select examples of software packages that helped address these challenges.

The first step of data analysis includes processing raw files obtained from MS analysis to identify proteins, peptides, or metabolic features. With ever-decreasing sample amounts to achieve spatial resolution at cellular and subcellular level, distinguishing true signal from noise is a fundamental challenge. New and next-generation bioinformatics aided the processing of low ion signals that could be mistaken for noise (false negative) or noise that could be mistaken for signal (false positive). The *Trace* software package employed machine learning on CE-HRMS data sets to create signal images from m/z and separation time data. These signal images effectively recognized true signal from noise with a record 95% confidence in single-cell metabolomics (Liu, Portero, et al., 2019). NPFimg recently extended the approach to metabolomics based on gas chromatography-MS (Jirayupat et al., 2021). OffsampleAI employed artificial intelligence for spatial metabolomics to recognize background interfering ions during MSI experiments. A machine learning tool was trained using ~2300 manually curated ion images to recognize background ions, thus improving metabolite identification from tissues (Ovchinnikova et al., 2020).

In parallel, a series of software developments increased speed, accuracy, and depth of coverage of the analyzed 'omes. To improve identifications from complex convoluted DIA data sets, newer machine learning tools, such as DeepMass (Tiwary et al., 2019), accurately predicted peptide fragment spectra to build spectral libraries in silico, essentially eliminating restrictions on laborious experimental reference libraries from precious, rare, or limited specimens. Similarly, PRESnovo, a de novo sequencing tool developed for neuropeptidomics, directly predicted peptide sequences (DeLaney et al., 2020), leading to the identification of 13 novel, putative neuropeptides from *Carcinus maenas* and 77 from *Cancer borealis* brain tissues. Most recently, several software tools were developed to overcome limitations in the processing of complex DIA and ion mobility data necessitating enhanced computational resources, which were recently discussed elsewhere (Allison et al., 2020; Rosenberger et al., 2017; Szymanska et al., 2016; F. F. Zhang et al., 2020).

As in other areas of HRMS, scientific rigor calls for stringent quality controls not just during measurements but also during data processing. After extracting qualitative (molecular identifications) and quantitative (abundance or intensities) information, data preprocessing uses normalization and advanced data checks to disentangle biological variations from artifacts (Slavov, 2022). Programs such as SCPCompanion helped credential the quality of single-cell proteomics data (Cheung et al., 2021) and suggest optimization of instrument

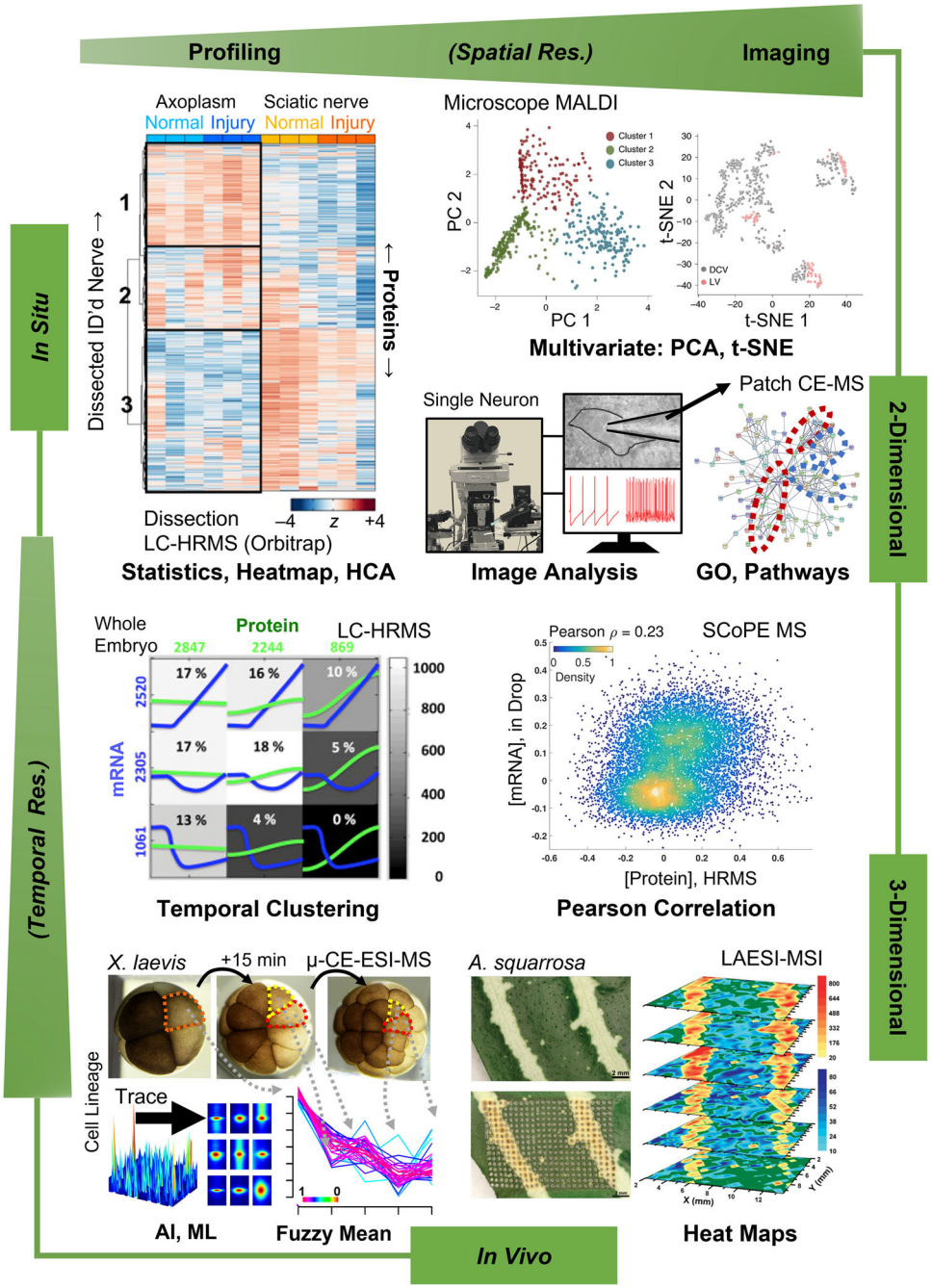

FIGURE 7 Chemometrics plays a pivotal role in analyzing metadata from spatiotemporal HRMS. Image analysis by optical microscopy and functional measurements, such as electrophysiology, help identify tissues and cells, guiding subsequent molecular characterizations to transcripts, proteins, peptides, and metabolites. Statistical and multivariate data analysis and knowledge bases are integral to interpreting molecular processes in specific biological contexts, as illustrated here for dissected rat sciatic nerves (HPLC), temporally developing whole *Xenopus laevis* embryos (HPLC), and dense core vesicles in *Autographa californica* (MALDI). Working in vivo, LAESI-MSI imaged chlorophyll and secondary metabolites in the variegated leaves of *Aphelandra squarrosa* and CE-HRMS enabled metabolomics-proteomics in single differentiating cells in *X. laevis* and neurons in the mouse brain. Models of artificial intelligence (AI) and machine learning (ML) assist in extracting trace-level signals (Trace) or uncovering relationships in the metadata. Technological abbreviations are in the text. Adapted with permission from (Budnik et al., 2018; Castro et al., 2021; Choi et al., 2022; Di Paolo et al., 2021; Y. F. Liu et al., 2019; Nemes et al., 2009; Onjiko et al., 2017; Peshkin et al., 2015). [Color figure can be viewed at wileyonlinelibrary.com]

parameters based on the preliminary data. In addition, single-cell proteomics readout of expression (SCeptre) and NOREVA provided normalization strategies to assist with reducing nonbiological variations in multiplexed single-cell 'omics (B. Li et al., 2017; Schoof et al., 2021). SpectralAnalysis was adapted to multiple instruments and provides a framework from data preprocessing to multivariate analysis within a single software package (Race et al., 2016).

Figure 7 represents examples of spatial profiling to imaging by biological HRMS. Chemometrics is crucial for the interpretation of metadata resulting from spatiotemporal HRMS (recall Figure 2). This field of research offers various solutions for statistical and multivariate analyses. For example, volcano plots are widely used to examine molecular differences between spatially or temporally distinct sample types. Unsupervised and supervised dimensionality reduction and clustering algorithms such as principal component analysis (PCA), linear discriminant analysis (LDA), t-distributed stochastic neighbor embedding (t-SNE), hierarchical cluster analysis (HCA), heatmaps, k- and fuzzy-mean clustering are broadly adaptable to exploring systematic molecular differences between samples, such as types of tissues or cells. Sciatic nerves dissected from the rat were surveyed using unsupervised HCA with LC-HRMS (Di Paolo et al., 2021), neuropeptides in dense core vesicles in *A. californica* neurons using unsupervised PCA/t-SNE with 2 D MALDI MSI (Castro et al., 2021), and chlorophyll and secondary metabolites in areas of the variegated *A. squarrosa* leaves with 3 D LAESI MSI (Nemes et al., 2009). Pearson correlation analysis and LC-HRMS permitted comparison of temporal expression between transcription and translation at the level of whole developing *X. laevis* embryos (Peshkin et al., 2015), single differentiating embryonic stem cells (Budnik et al., 2018). Live stereomicroscopy and micro-probe CE-MS used fuzzy means cluster analysis to find reorganization of the single-cell proteome as cells formed a neural-tissue-fated clone in the live *X. laevis* embryo (Onjiko et al., 2017). STRING was used to predict functional association networks on proteins that were quantified in single dopaminergic neurons in the mouse brain after electrophysiology using patch CE-MS (Choi et al., 2022). Software packages like MetaboAnalyst (Pang et al., 2021), Perseus (Tyanova et al., 2016), Orange (Demšar et al., 2013), and IOAT (Wu et al., 2021) provide user-friendly platforms for performing such analyses. EXIMS (Wijetunge et al., 2015) is another such tool developed to recognize spatial distribution patterns in MSI data sets.

A broadening number of software tools enable systems biology for spatiotemporal HRMS. Apart from classical tools from chemometrics, freeware programs, such as MetaboAnalyst, helped integrate transcriptomic to proteomic to metabolomic data sets, with added capabilities for enrichment studies and analyses of pathway networks. STATegra, another open-source tool, merged and identified important features from multi-'omics data in a stepwise manner (Planell et al., 2021). Similarly, Mergeomics tailored to extracting information on key biological pathways and networks, with demonstrated utility in aiding the understanding of disease progression (Ding et al., 2021). These and other statistical and data visualization pipelines help generate and test new hypotheses at the levels of proteomes, peptidomes, and metabolomes in biological processes occurring in space and time.

3 | CONCLUSIONS

Interdisciplinary efforts molded HRMS 'omics into a powerful tool to advance basic and translational research for molecular processes occurring in space and time. These developments are the result of still rapidly progressing interdisciplinary and collaborative efforts innovating essentially all steps of biological HRMS. Inventions of specialized strategies, equipment, and miniaturization to collect and process samples with speed and high fidelity is pivotal for studies on limited and dynamic biological systems. Advances in chemical separations, ionization, ion transfer and accumulation, and HRMS detection increase detection sensitivity and measurement throughput, thus ushering 'omics to increasingly finer spatial and temporal resolutions. Continued developments in data acquisition and user-friendly software packages to handle the resulting complex data promise to further deepen the detectable and quantifiable portion of proteomes, peptidomes, and metabolomes from trace amounts of specimens. Grand challenges associated with the acquisition, integration, and distribution of complex data from spatiotemporal multi-'omics pose exciting opportunities to move science forward toward a holistic understanding of life processes.

Sustaining the scientific momentum is as important as using the new knowledge that we learn to best benefit society at large. Collection of proven protocols, formulation of community standards and best practices, and deposition of primary and secondary data into public data repositories—such as pioneering works in metabolomics (Sansone et al., 2007), proteomics (Taylor et al., 2008), MS informatics (Binz et al., 2008), and now also in single-cell analyses (Gatto et al., 2022)—is pivotal for stringent, reproducible, and rigorous science. Investigators continue to break down classical boundaries in

bioanalyses to reach ever-deeper into the molecular world. In parallel, it is equally important to make these developments available and adaptable to broad users, including but not limited to investigators in the basic life sciences, translational research, pharmaceutical industry, and healthcare. Furnishing methods with versatility and compatibility across scientific disciplines, automation, and cost-efficiency are necessary and proven paths toward addressing pressing biological questions at present and to pose new ones to continue leading us to the future.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and revision of this work.

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