Advances in mass spectrometry-enabled multiomics analyses at single-cell resolution

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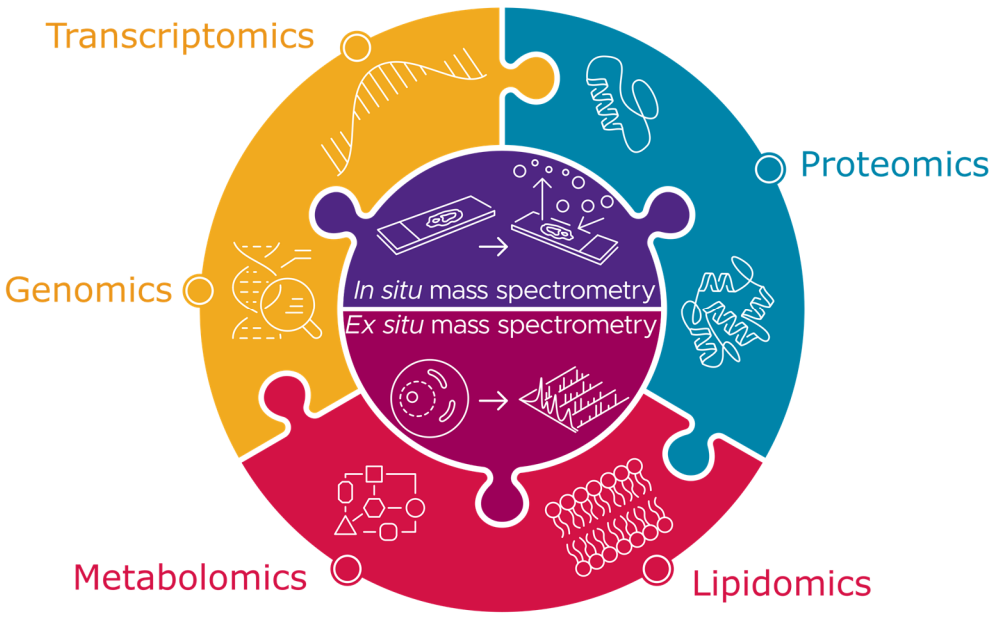
Abstract Biological organisms are multifaceted, intricate systems where slight perturbations can result in extensive changes in gene expression, protein abundance and/or activity, and metabolic flux. These changes occur at different timescales, spatially across cells of heterogenous origins, and within single cells. Hence multimodal measurements at the smallest biological scales are necessary to capture dynamic changes in heterogenous biological systems. Of the analytical techniques used to measure biomolecules, mass spectrometry has proven to be a powerful option due to its sensitivity, robustness, and flexibility with regards to breadth of biomolecules that can be analyzed. Recently many studies have coupled mass spectrometry to other analytical techniques with the goal of measuring multiple modalities from the same single-cell. It is with these concepts in mind that we focus this Review on mass spectrometry-enabled multiomic measurements at single cell or near single cell resolution.

Graphical Abstract



Introduction

Understanding health and disease mechanisms in animals, plants and microbes requires a systems-level investigation of regulatory networks at the levels of genes, transcripts, proteins, and metabolites. The majority of multiomic studies performed to date have been bulk analyses on blood[1], plasma[2], cell pellets[3] or whole tissues. While bulk analyses are often cost effective and technologically less demanding, such analyses cannot measure cell heterogeneity and biologically significant rare cell populations are often missed. Several ongoing initiatives, with a loosely shared goal of characterizing tissues at single-cell resolution, such as the Human Biomolecular Atlas Program (HuBMAP)[4], Human Tumor Atlas Network (HTAN)[5], Kidney Precision Medicine Project (KPMP)[6], Human Cell Atlas[7], and Cellular Senescence Network (SenNet)[8] are supporting the development of novel single-cell -omic techniques. Recently developed single-cell RNA sequencing (scRNAseq) techniques have revolutionized our understanding of developmental trajectories,[9] identified new cell types,[10] and enabled the mapping of entire organs.[11] Towards holistic understanding of biological processes, these sequencing methods have also been extended to multimodal measurements from the same single-cells.[12][13]

Although sequencing-based -omic approaches have many advantages (e.g., fidelity, cost, and throughput), there are limitations for protein measurements (e.g., intermediate antibody probes and limited access to intracellular targets)[14–16] and metabolites/lipids cannot be directly measured. Fortunately, it is within these classes of molecules that mass-spectrometry (MS) thrives, demonstrated by decades of studies performed at tissue scale. Recent advancements in MS techniques have pushed the envelope further, enabling cellular and subcellular measurements. When combined with orthogonal techniques, measurements across modalities from same single cells becomes possible.[17] It is with this in mind that we focus this Review on same single-cell (or near single-cell) MS-enabled multiomics.

State-of-the-art approaches

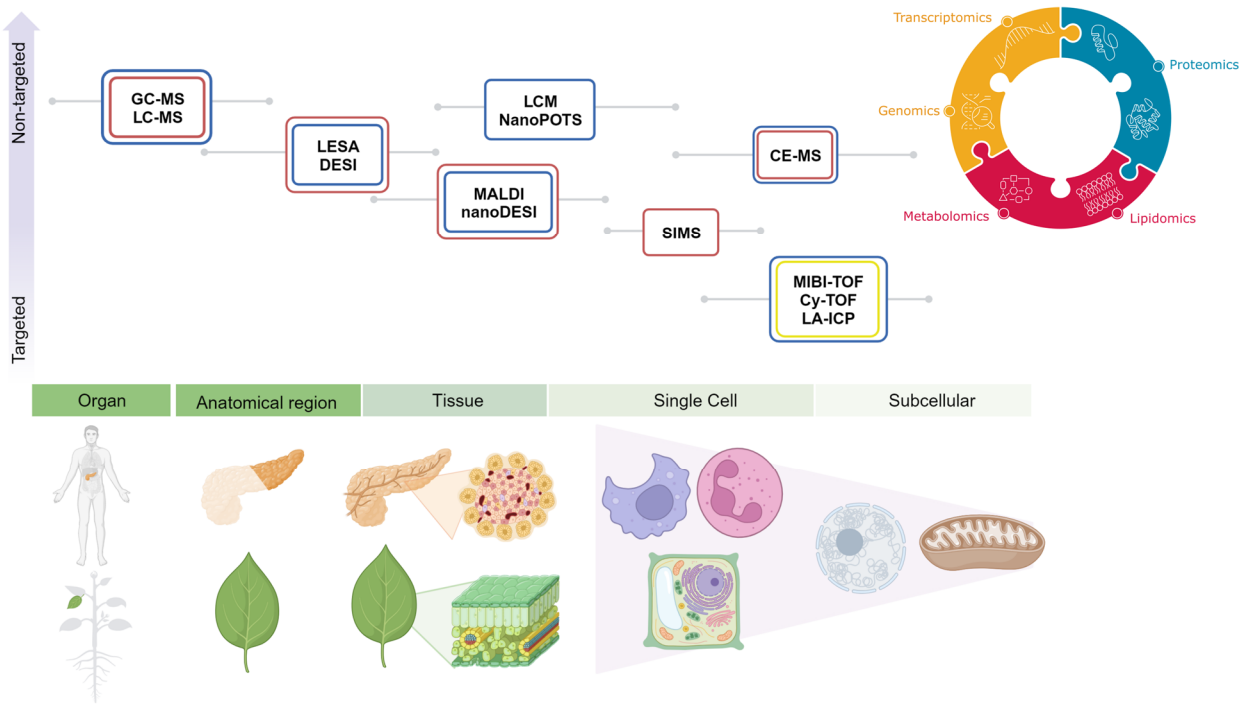


Figure 1. Overview of mass spectrometry approaches (both *in situ* and *ex situ*) applied to multimodal analyses. The color of the box surrounding each technique represents modalities measured (reflected in the upper right corner), while spatial resolution is reflected along the x-axis and breadth of molecular coverage (targeted to non-targeted) along the y-axis. Figure created with Biorender.com

Significant technological advances in MS over the past decades have enabled single-cell and even subcellular analyses (**Figure 1**). These techniques with single cell or near single cell resolution can broadly be classified as 1) *In situ* or 2) *Ex situ* MS-enabled single-cell multiomics.

Techniques classified under *in situ* are largely based on MS imaging (MSI), which directly analyzes biomolecules from thin tissue sections to retain spatial context. On the other hand, *ex situ* techniques largely require removal of single cells from their spatial context. These techniques often include the use of chromatographic separations on extracted single-cells prior to MS analysis. Further distinction of multimodal MSI applications can be made regarding the breadth of measurements, which we refer to as "targeted" or "non-targeted".[18] These terms refer to the number of features being measured, with targeted approaches typically focusing maximally on several dozen or fewer biomolecules and non-

targeted capturing hundreds or thousands (**Figure 1**). Also, note that several MS-only MSI techniques exist that can measure multiple modalities simultaneously or sequentially (such as matrix-assisted laser desorption/ionization [MALDI] and gas cluster ion beam secondary ion MS [GCIB-SIMS]), [19–21] however the principal focus in this Review is on MS techniques alone or combined with orthogonal, non-MS methods for measuring multiple biomolecules with single-cell or near single-cell resolution.

In situ MS-enabled single-cell multiomics

Numerous *in situ* MS-enabled multimodal approaches have recently been demonstrated at single-cell or near single-cell resolution on the same sample (**Figure 2a** and **Table S1**; a summary of MSI-enabled multiomics studies performed on different samples is also presented in **Figure S1**). Among MSI techniques at single-cell level, MALDI is the most versatile, having been widely used for the analysis of proteins, glycans, peptides, lipids, and metabolites.[22,23] It should be noted, however, that there is an inverse relationship between analyte size/ionizability and sensitivity with MALDI. For example, metabolites are routinely detected at trace amounts (low attomole)[24] whereas intact proteins typically have limits of detection orders of magnitude higher. Furthermore, sensitivity is intrinsically tied to the spatial resolution, where lower resolution (e.g., 100 µm) is more sensitive than high spatial resolution (e.g., 10 µm) due to the size of the area being sampled. Last, MALDI-MSI is completed at modest spatial resolutions of 5-50 µm and in a non-targeted manner.

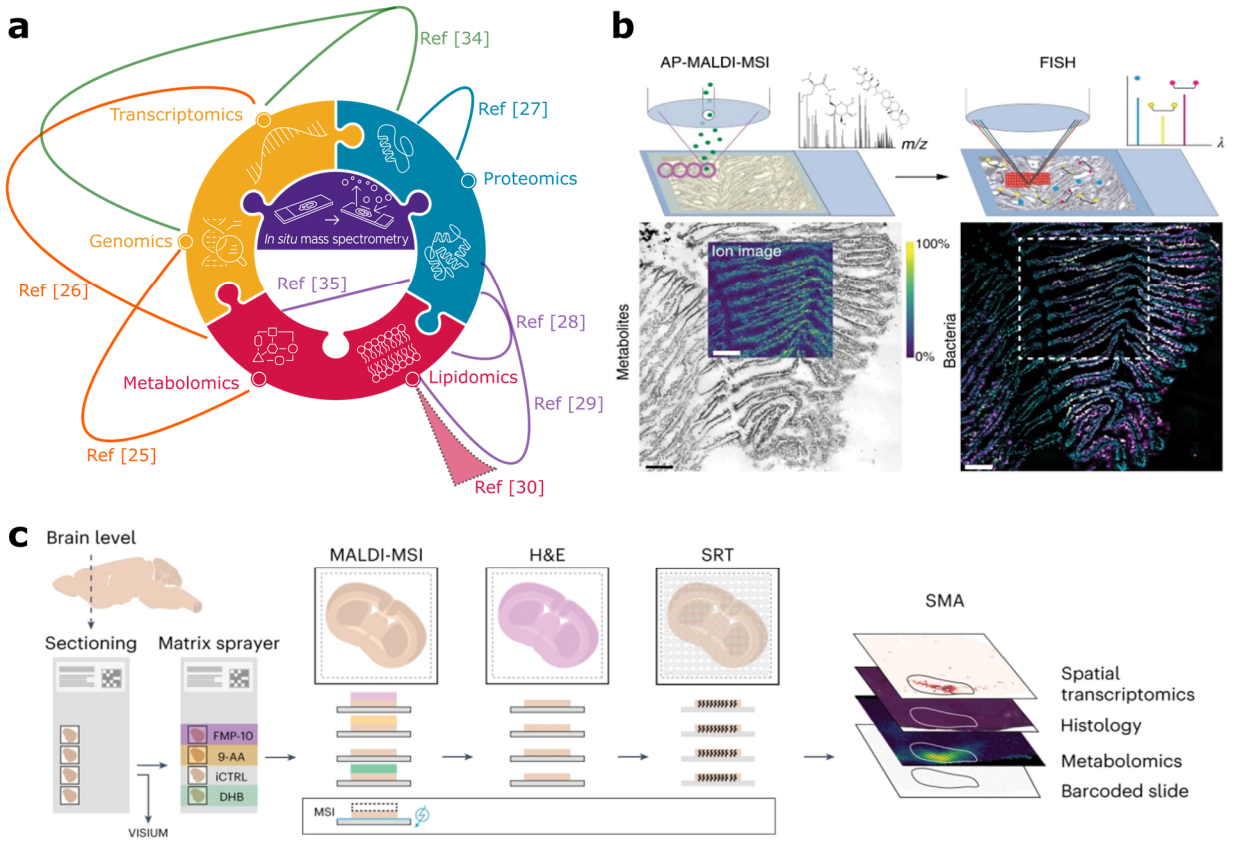


Figure 2. Summary and examples of *in situ* mass spectrometry-enabled single-cell multiomics**.** (**a**) Chord diagram showing the connections (*i.e.* studies) between modalities enabled by *in situ* techniques. (**b**) Left panel; MALDI-MSI metabolite mapping of bacteriocyte-containing deep-sea mussel *Bathymodiolus puteoserpentis* cryosection. Ion image is overlaid onto bright-field image of the same cryosection. Right panel; FISH after MALDI-MSI on the same cryosection. Colors indicate host nuclei (cyan) and two bacteriocyte phylotypes (magenta and yellow). (**c**) Overview of spatial multimodal analysis (SMA) showing cyrosectioning onto barcoded Visium Gene Expression arrays.

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Co-measuring of genomes and metabolomes from the same section have been demonstrated with non-targeted MALDI-MSI (spatial resolution of 3 µm) and FISH (termed metaFISH, **Figure 2b**), providing greater understanding of host/symbiont relationships between deep-sea mussel and their intracellular bacteria.[25] Another recent study demonstrated MALDI-MSI based metabolomics and spatial transcriptomics on the same tissue sections by mounting them on the Visium Spatial Gene Expression array, performing MALDI-MSI metabolomics, and finally spatial transcriptomics with Visium (**Figure 2c**).[26] This technique, termed spatial multimodal analysis (SMA) protocol, enabled the

identification of unique spatial correlations between dopamine and mRNA from *SCG2* in mouse and human brain samples. Notably, SMA is currently the only MSI enabled multimodal approach at near single cell resolution where both modalities are non-targeted.

MALDI-MSI can be also used to map post translational modifications such as N-glycans in combination with spatial analysis of target proteins. In a recent study, systematic optimization was done for sequential MALDI-MSI imaging of N-glycans or collagen peptides (non-targeted), AmberGen photocleavable mass-tags (MALDI technique leveraging photocleavable mass tags on antibodies, targeted), imaging mass cytometry (IMC, targeted), and GeoMx digital spatial profiling (targeted).[27] This study is noteworthy because the authors demonstrated the compatibility of MALD-MSI with antibody-directed techniques and determined optimal order of different techniques for achieving greatest sensitivity. Similarly, MALDI-MSI based lipidomics has also been combined with protein measurements such as antibody-based fluorescence imaging techniques (*i.e.* CODEX) on same tissue sections or with immunocytochemistry on dispersed cells.[28][29] Finally, MALDI-MSI of lipids has also been sequentially combined with Raman spectroscopy (which identifies functional groups as biomolecular fingerprints) in the analysis of same kidney and brain tissue sections.[30] Although Raman cannot provide annotations of molecules in complex mixtures such as tissue, this approach uniquely allowed for correlation between MALDI-MSI lipid species and Raman spatial patterns to identify similar distributions. Taken together, these studies demonstrate the versatility of non-targeted MALDI-MSI in single-cell, multimodal data acquisition.

Targeted MSI techniques include imaging mass cytometry (IMC), nanoSIMS, multiplexed ion beam imaging by time-of-flight (MIBI-TOF), and laser ablation inductively coupled MS (LA-ICP). Applications of these techniques typically involves the addition of an antibody, oligo probe, or ligand labeled with heavy elements (e.g., lanthanides) to provide a secondary measurement of the analyte of interest. Notably, the use of these ionization methods and elemental measurements have some of the highest spatial resolution (submicron) and sensitivity (down to 5 atom detection limit),[31] however they

are often restricted to targeted elemental isotope analysis to infer molecular identifications**.**[32,33] Protein and nucleic acid *in situ* imaging (PANINI), which uses the MIBI-TOF imaging platform, was shown to be compatible with CODEX. The PANINI technique provided the ability to measure simian immunodeficiency virus DNA and RNA alongside host immune protein markers in FFPE tissues.[34]

The above approaches all rely on MSI; however, a recently developed *quasi in situ* approach permits *in vivo* microsampling. Microsampled lysates can be analyzed with downstream chromatographic techniques for non-targeted multimodal analyses. Specifically, this approach has been demonstrated with *Xenopus laevis* embryos that were microsampled to withdraw ~5% of cellular volume before downstream proteometabolomic analysis with ultrasensitive capillary electrophoresis MS.[35] With as little as 0.01% of the cell's volume, 738 proteins and ~150 metabolite features could be identified per single-cell. Although *X. laevis* embryo cells are very large (180 nL volume / cell in the 8-cell embryo), there are several notable advantages to this approach. First, microsampling is relatively non-destructive and permits early-stage embryos to continue development into later stages. Second, because every cell in early-stage embryos can be identified based on pigmentation, microsampling permits sampling of specific cells with distinct trajectories. Therefore, microsampling is uniquely a subcellular, spatiotemporal method for sampling from embryo cells without impacting their viability.

Ex situ MS-enabled single-cell multiomics

Single-cell multimodal studies that utilize *ex situ* MS typically include downstream chromatographic methods, and therefore have a different set of challenges that have limited widespread adoption (**Figure 3a**).

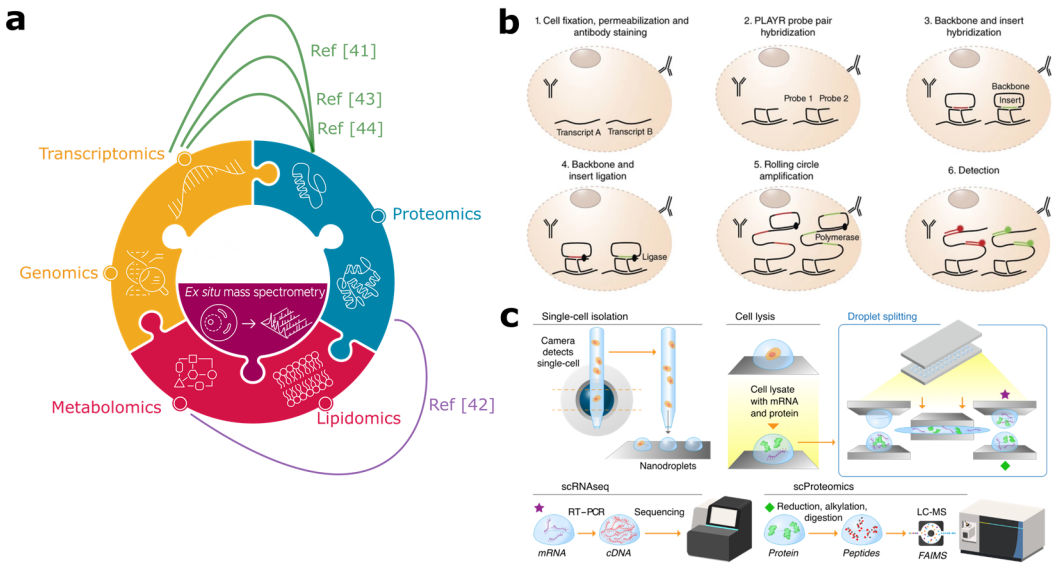

Figure 3. Summary and examples of *ex situ* MS-enabled single-cell multiomics. (**a**) Chord diagram showing the connections (studies) between modalities enabled by *ex situ* techniques. (**b**) Overview of the PLAYR protocol showing the major steps before FACS and CyTOF measurements of single-cells. **(c)** Overview of the nanoSPLITS multiomics approach

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The sampling of biomolecules from single-cells required for these techniques often leads to the loss of analytes due to non-specific surface adsorption.[36] Fortunately, recently developed microfluidic techniques for manipulating picoliter to nanoliter volumes have helped to overcome these issues[37,38]. Furthermore, the last few decades have seen significant improvements in MS -omics data analysis tools, sample preparation, and large increases in MS analyzer sensitivity making MS-enabled single-cell -omics feasible.[35,37,39] Nanoflow reverse phase liquid chromatography (RPLC) and capillary electrophoresis (CE) are most common liquid phase separation methods for single-cell omics. Detection of peptides from as little as 700 zmol have been demonstrated with CE-ESI, [35] and nanoflow RPLC has been

demonstrated down to 30 zmol.[40] In some demonstrations, *ex situ* MS-enabled multiomics can be performed directly on single-cells, thereby avoiding issues related to analyte loss during handling.

One such technique that has utilized sorted cells directly is proximity ligation assay for RNA (PLAYR), a targeted RNA and protein technique that permits simultaneous measurements through mass cytometry (**Figure 3b**).[41] PLAYR utilized permeabilized cells labeled with metal-conjugated antibodies and metal-conjugated oligonucleotides to simultaneously quantify targeted protein and mRNA molecules, respectively. Generally, CyTOF multiplexing is limited to a maximum of 50 targets. Another multimodal study utilized specialized microwells with polyethylene glycol-modified surfaces to divide metabolites and proteins from smaller single-cells through differential adsorption for downstream non-targeted analysis by ESI-MS.[42] This strategy enabled quantitation of 132 metabolites and over 1,200 proteins from same single-cells.

The combination of non-targeted scRNAseq with non-targeted MS-based scProteomics has also been an area of recent development. Two such techniques have been demonstrated thus far: single-cell simultaneous transcriptome and proteome (scTAP)[43] analysis and nanodroplet splitting for linked-multimodal Investigations of trace samples (nanoSPLITS)[44]. scTAP has been demonstrated with single oocytes by splitting the cell lysate using a capillary probe. This enables half of the lysate to be analyzed with LC-MS/MS proteomics and the other half to be analyzed with RNA sequencing. With the scTAP technique 19,948 genes and 2,663 proteins on average could be identified from single mouse oocytes. However, throughput of scTAP is limited and has not been demonstrated in cultured or primary cells, which are considerably smaller in size and molecular content than oocytes. Alternatively, nanoSPLITS was also recently developed for co-measuring the transcriptome and proteome from same single-cells (**Figure 3c**).[44] The nanoSPLITS approach is accomplished by sorting single-cells onto a nanodroplet array that contain a mild permeabilization buffer, after which a separate array of blank droplets without cells is brought in proximity to enable merging and splitting of the cell lysates, thus allowing for parallel scRNAseq and scProteomics. The nanoSPLITS approach confidently identified and quantified ~5000

genes (using scRNAseq Smart Seq) and ~3000 proteins (using nanoPOTS-based LC-MS proteomics) from cultured single-cells.[44]

Prospects and Conclusion

MS-enabled multimodal studies performed on same single cells or same tissue sections are still in their infancy. When considering MS-only multimodal studies, *in situ* MSI approaches have seen considerably more single-cell or near single-cell applications relative to *ex situ* MS techniques. As MSI retains spatial context, this begs the question why are *ex situ* MS techniques needed? One answer lies in the quantitative limitations of MALDI (e.g., ionization biases across all ionizable molecules),[45] and the need for intermediate antibodies or probes for other MSI techniques. It is also interesting to note that many MSI multiomic studies rely on consecutive tissue sections to measure different modalities. As each tissue section is essentially composed of different cells, many of the casual relationships between biomolecules will be lost. Furthermore, image alignment and co-registration are non-trivial, further reducing the connectedness between modalities. Fortunately, recent studies have begun to optimize and integrate disparate sample preparation methods to achieve multimodal measurements on the same tissue section[26–28]. We anticipate this trend will continue to grow, and ideally obviate the need for consecutive tissue sectioning in the future for performing multiomics.

Figures 2a and **3a** suggest that connections between some modalities are more represented than others. Particularly for *ex situ* MS approaches, multimodal lipid measurements are underrepresented. One reason may be that *ex situ* techniques enabling single-cell lipidomics only recently become available. [46,47] It is also noteworthy that no studies have yet accomplish measuring all three major classes of biomolecules in a non-targeted manner from the same single-cell using MS methods alone or in combination with other methods. Such a feat, if performed in high enough throughput, would undoubtedly prove useful for modeling of cellular processes. Conceivably, techniques that sample a portion of the cellular contents (such as scTAP, nanoSPLITS, and microsampling) could be extended to include additional modalities. The MS-enabled non-targeted scRNAseq approaches are also notable due

to the utility of transcriptomics data with assigning cell types in heterogenous populations. The past few years have seen an explosion in large scale scRNAseq studies that have provided millions of cells for reference mapping across almost all major organs in human and mouse, and even whole model organisms such as *C. elegans*.[48,49] In cases where such data is available, these reference maps provide incredible power for discriminating cell types due to the clustering across large, diverse populations of cells which often cannot be easily accomplished in other modalities such as proteomics. Furthermore, by relying on one modality (e.g., transcriptomics) to cluster and determine cell types, the other modality is free from statistical "double dipping" that inflates false positives when trying to cluster and perform differential expression analysis on the same data.[50]

In this Review, we have focused on single-cell or near single-cell MS-enabled multimodal studies alone or in combination with orthogonal techniques for comprehensive understanding of biological systems. MS-based approaches provide excellent orthogonality to sequencing in the context of discovery-based experiments at the single-cell level, and studies discussed in this review have successfully exploited these different approaches to great effect. As this field is still in its infancy, we anticipate the next decade will experience significant progress and innovation leading to novel discoveries enabled by MS-enabled single cell multiomics.

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