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Single cell metabolomics using mass spectrometry: Techniques and data analysis

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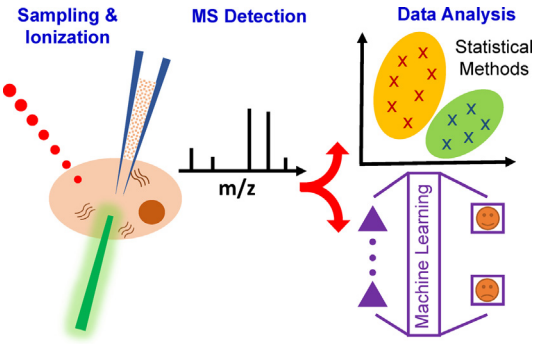
HIGHLIGHTS

- Recent experimental techniques for single cell mass spectrometry metabolomics.
- Current single cell metabolomics data analysis methods.
- Prospects of future developments in experimental technologies and data analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Mass spectrometry (MS) based techniques are gaining popularity for metabolomics research due to their high sensitivity, wide detection range, and capability of molecular identification. Utilizing such powerful technique to explore the cellular metabolism at the single cell level not only appreciates the subtle cell-to-cell difference (i.e., cell heterogeneity), but also gains biological merits corresponding to individual cells or small cell subpopulations. In this review article, we first briefly summarize recent advances in single cell MS experimental techniques, and then emphasize on the single cell metabolomics data analysis approaches. Through implementation of statistical analysis and more advanced data analysis methods, single cell metabolomics is expected to find more potential applications in the translational and clinical fields in the future.

1. Introduction

Metabolomics, as a novel branch of current "omics" family, becomes an emerging field that explores the nature of biological entities. Studies in this field include identifying metabolites, measuring metabolites' abundances, revealing the dynamics of biological activities, and ultimately illustrating metabolic mechanisms [1–3]. To date, metabolomics studies have been conducted using a variety of different approaches, primarily based on two

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prevalent platforms: nuclear magnetic resonance (NMR) and mass spectrometry (MS). In spite of the nondestructive detection and high reproducibility demonstrated using NMR [4–6], most metabolomics research relies on MS platforms due to their unique features, including high detection sensitivity, broad detection range, and the power of molecular structure identification. In these regards, MS based metabolomics is gaining popularity, and numerous articles have been published to elaborate both the "untargeted" [7,8] and "targeted" [9,10] approaches using liquid chromatography-MS (LC-MS) to analyze metabolites from difference sources and further identify and quantify molecules of interests [11–13]. In addition, as a subset of metabolomics, lipidomics is of great interest in many studies of the structure, function, and energy of cell, human health, and diseases [14–16]. Similar to metabolomics, lipidomics studies are predominately performed using LC-MS analysis for both qualitative and quantitative analysis [17,18].

Cells are essential components of living organisms. Cellular metabolome represents the downstream products of genome, transcriptome, and proteome, and results from these studies carry valuable biological information [19,20]. Given their intrinsic complex biological nature, cells are commonly used as the model systems in numerous metabolomics studies [21–23]. Despite the success of the research based on the conventional LC-MS platforms, those results are concluded from populations of cells. However, each cell is an individually functional unit, and subtle cell-to-cell difference (i.e., cell heterogeneity) has been realized to be responsible for critical biological process such as the development of drug resistance [24–26], induction of tumor metastasis [27–29], and determination of cell fate [30,31]. Due to the presence of cell heterogeneity in all cell populations, the collectively measured results in traditional studies cannot represent the characteristics of individual cells [32,33]. In addition, rare cell types (e.g., circulating tumor cells, cancer stem cells, and antigen-specific T cells) exist at low abundances among homogenous population; however, they play important roles in disease mechanisms, immune responses, and angiogenesis [34]. Traditional bulk analysis cannot provide meaningful results of rare cell types due to their low populations. Therefore, metabolomic analysis at a higher resolution, the single-cell level, becomes necessary, and using MS for single cell metabolomics studies is an inevitable choice. In general, a typical single cell MS metabolomics study includes multiple steps such as sample preparation, MS measurement, and data analysis. In this review, we intend to cover these aspects based on recent advancement of both technology development and data analysis.

2. Single cell sampling and ionization techniques

In single cell MS studies, sampling analytes from a single cell is one of the most challenging steps due to the size of a cell (typically several tens of micrometer in diameter for a mammalian cell) [35,36], limited volume of cellular contents [37], intrinsic complexity of intracellular species [38], and rapid turnover of cellular metabolites [39,40]. Thus, an effective sampling technique generally requires high magnification microscope for monitoring cells, precisely controlled mechanical system for sample motion, and efficient extraction or desorption method for cellular content acquisition. The acquired cellular species are then ionized by different mechanisms for MS analysis.

Up to date, a collection of techniques have been reported to sample and ionize molecules from individual cells in both vacuum and ambient conditions. According to their sampling approaches, these techniques are based on ion beam [41,42], laser [43–45], probes [46–49], microfluidic devices [50–52], and other methods (Fig. 1) [53,54]. Sampled cellular molecules are ionized (e.g., by ion beam or laser) during the desorption process or after the sampling step (e.g., by ESI) for qualitative and even quantitative studies. In addition to the development of sampling and ionization techniques, versatile approaches have been investigated to incorporate separation processes and chemical reactions of metabolites prior to MS analysis. More detailed descriptions of the mechanisms of reported methods are summarized in previously published review articles [36,55–59], and these techniques have been widely applied to probing the metabolomic profiles of single cells [60,61], visualizing cell heterogeneity [62], quantifying compounds of interest [63,64], localizing cell subpopulations [65], and investigating certain biological pathways [31,66]. Here, we will cover specific examples in each category of techniques to demonstrate their features and recent advancements.

2.1. Vacuum-based methods

Currently, there are two major types of mechanisms (i.e., ion-beam and laser) for sampling and ionization under vacuum environment. The sampling and ionization processes of these techniques simultaneously occur. The ion-beam based techniques, such as time-of-flight secondary ion mass spectrometry (TOF-SIMS) and nanoscale SIMS (nanoSIMS), can achieve high spatial resolution (100 nm−1 μm) [67]. Because these techniques provide high spatial resolution and excellent sensitivity, they are powerful tools for single cell imaging and subcellular analysis [68,69]. Utilizing TOF-SIMS, the Winograd group demonstrated the identification of neuronal lipids through tandem MS (MS/MS), and further depicted the distribution of multiple species, including PC (16:0e/18:1), vitamin E, and cholesterol from single neurons [70]. Furthermore, recent advancement of TOF-SIMS methods enabled 3D imaging of single cells with more than 500 nm lateral depth. In a particular study, the spatial localizations of a drug target, amiodarone, and other co-localized species in single cells were mapped in a 3D space by Passarelli et al. [41] However, SIMS-based methods are relatively more energetic, and large number of fragments are generally produced, resulting in challenges in data analysis of most biomolecules. As a major laser-based technique, matrix-assisted laser desorption-ionization (MALDI) MS relies on interactions between laser beam and organic matrix molecules to initiate desorption and ionization of analytes, and it is commonly used in single cell MS experiments. This technique has excellent tolerance for salts and provides high sensitivity and throughput measurements [71,72]. In addition, combined with flash-freezing sample preparation method, MALDI-MS can provide high-fidelity results reflecting the native distribution of cellular analytes [73]. The Sweedler group employed MALDI-MS to screen over 1500 single cells, and discovered multiple cellular lipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), that are significantly different between GFAP- and NF-L-positive cells [44]. Another example by the Lee group demonstrated the distribution of cellular lipid species in individual zebrafish embryos [71]. In recent advancement, matrix-free laser-based ionization techniques have been applied to analyze molecular species within single cells. Siuzdak's group utilized Nanostructure-initiator MS imaging (NIMS) to explore change of cellular metabolism by monitoring xenobiotic metabolites after docetaxel treatment in lymphoma cells [74], and Vertes' team successfully applied nanopost array-laser desorption ionization (NAPA-LDI) to detect over 80 unique species in lamellipodia, a subcellular compartment of human hepatocarcinoma cells [75]. In a recent report, Sweedler and co-workers demonstrated that a MALDI/C_{60}-SIMS hybrid Q-TOF instrument can achieve 1 μm of spatial resolution to imaging multiple lipid species in cultured neurons [76]. Despite their mature instrumentation and broad applications, the vacuum working

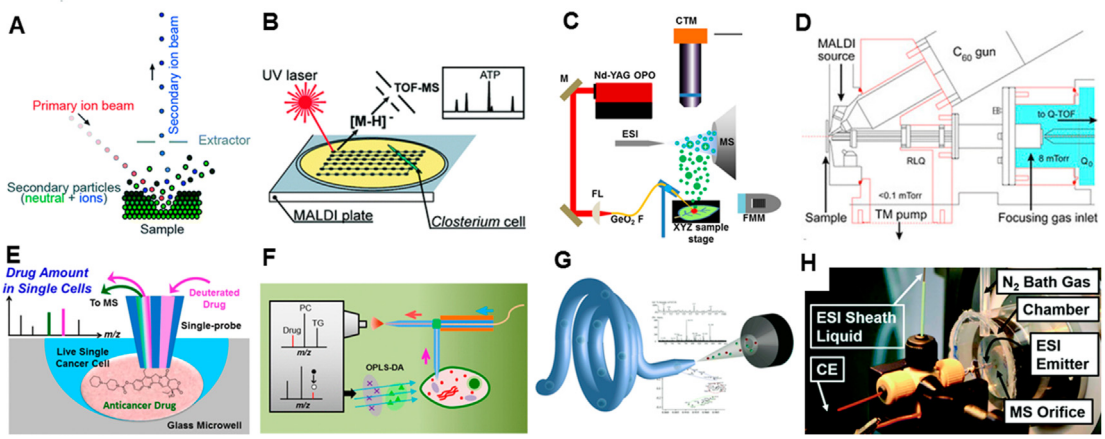

Fig. 1. Examples of single cell MS metabolomics techniques. (A) Secondary ion MS (SIMS). Reproduced from Ref. 42, with permission from The Royal Society of Chemistry. (B) Matrix-assisted laser desorption/ionization (MALDI) MS. Reproduced from Ref. 43, with permission from American Chemical Society. (C) Laser ablation electrospray ionization (LAESI) MS. Reproduced from Ref. 45, with permission from Frontiers Media. (D) MALDI/C_{60}-SIMS hybrid Q-TOF platform. Reproduced from Ref. 76, with permission from American Chemical Society. (E) Single-probe MS. Reproduced from Ref. 63, with permission from American Chemical Society. (F) T-probe MS. Reproduced from Ref. 49, with permission from American Chemical Society. (G) Dean flow assisted cell ordering system. Reproduced from Ref. 62, with permission from The Royal Society of Chemistry. (H) Major components of CE-ESI-MS interface. Reproduced from Ref. 31, with permission from The Royal Society of Chemistry.

conditions for SIMS- and MALDI-based methods require complex sample preparation, which may affect the chemical compositions of cells compared with those in the native biological environment.

2.2. Ambient methods

In contrast to vacuum-based methods, ambient sampling techniques can largely preserve the cellular microenvironment when sampling *in situ*. This unique feature becomes increasingly advantageous for metabolites with rapid turnover rate [36]. Due to the flexibility in ambient working conditions, a large number of innovative methods have been developed. In general, these techniques have separate steps of sampling and ionization. Sampling cellular molecules can be conducted using laser, probes, or microfluidics, whereas ionization mechanisms are primarily based on nano-electrospray ionization (nanoESI).

Laser ablation electrospray ionization mass spectrometry (LAESI-MS), which utilizes IR laser for desorption and ESI for ionization, represents a well-known laser-based method. The Vertes' group used LAESI-MS to detect and identify cellular metabolites, such as monosaccharide, disaccharide and trisaccharide, from *A. Cepa* cells [77]. Furthermore, the same group employed LAESI-MS for subcellular analysis, and significant difference of lipid profiles was observed between the animal and vegetal poles of *Xenopus laevis* eggs [78].

Probe-based methods have a more variety of designs. For example, the Lanekoff group employed nano-desorption electrospray ionization (nano-DESI), which utilizes a microscale liquid junction between two fused silica capillaries for analyte extraction, to detect lipids and amino acids from single human cheek cells. The Yang group introduced multiple devices, such as the Single-probe and T-probe [46,49], to detect intracellular metabolites as well as drug compounds from single cells in their native microenvironment. Particularly, the Single-probe was recently used to quantify xenobiotics, such as antitumor drug compound, in the single cells residing in microwells [63], and the T-probe was modified to analyzed live non-adherent cells [61].

Microfluidics-based methods take the advantage of microfabrication techniques to design microscale devices that allow for higher throughout analysis. The Lin group designed a Dean flow assisted cell ordering system to detect multiple cellular lipids, including PC and PE, and further distinguished the difference of cellular metabolomic profiles between different types of cells [62]. In another example, a drop-on-demand inkjet printing device was fabricated and applied to analyze single cells from cell suspension, and the cellular profiles of four different types of cells were compared [79].

Despite a variety of sampling techniques that directly extract cellular contents from single cells of interest followed by immediate analysis, molecular separation or cell sorting has been incorporated in a number of studies. For example, capillary electrophoresis (CE) has been coupled to microdissection and microextraction to enable online single cell analysis. The Nemes group successfully detected and identified more than 80 of unique metabolites such as amino acids from *Xenopus* embryos [80]. The same group further optimized the solvent composition involved in the microextraction procedure to achieve detections with higher selectivity of metabolites [66], and utilized dual-polarity to increase metabolite coverage [31]. Alternately, ion mobility separation (IMS) can be seamlessly coupled to the sampling process to alleviate the influence of intrinsic matrix effect, which is caused by the complexity of cytoplasm, on detection sensitivity as well as differentiate isomers, promoting more sensitive detection and accurate identification of cellular species. As a result, twice as many species can be detected from *A. thaliana* cells through orthogonally coupled IMS than conventional ESI-MS [81]. It is also worth noting that both CE-MS and IMS-MS have advantages in identifying detected cellular species due to a second dimension of information (i.e., migration time for CE and collision cross section for IMS) in additional to merely *m/z* values. Involving separation in single cell metabolomics studies becomes advantageous as more data are being collected to construct larger databases. In addition to the separation of molecular species, cells can be selected for analysis. For example, fluorescence-activated cell sorting (FACS) has been conventionally used to separate certain type of cells according to their surface protein markers. The Connolly group utilized this method to measure cellular metabolic profiles of human peripheral blood mononuclear (PBMC) cells, and compared abundances of metabolic transporters before and after glycolytic inhibition [82]. However, the process of FACS introduces excessive stress to the examined cells and alters the metabolomic dynamics in single cells [83].

Due to the complex chemical compositions and extremely limited sample amounts, single cell MS studies are generally focused on qualitative analysis. With precise control of the sampling process, several ambient techniques have been reported for quantitative single cell studies. The Laskin group demonstrated electroosmotic extraction of 2–40 pL of cytoplasm from *A. Cepa* cells followed by quantification of glucose (3.2–16 mM) using nanoESI [47]. In addition, quantification of selected lipid species in human cheek cells have been measured using nanoDESI, and the amount of phospholipids can vary between 100 and 300 fmol in a cell [64]. Also, the anticancer drug uptake in single HeLa and HCT-116 cells was quantified using the Single-probe, and the results obtained at the single-cell level were systematically compared with those obtained from cell lysates prepared using populations of cells [63].

To further increase detection sensitivity and acquire more structural information, chemical reactions with single cells can be conducted prior to MS analysis. The Huang group combined CBT-Cys click reaction with induced nanoelectrospray ionization (InESI) to derivatize cellular species *in situ* with low response, and therefore enhanced detection sensitivity [84]. Recently, the Yang group introduced a micropipette needle to initiate Paternò-Büchi (PB) reaction of unsaturated lipids in non-adherent cells, and further assigned the carbon-carbon double bond positions [85]. In another example, the Guo group performed on-probe derivatization of fatty alcohol and sterol metabolites extracted from single L-02 and HepG2 cells, and further induced quaternization reactions to convert molecules with low ionization efficiencies into pre-charged species for enhanced detection sensitivities [86].

3. Single cell metabolomics data analysis

For most single cell metabolomics studies, the acquired raw data, consisting of all detected species and their corresponding ion intensities, are tremendous in size and complex in nature. Therefore, a systematic data analysis approach is required to eliminate redundancy and confusions while retaining the underlying biological information. Different from conventional LC-MS metabolomics, the data analysis methods are not standardized in current single cell metabolomics studies, and individual research groups may have their preference of using different data analysis pipelines. This review will focus on critical steps from reported data analysis approaches to uncovering the biological information from single cell metabolomics datasets. Conventionally, LC-MS based metabolomics data analysis involves multiple steps, including data conversion, normalization, scaling and transformation, feature selection, biomarker determination, and metabolite annotation [87,88]. Although those concepts and practice still apply to the single cell metabolomics data analysis, adaptions and customizations are generally needed. Thus, the workflow of current single cell metabolomics data analysis primarily includes data pre-processing, univariate analysis, multivariate analysis, and more advanced data analysis, if applicable. Together, they are integrated into multiple software packages that have been used by different labs to promote deeper understanding of cellular metabolism. However, due to the characteristics of single cells (e.g., extremely limited amounts of analytes, complex chemical compositions, and heterogeneous cell populations), ion signals obtained from single cells generally have relatively low signal-to-noise ratio. Thus, ones should be cautious when analyzing their data.

3.1. Data pre-processing

Single cell metabolomics data pre-processing is a comprehensive procedure to extract useful metabolomic information from the raw data (Fig. 2) [89]. Although different data pre-processing approaches have been reported, this review will cover essential steps commonly involved in most studies.

First, noise and background signals need to be removed. Both random noise and background signal from ambient environment may interfere with the "true" metabolomic fingerprints of single cells. To alleviate such interference, the random noise generated by the instrument can be excluded by filtering all acquired signals with a set threshold. Because most of current signal cell metabolomics studies are conducted using high-resolution MS platforms (e.g., Orbitrap, Q-TOF, and FT-ICR), this step eliminates a majority of MS signals not corresponding to cellular metabolites. However, cautions should be taken when choosing the noise threshold. Unnecessarily high thresholds may discard useful metabolomic information. In other words, peaks with ion intensity lower than the set threshold will be treated as noise and then discarded, resulting in information loss of metabolites with low abundances or ionization efficiencies. On the contrary, excessively low threshold may include noise and sporadic ion signals, which will lead to data redundancy. In addition to noise removal, background signals need to be eliminated. Generally, a "blank" sample (e.g., fresh cell culture medium or solvents) containing molecules simultaneously detected along with cellular contents can be analyzed using the same experimental setup, and the acquired ions are then treated as background signals and subsequently subtracted from the raw data. However, one should be aware that molecules present in the culture medium can be accumulated or metabolized by cells, and therefore, potential bias may be introduced when completely eliminating these species and their metabolites when they are of interest. After noise removal and background reduction, the dimensionality of the datasets can be tremendously reduced. The remaining peaks that correspond to cellular metabolites can be subjected to the second step, normalization and peak alignment.

Second, normalization of ion intensity needs to be conducted, and the normalized ion intensities can then be correlated to their relative abundances among different cells. In the current single cell metabolomics studies, each peak is either normalized to the total ion counts (TIC) of all detected metabolites or the most abundant peak detected (the base peak). Similar to LC-MS/MS metabolomic data analysis, all normalized peaks are then aligned across all examined cells to reflect both mutual and unique species present. In addition, internal standard (e.g., isotopically labeled analog) can be used for normalization to quantify the absolute amounts of molecules in single cells [47,63,64,90].

Last, to reduce resource demand for data analysis and avoid misleading results, it is necessary to select metabolites that can be commonly detected from majority of single cells. Typically, only those metabolites that present in most of cells are included and subjected to following statistical analysis. A variety of software packages, although a majority of them were originally designed to perform the conventional LC-MS/MS metabolomics, can be adapted to perform these tasks for the single cell metabolomics datasets. Examples of popular tools include, but not limited to, MZmine (http://mzmine.github.io/) [91], MetaboAnalyst (https://www.metaboanalyst.ca/) [92], Geena2 (http://bioinformatics.hsanmartino.it/geena2/) [93], NOREVA (http://idrblab.cn/noreva/) [94], MetAlign (https://www.wur.nl/en/show/MetAlign-1.htm) [95], mMass (http://www.mmass.org/) [96], and other customized packages written in R (e.g., MALDIquant [60]) and Python (e.g., microMS [97]). After data pre-processing, complex raw data become simplified data matrices that consist of the detected cellular metabolites along with their normalized intensities. Additional data scaling and transformation may be conducted to smooth the datasets, and potentially reduce the effect from extreme "outliers" (i.e., metabolites with extremely high or low abundances).

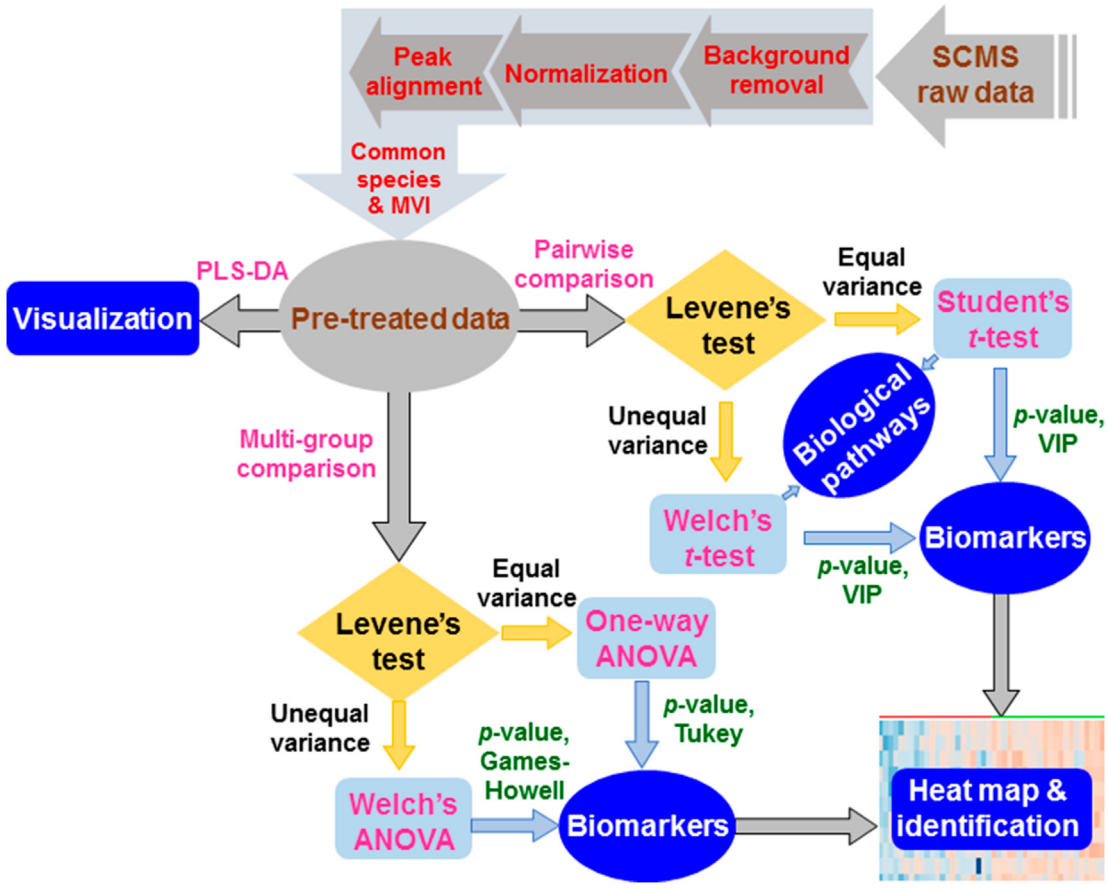

Fig. 2. A generalized single cell metabolomics data analysis workflow that consists of raw data pre-processing, univariate analysis and multivariate analysis. Pathway enrichment analysis may be performed based on significantly altered cellular metabolites (biomarkers) as needed. Reproduced from Ref. 89, with permission from Elsevier.

3.2. Univariate analysis

To reveal the change of cellular metabolites corresponding to certain biological process (e.g., drug treatment and environmental stress), statistical analysis that focuses on one variable at a time (i.e., univariate analysis) is needed. This approach discerns the difference (i.e., upregulation or downregulation) of metabolites, and it has been constantly performed in single cell metabolomics studies to discover potential metabolomic biomarkers, which are characteristics of groups of cells with certain biological traits. Similar to conventional LC-MS/MS metabolomic data analysis, *t*-test is broadly used to locate those metabolites with significantly different relative abundances between two groups. The resulting metabolites with positive test results (i.e., test p-value < 0.05) are regarded as metabolomic biomarkers. For example, the level of glutathione (GSH)/oxidized glutathione (GSSG) is significantly different following two dissection methods of single embryos, which may further indicate different cellular reaction mechanisms towards such environmental stress [53]. A collection of lipids in single algae cells, including phosphatic acid (PA), phosphoethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI) phosphatidylcholine (PC), monoglycerides (MG), diglycerides (DG) and triglycerides (TG), have significantly different abundances in light or dark environment. Such observation is likely related to the availability of nutrition [98]. Some lipid species, such as PC 34:1, PC 34:2, PC 36:2, PC 36:3, possess significantly different abundances in single cells obtained from different donors, and such differences may be attributed to different diets [64]. It is worth noting that data distribution needs to be evaluated prior to univariate analysis. Specifically, datasets having Gaussian or near-Gaussian distribution (determined by data normality tests) [99] may be subjected to parametric *t*-test (Student's *t*-test); whereas those having skewed distribution may be subjected to non-parametric *t*-test (Welch's *t*-test) instead [89]. When exploring complex systems with more than two groups of single cells, analysis of variance (ANOVA) is the statistical method of choice to reveal biomarkers among multiple study groups. Similar to *t*-test, data normality needs to be thoroughly examined before conducting either parametric (i.e., One-way) or non-parametric (i.e., Welch's) ANOVA. It is worth noting that biomarkers resulting from ANOVA correspond to all groups of cells rather than two specific groups of cells.

3.3. Multivariate analysis

Although univariate analysis is a powerful and predominant approach to discovering metabolomic biomarkers, it inherently lacks the capability to collectively handle metabolomic profiles from all cells. Multivariate analysis can handle the overall metabolomics profiles of single cells through the analysis of all variables (i.e., metabolites and their abundances). The principle of any multivariate analysis method is projecting the high dimensional raw data into lower dimensional space (e.g., 2D or 3D space) with preservation of most information using fewer numbers of variables. Such manner reduces the dimensionality of the raw data, but the key information reflecting biological functions in cells can be retained. Additionally, one can visualize the complex cellular metabolomic profiles in a 2D or 3D space, which enables intuitive

visualization of cell heterogeneity.

There are two types of multivariate analysis, namely, unsupervised and supervised methods (Fig. 3). Unsupervised methods, such as k-means clustering, Principal Component Analysis (PCA), and *t*-Distributed Stochastic Neighbor Embedding (*t*-SNE), reduce the dimensionality of the original data by grouping objects with higher similarities in the high dimensional space through linear or non-linear fashion. On the contrary, supervised methods, such as partial least squares discriminant analysis (PLS-DA), orthogonal projections to latent structures discriminant analysis (OPLS-DA), and random forest, require prior knowledge of the group attributes of single cells. The grouping information will be used to properly assign group labels for all subsequent single cells in the training data set.

Due to this fundamental difference between these two types of methods, supervised methods offers more definitive clustering results, which become critical when the induced change of cellular metabolism is subtle compared with intrinsic cell heterogeneity (regarded as "noise") [100]. However, as bias may be introduced when constructing supervised models, it is imperative to validate each supervised model using cross-validation and permutation tests. A robust supervised model with a small permuted *p*-value (e.g., <0.05) may truly suggest significant difference between two groups of single cells. On the contrary, unsupervised methods present unbiased group clustering, and additional model validation is not required.

Currently, both types of multivariate analysis have been applied to single cell metabolomics studies as described in the following examples. PCA was conducted to visualize the metabolomic profiles of osteosarcoma cells and normal human osteoblasts after mannose stimulation [101], and it was also used to demonstrate the metabolomic difference of bacteria infected and uninfected plant cells [102]. *t*-SNE was performed to present the discrimination of four distinct subtypes of breast cancer cells, BT-474, MCF7, MDA-MB-468, and SK-BR-3 [103]. PLS-DA was applied to distinguish cancer stem cells (CSCs) and non-stem cancer cells (NSCCs) based on cellular metabolomic profiles acquired from positive and negative ion modes [104]. OPLS-DA was conducted and clear discrimination was observed for two groups of single cells [105].

3.4. Biological variance vs technical variance

Although the statistical methods mentioned above can be used to compare abundances or overall profiles of metabolites in cells from different groups, caution needs to be taken when studying biological variance of cells under the influence of technical variance at the single-cell level. Similar to other analytical techniques, it is necessary to investigate the biological variance (e.g., due to cell type difference, cell heterogeneity, or environment variation) under the influence of technical variance (e.g., fluctuation of ion signal due to inconsistent ionization efficiency, variability of the experimental process, and run-to-run instrumental variation) at the

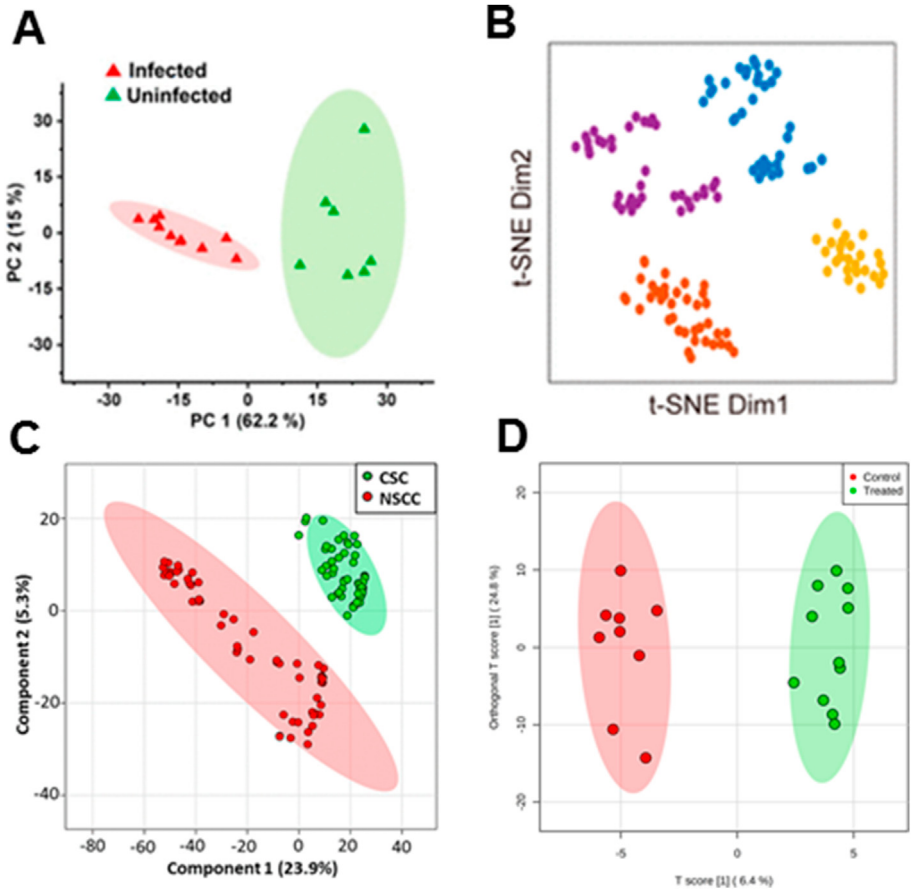

Fig. 3. Examples of multivariate analysis methods for single cell metabolomics data analysis. (A) PCA to visualize two groups of single cells with or without bacterial infection. Reproduced from Ref. 102, with permission from American Chemical Society. (B) *t*-SNE to visualize multiple subtypes of single breast cancer cells. Reproduced from Ref. 103, with permission from American Chemical Society. (C) PLS-DA to visualize the difference of cellular metabolomic profiles of cancer stem cells and non-stem cancer cells. Reproduced from Ref. 104, with permission from American Chemical Society. (D) OPLS-DA to visualize the difference of control (untreated) and antitumor drug treated single cells. Reproduced from Ref. 49, with permission from American Chemical Society.

single-cell level. However, this becomes more challenging in single cell studies due to multiple factors, including extremely limited amount of materials, complex composition of analytes, intrinsic cell heterogeneity, rapid turnover rate of metabolites, and relatively larger influence from technical variance. Particularly, repeated experiments using the same cell cannot be performed, as single cell MS techniques are generally invasive, and results from individual cells are intrinsically irreproducible. These challenges can be largely alleviated through carefully designed experiments and comprehensive data analysis [45,65,106]. First, measurements of all cells in different groups should be accomplished as closely as possible to avoid unnecessary biological variance of samples [89,104,106,107]. Second, the relative standard deviation (RSD) of cellular species in single cell MS experiments can be utilized to represent the combined technical and biological variance. Third, solutions containing standard compounds need to be measured using the same single cell MS experimental setup, and the calculated RSD of ion intensities of these compounds can be used to evaluate technical variance. Last, sizes of these two types of RSD will be compared, and a significantly larger RSD from the single cell MS measurement indicates a valid biological variance [45,65,106].

3.5. Molecular identification

The molecular identification in single cell MS experiment is challenging, primarily due to limited sample amount and complex composition of contents within single cells. Although mass scan combined with database searching (e.g., METLIN [108] and HMDB [109]) can provide potential labels of ions, MS/MS analysis is necessary to acquire more reliable identifications. Different from traditional bulk analysis, in which adequate sample amount allows for detailed MS/MS analysis of a large number of ions, identifying ions of interest from single cells is nontrivial. A number of approaches have been developed in previous studies. Online MS/MS has been applied to directly identify species from single cells, particularly for those relatively abundant ions such as phospholipids and certain metabolites [49,104]. In addition, reactions can be carried out for single cells to enhance molecular detection and identification. For example, dicationic reagents, such as $[C_5(bpyr)_2]^{2+}$ and $[C_3(triprp)_2]^{2+}$, have been introduced to the sampling solvent to detect and identify negatively charged species (e.g., AMP and palmitic acid) in positive ion mode with significantly improved sensitivity [110]. In another example, PB reactions are induced by UV irradiation to determine the locations of C=C bonds in unsaturated lipids assisted by R programs [85]. Second, single cell MS scan can be combined with traditional LC-MS. In this approach, single cell MS scan results from different groups are analyzed using statistical methods (e.g., *t*-test, ANOVA, and multivariate statistical models) to generate the list of ions of interest (e.g., those with significantly different abundances). Cell lysates are then prepared for LC-MS experiments, and MS/MS analysis is focused on ions of interest, including those with low intensities in single cell mass spectra. Third, separation can provide orthogonal information to assist identification. Molecular separation can be performed before MS ionization. For example, CE provides excellent separation of small amounts of samples. Coupled with MS, CE-ESI-MS has been utilized for single cell studies. Both temporal information (i.e., migration time) and molecular information (i.e., *m/z* value) are obtained for the parent ions for more confident identification. For example, serotonin and acetylcholine were identified in single MCC and R2 neuron, respectively, using this technique [111]. Separation can be also achieved after molecular ionization. As an example of post-ionization separation, ion mobility separation (IMS) offers a second dimension of molecular information (i.e., collision cross-section value). For example, ESI-IMS-MS has been successfully utilized to identify over 50 unique species in mitotic HepG2/C3A cells [65].

3.6. Biomarker and pathway analysis

Metabolic pathways link complex chemical reactions of metabolites within a cell. Discovery of metabolite biomarkers is extremely useful to further compute the biological pathways involved in the biological process. Although *t*-test is widely used as a common method to locate biomarkers, other statistical methods have been explored and applied. Among them, the loading score of multivariate analysis [106,112] and the VIP (variable importance on projection) obtained from statistical models have been utilized to determine metabolite biomarkers. In the studies of young and old algae cells, pigments fucoxanthin, Chl α, carotene, and alloxanthin were designated as metabolomic biomarkers of age-related biological process by ranking their VIP scores of a PLS-DA model [60]. In addition, pathway analysis tools, which are originally developed for classic LC-MS metabolomics studies, can be extended to single cell metabolomics. For example, by mapping the discovered biomarkers against known metabolic pathways (e.g., Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolomic database), it was found that arginine–proline metabolism and glutathione metabolism were enriched in certain regions of *Xenopus* embryos by retrieving cellular metabolites with different solvents [66]. In addition, our previous work indicates biopterin metabolism, glycerophospholipid metabolism, bile acid biosynthesis, *de novo* fatty acid biosynthesis were significantly altered after treating single HCT-116 cells with anti-tumor compounds, paclitaxel and vinblastine. The pathway enrichment analysis was performed using Mummichog, which only utilizes measured accurate *m/z* values (not identified metabolites) [89].

3.7. Advanced data analysis

Although univariate and multivariate analysis are extensively used in current single cell metabolomics research, more advanced data analysis approaches have been recently introduced. For example, artificial intelligence (AI) has been applied to process single cell metabolomics data due to its high efficiency. AI relies on computational resource to “teach” models through a variety of algorithms, and gradually approaches the underlying complex nature of the studied object, which is usually difficult to explore by human, during each interaction of “training”. Among all AI methods, machine learning (ML) has been utilized to analyze single cell metabolomics data. By incorporating conventional statistical analysis with advanced algorithms, complex mathematical models can be established. These models can be generally classified into supervised and unsupervised models. To date, supervised ML methods have been used in single cell metabolomics studies. In those models, the acquired datasets are divided into two sets: the training set and the validation set. The training set is used to construct different ML models using a variety of strategies, including random forest (RF), support vector machine (SVM), logistic regression (LR), and artificial neural network (ANN), through an iterative fashion. The validation set is used to evaluate the model performance in predicting the group attribute of foreign objects. Provided that the training set contains sufficient numbers of single cells, the ML models could generate high predictive accuracy of the phenotype of an unknown single cell based on its integral metabolomic profile.

In a recent publication, ML models were reported to achieved high (>80%) classification accuracy between neurons and astrocytes, and the models were further evaluated using area under sensitivity-specificity curve (AUC) [113]. In another study, multiple

ML models were established and systematically evaluated to predict single cells with primary drug resistance (Fig. 4). As a result, the ANN model, which contains cellular metabolites detected from more than 40% of all examined cells, achieved high predictive accuracy within relatively short time. This model was further validated using MS data acquired from other sets of single cells with comparable performance [107]. Furthermore, ML models were constructed and applied to predict the degree (i.e., none, low, or high) of chemotherapy-induced drug resistance based on metabolomic profiles [106]. Such multi-group predictive model demonstrated high predictive accuracy in both the validation set and new batches of single cells. Interestingly, it is found that ML models containing more variables (i.e., ions and their intensities) tend to yield higher predictive accuracy. However, excessive data redundancy should be avoided to save computational time and resource. For datasets containing metabolites with low abundances, the Trace framework, developed by Nemes et al., is capable of extracting trace-level signals from high-resolution mass spectra using combined ML algorithms and advanced feature categorization [114]. As an emerging and powerful technique, ML-based single cell metabolomics approaches can be adopted to assist decision making or initiation of related mechanistic studies, and they have the potential towards future translational applications in the clinical field in the long run.

4. Future perspectives

As an emerging research field, single cell metabolomics is gaining popularity recently. Despite the success of current studies, the next generation of single cell analytical techniques are under development, likely focusing on multiple aspects. First, push the limit of detection sensitivity, and therefore broaden the detection spectrum of the cellular metabolites, especially for those with low abundances or poor ionization efficiencies. Second, investigate subcellular compartments and precisely analyze metabolites corresponding to specific organelles. Third, sample live single cells in their native microenvironment representing their living conditions. In terms of data analysis, statistical and more advanced data analysis approaches have been utilized to handle complex data matrix to generate critical biological merits. However, standardized single cell metabolomics data analysis pipeline is not available at this stage, hindering the direct comparison of experimental results between different labs. Therefore, it is necessary to construct the standardized data analysis methods that integrates essential functions for data processing. On the other hand, ML methods tremendously boosted the efficiency of single cell metabolomics data analysis, and they uncovered the underlying nature of cellular metabolism that is difficult to explore by human. Although still in the infancy, more applications of sophisticated ML models are expected towards future translational and clinical fields. Last, the data integration of multi-omics, including genomics, transcriptomics, proteomics, and metabolomics at the single cell level is receiving increasing interest (Fig. 5) [115,116]. This integration requires not only novel experimental techniques, but also new bioinformatics tools. The success of integrated multi-omics studies allows to explore the inner dynamics of cells from a more comprehensive perspective, and ultimately promote profound understanding of the biological nature of cells. As an ultimate goal, some of these advanced experimental techniques and data analysis methods will go through iterative development, and eventually become mature enough for standardization or commercialization. We can expect that, in the near future, single cell MS metabolomics will be readily

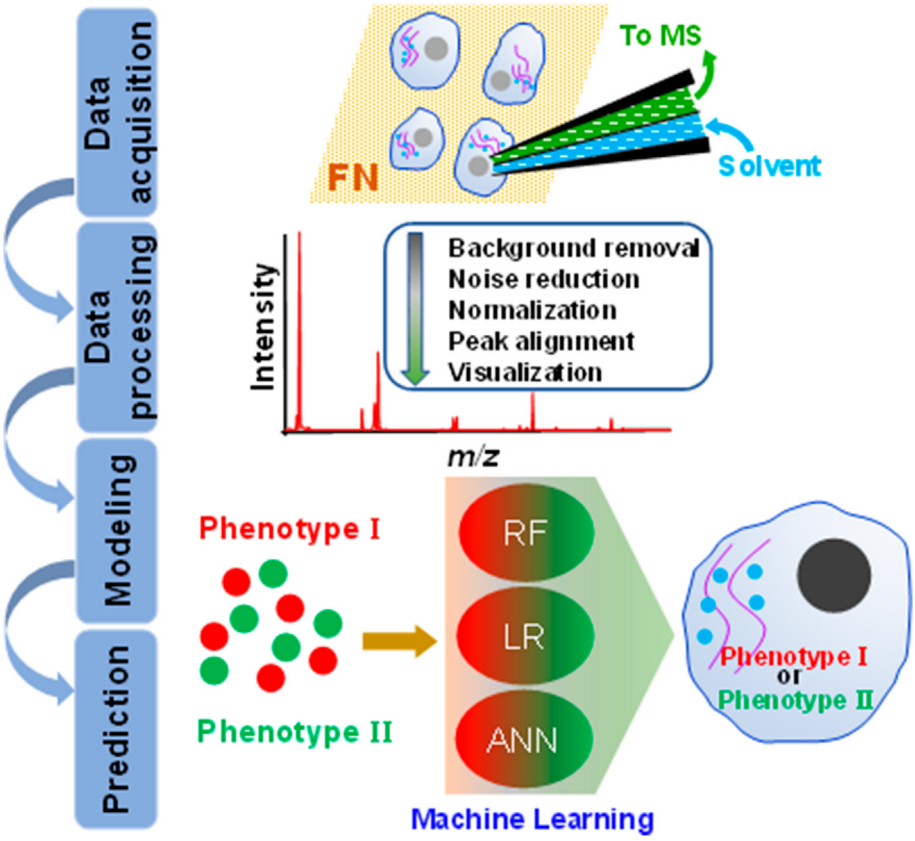

Fig. 4. Workflow of single cell data acquisition and machine learning model construction. Typical steps include data acquisition, data pre-processing, model construction, model evaluation, and model application. Reproduced from Ref. 107, with permission from The Royal Society of Chemistry.

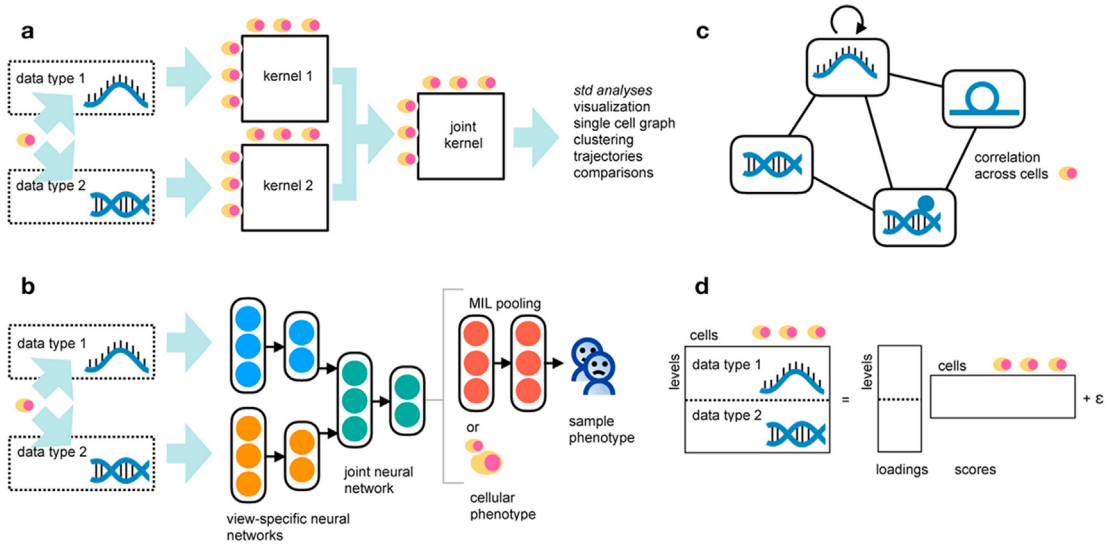

Fig. 5. An example of multi-omics data integration workflow. Machine learning models are used to efficiently integrate the data and generate grouping information of single cells. Reproduced from Ref. 116, with permission from Elsevier.

utilized, particularly by non-expert users, for a broad range of applications in fundamental studies and clinical applications.

CRediT authorship contribution statement

Renmeng Liu: Writing - original draft. **Zhibo Yang:** Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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