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CRITICAL REVIEW

Chemical tagging mass spectrometry: an approach for single-cell omics

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Abstract

Single-cell (SC) analysis offers new insights into the study of fundamental biological phenomena and cellular heterogeneity. The superior sensitivity, high throughput, and rich chemical information provided by mass spectrometry (MS) allow MS to emerge as a leading technology for molecular profiling of SC omics, including the SC metabolome, lipidome, and proteome. However, issues such as ionization suppression, low concentration, and huge span of dynamic concentrations of SC components lead to poor MS response for certain types of molecules. It is noted that chemical tagging/derivatization has been adopted in SCMS analysis, and this strategy has been proven an effective solution to circumvent these issues in SCMS analysis. Herein, we review the basic principle and general strategies of chemical tagging/derivatization in SCMS analysis, along with recent applications of chemical derivatization to single-cell metabolomics and multiplexed proteomics, as well as SCMS imaging. Furthermore, the challenges and opportunities for the improvement of chemical derivatization strategies in SCMS analysis are discussed.

Keywords Chemical derivatization · Single-cell analysis · Metabolomics · Proteomics · Mass spectrometry imaging · Lipidomics

Introduction

Cells are the basic building blocks of living organisms, and an individual cell governs the basic function of a biological system [1, 2]. Cellular heterogeneity has been recognized to be responsible for critical biological processes, such as the development of drug resistance, tumor initiation, progression, metastasis, and determination of cell fate [3, 4]. Therefore, single-cell (SC) analysis will provide meaningful data to improve our understanding of important biological processes and fundamental mechanisms of disease [5, 6]. Considering single cells are featured with the composition complexity, limited size, low concentration, and structural diversity for certain compounds, analytical technologies have surged toward improving SC sensitivity and high information coverage [7]. Mass spectrometry (MS) is growing as a powerful tool for the analyses of single cells, owing to its superior sensitivity and specificity, wide molecular coverage, quantitation, and structural elucidation capabilities [8]. Advances in MS technologies have enabled investigation of SC omics at unprecedented resolution and scale [9]. However, analytes with low ionization efficiency in single cells are not readily detected by MS. In addition, certain mass analyzers have limitations at lower mass ranges; this becomes an issue for the small-molecule detection in single cells [10]. Furthermore, the diversity of metabolite structures is immense, yielding a complex mixture of isomers and isobars [11]. These limitations make sensitive molecular characterization of certain compounds in single cells challenging.

To circumvent the above-mentioned challenges, emerging studies demonstrate that chemical tagging/derivatization

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offers a bright avenue for SC omics analysis. Chemical tagging modifies target analytes using a derivatization reagent; the labeled derivatives result in improved chemical and physical properties for MS analysis. Chemical derivatization overcomes problems associated with low ionization efficiency, poor selectivity, and compound instability, with widespread applications in many areas, such as chemistry, medicine, food science, forensic science, and environmental sciences [12]. Chemical derivatization has been increasingly adopted in single-cell mass spectrometry (SCMS) analysis to improve the detection sensitivity and acquire in-depth structural information. For instance, monobromobimane derivatization and MS-based method has been developed for sensitive detection of glutathione content in a single human erythrocyte [13]. The quaternary ammonium salt group-based charge tag was combined with click reaction to increase MS sensitivity for the detection of cysteine in single HeLa cells [14]. A micropipette needle is used to initiate the Paternò-Büchi (PB) reaction to assign the carbon-carbon double-bond positions at the SC level [15]. Chemical tags carrying diazo groups were designed to selectively react with the phosphate group on polar phosphorylated metabolites in single cells [16]. Additionally, sensitive quantitative and qualitative analysis of low-molecular-weight thiols in a single HeLa cell before and after oxidative stress treatment was obtained by chemical derivatization-assisted liquid chromatography-mass spectrometry (LC-MS) [17]. These examples demonstrated that chemical derivatization coupled with MS provides a versatile platform for sensitive interrogation of chemical components in single-cell samples.

Therefore, a suite of SCMS techniques has been developed for different types of biomolecules, including metabolites, lipids, proteins, and nucleic acids. Compared with single-cell sequencing, SC metabolomics and proteomics are less established due to the lack of amplification technologies on metabolites and proteins [18]. Thus, the development of chemical derivatization strategies opens a new avenue for SC metabolomics, multiplexed proteomics, and even SCMS imaging. Herein, we review the principles of chemical tagging strategies in SC analysis, and the applications of chemical derivatization in SC metabolomics, multiplexed proteomics, and spatial omics. Moreover, the current limitations and future directions of chemical tagging strategies in SCMS analysis are discussed.

The principle of chemical derivatization

Ideally, for untargeted SCMS approaches, SC components should be extracted from an individual cell with minimal sample loss and dilution [19]. However, SC sampling is extremely challenging, with issues including limited cell size (typical mammalian cells have diameters around 10 μm with a volume of approximately 1 pL), wide dynamic range of concentration (from a few molecules per cell to 10^{10} molecules for major metabolites in larger cells) [20, 21], and a high complexity of cellular compositions [11], as well as rapid turnover of cellular metabolites [22, 23]. Particularly, cells are usually cultured in a complex medium with high abundance of molecules that are similar or even identical to metabolites, making it challenging to differentiate endogenous metabolites from the surrounding medium and within a cell [20]. To circumvent these challenges, various techniques, based on ion beams [24], laser [25], probes [26–28], and microfluidic devices [29], have been reported for SC sampling. Sampled molecules are further ionized by ion beam/laser during the desorption process or by ESI for qualitative and quantitative analysis [4, 18, 30–32]. To improve the sensitivity of analytes, a chemical derivatization strategy could be adopted. The process of chemical derivatization is usually conducted offline, which is briefly shown in Fig. 1A; the reaction group of derivatization reagent firstly reacts with the functional group of targeted analytes, generating a derivative which has better ionization efficiency for subsequent MS analysis. Figure 1B illustrates the general reactions for various targeted functional groups, such as carboxyl [33], aldehyde [34], diazo group [16], hydroxyl [31], carbon-carbon bond [35], thiol [13, 14], and amine [33, 36]. The chemical structures of reactants and products are provided. Among the functional groups mentioned, diazo group [16], thiol [13, 14], hydroxyl [31], carbon-carbon bond [37], and amine [38] can be utilized for derivatization on metabolites, lipids, and proteins within a single cell. While the chemical derivatization strategy has been successfully employed for metabolites, lipids, and proteins, its application in single-cell glycan analysis is still in its nascent stage. This can be attributed to the lack of appropriate sample preparation methods and the minute quantities of glycans present within a single cell. It is noted that recent advancements in MS-based glycomics offer higher sensitivity, providing promising prospects for the detection of glycan signatures at the single-cell level [39]. As a result of these advancements, we are optimistic that the analysis of glycans in single cells will soon become feasible. It should be noted that the following considerations should be taken for the application of chemical derivatization in single-cell omics: (1) selecting the appropriate derivatization reagent is dependent on the specific application; (2) considering the availability, purity, and cost of selected derivatization reagents; (3) conducting chemical derivatization under optimal conditions to ensure the maximal derivatization efficiency; (4) using proper internal standards to correct for possible target analyte loss during sample preparation; (5) evaluating potential metabolic pathways of target analytes during chemical derivatization; and (6) avoiding the conversion of derivatization products back to the parent molecule.

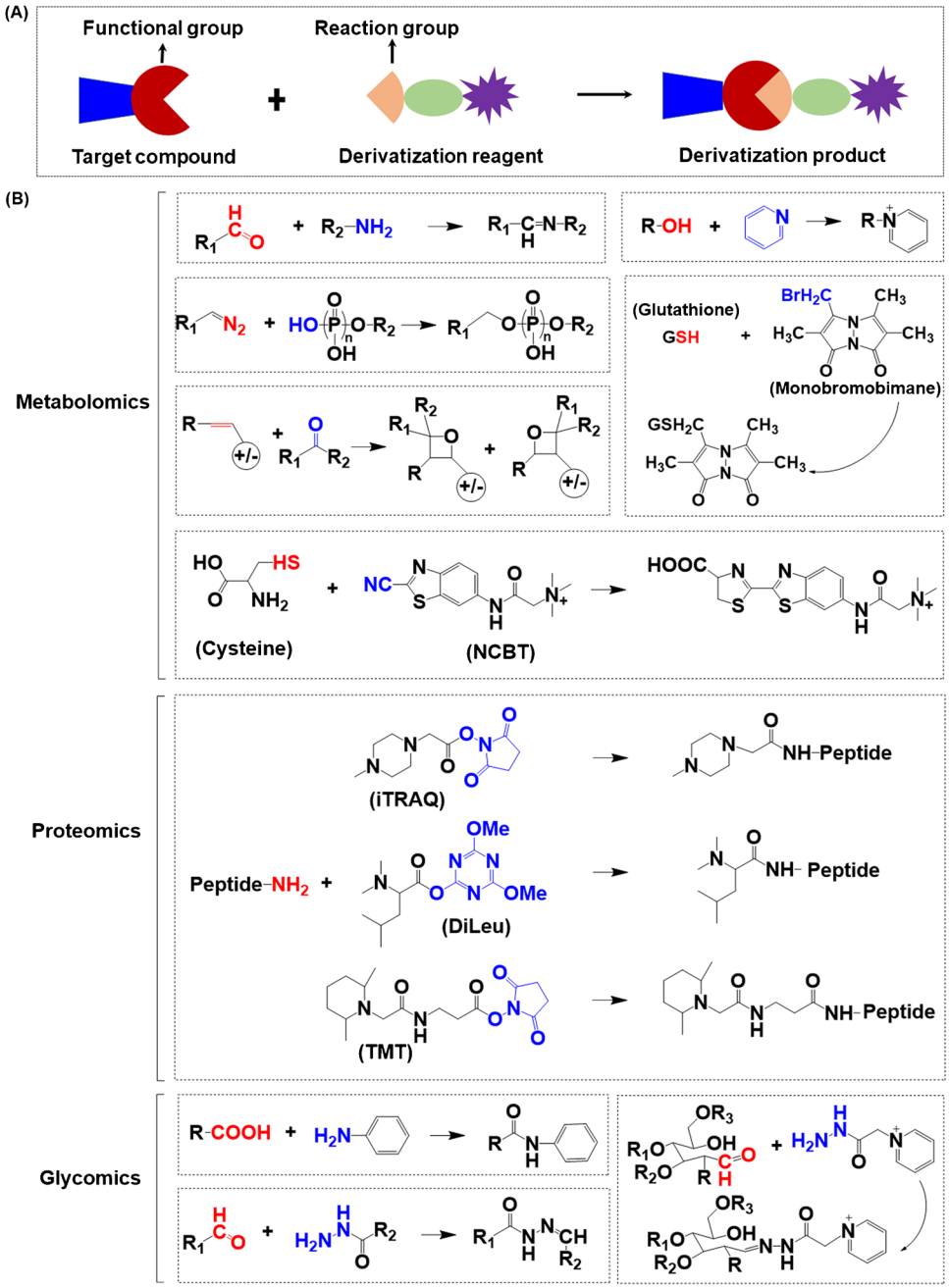

Fig. 1 **A** The schematic diagram of chemical derivatization of target components. **B** A summary of representative reactions for each targeted functional group with chemical structures of reactants and products (functional group highlighted in red, reaction group highlighted in blue)

The application of chemical derivatization methods in SC analysis

Metabolomics

The cellular metabolome represents the downstream products of genome, transcriptome, and proteome, which conveys valuable biological information. However, cellular activity and unique microenvironment could result in distinct cellular metabolomes [11, 40], which makes SC metabolomics study necessary. Since SC metabolomics offers many opportunities in clarifying the mystery behind cellular heterogeneity [41], SC metabolomics has become a hot area in SCMS studies. For example, live-single-cell MS has been established to directly characterize a large number of metabolites from a single plant cell [42]. Subsequently, this method has been successfully applied to the analysis of metabolites in circulating tumor cells [43]. Interestingly, a pulsed direct current electrospray ionization mass spectrometry (Pico-ESI-MS) was reported to enable systematic profiling of components in a small-volume sample; Pico-ESI-MS not only allowed the collection of abundant and valid metabolomics data from single *Allium cepa* and HeLa cells [32], but also allowed the identification of metabolites from single cancer cells [44].

In addition, quantitation of glucose-phosphate in single cells has been achieved by combing microwell-based nanoliter droplet microextraction with Pico-ESI-MS analysis [45].

Molecular analysis for either selected molecules or untargeted metabolomics at the cellular or even subcellular level is challenging. A method based on probe ESI mass spectrometry (PESI-MS) was established to detect different kinds of metabolites, including fructans, lipids, and flavone derivatives in single *Allium cepa* cells, revealing that different cell types of *A. cepa* bulb and different subcellular compartments of the same cell have significant diversity of metabolites [26]. Compared to the offline SCMS methods (e.g., Pico-ESI-MS and PESI-MS), real-time SCMS techniques enable the analysis of live single cells. T-probe, an integrated microscale device for cellular content extraction and immediate ionization, allows in situ SCMS analysis under ambient conditions with minimal sample preparation [27]. Similarly, a single-probe device was reported to enable rapid, real-time analysis of intracellular species from a suspension leukemia cell line [28]. Though a great many SC metabolites have been detected and investigated in detail, methods for SC metabolomics usually report on the detection of high-abundance compounds (such as phospholipids). For example, the combination of droplet extraction with Pico-ESI-MS allows the successful identification of more than 300 phospholipids from single cancer cells [44]. Additionally, sensitive and rapid determination of phospholipids in single human cell samples has been achieved via coupling of dispersed solid-phase microextraction combined with extractive electrospray ionization mass spectrometry [5].

In SC metabolome measurement, the limited sample volume, low analyte amounts, rapid metabolic dynamics, structural diversity of molecules, and inability to amplify the metabolites pose significant challenges in the detection of certain types of SC metabolites [20]. Taking fatty alcohols and sterol metabolites as examples, their low solubility in ESI-compatible solvents and low ionization efficiency during the ESI process produce low MS signal response. To address these problems, on-probe derivatization coupled with a noncontact nanocarbon fiber ionization strategy was proposed for sensitive analysis of fatty alcohol and sterol metabolites at the SC level (Fig. 2A), in which seven kinds of even-numbered-carbon fatty alcohols (C12-C22) and five types of sterols were successfully detected in single L-02 and HepG2 cells [31]. Similarly, a SC on-probe enzymatic dehydrogenation derivatization method was exploited for chiral analysis of lactate during direct-contact cancer-normal cells coculture, producing results that enabled unraveling metabolic alterations caused by D-lactate [46].

Due to the complexity of the intracellular metabolome, separation techniques have been adopted to further improve the detection sensitivity. Capillary electrophoresis (CE) has demonstrated encouraging results in metabolomics owing to its compatibility with small-volume samples and tolerance for high salt concentrations [47, 48]. Single-cell capillary electrophoresis microflow ESI-MS (CE-μESI-MS) was reported to reveal that small molecules could affect cell fate in the 16-cell embryo, providing new insights into the role of the metabolome in early embryo development [49]. To further address the sensitivity issue of CE-MS in SC metabolomics, coupling an ionization emitter (nanoCESI) with large-volume dual-sample preconcentration by isotachophoresis and stacking (LDIS) (Fig. 2B) realized the quantitation of 20 amino acids and identification of 40 metabolites in single HeLa cells [50]. Furthermore, an intracellular derivatization method was propsed to introduce naphthalene-2,3-dicarboxaldehyde into living cells for amino acid derivatization by capillary zone electrophoresis with electrochemical detection, allowing the quantification of six amino acids in a single erythrocyte [51]. To obtain the maximal sensitivity of cysteine detection in living single cells, a quaternary ammonium salt group-based charge tag [(2-cyano-benzothiazol-6-ylcarbamoyl)-methyl]-trimethylammonium (NCBT) was designed to increase induced nanoelectrospray ionization mass spectrometry (nanoESI-MS) sensitivity for cysteine detection in living single HeLa cells (Fig. 2C), the method provides an ideal strategy for in situ chemical derivatization for signal amplification [14]. Surprisingly, when aqueous droplets containing sugar and an amine as reagents are allowed to evaporate, they may form long-lasting micro thin films in which derivatization reaction occurs rapidly relative to reaction rates in bulk solution. A nonvolatile amine (N, N-Dibutyl-1,3-propanediamine) was used to derivatize reducing sugars from the intracellular fluid (ca. 1 nL) of a single *Allium cepa* cell via the micro thin film reactor (Fig. 2D); 29 reducing saccharides were successfully identified. The success of using a micro thin film reactor for single-cell derivatization shows its powerful potential for the rapid derivatization and identification of lower-nanogram-level analytes in single cells [34].

Lipidomics is one of the most important branches in metabolomics research [21]. Although SC lipidomics is less characterized compared to other small metabolites, previous studies have demonstrated the importance of lipid heterogeneity and diversity in the developing human brain at the single-cell level [52]. Emerging studies have shown that a more detailed molecular structure interpretation of isomeric lipids is crucial to better understand the role of lipid structural variations in lipid homeostasis and diseases such as cancers, type II diabetes (T2D), Alzheimer's, and other neurodegenerative diseases [35, 53]. Nevertheless, the occurrence of isomeric interference factors, such as variations in lipid headgroups, fatty acyl chain lengths, cis/trans configuration, and carbon-carbon double-bond (C=C) locations, results in the complex structural diversity and isomerism of lipids [54]. Therefore, the development of more specialized

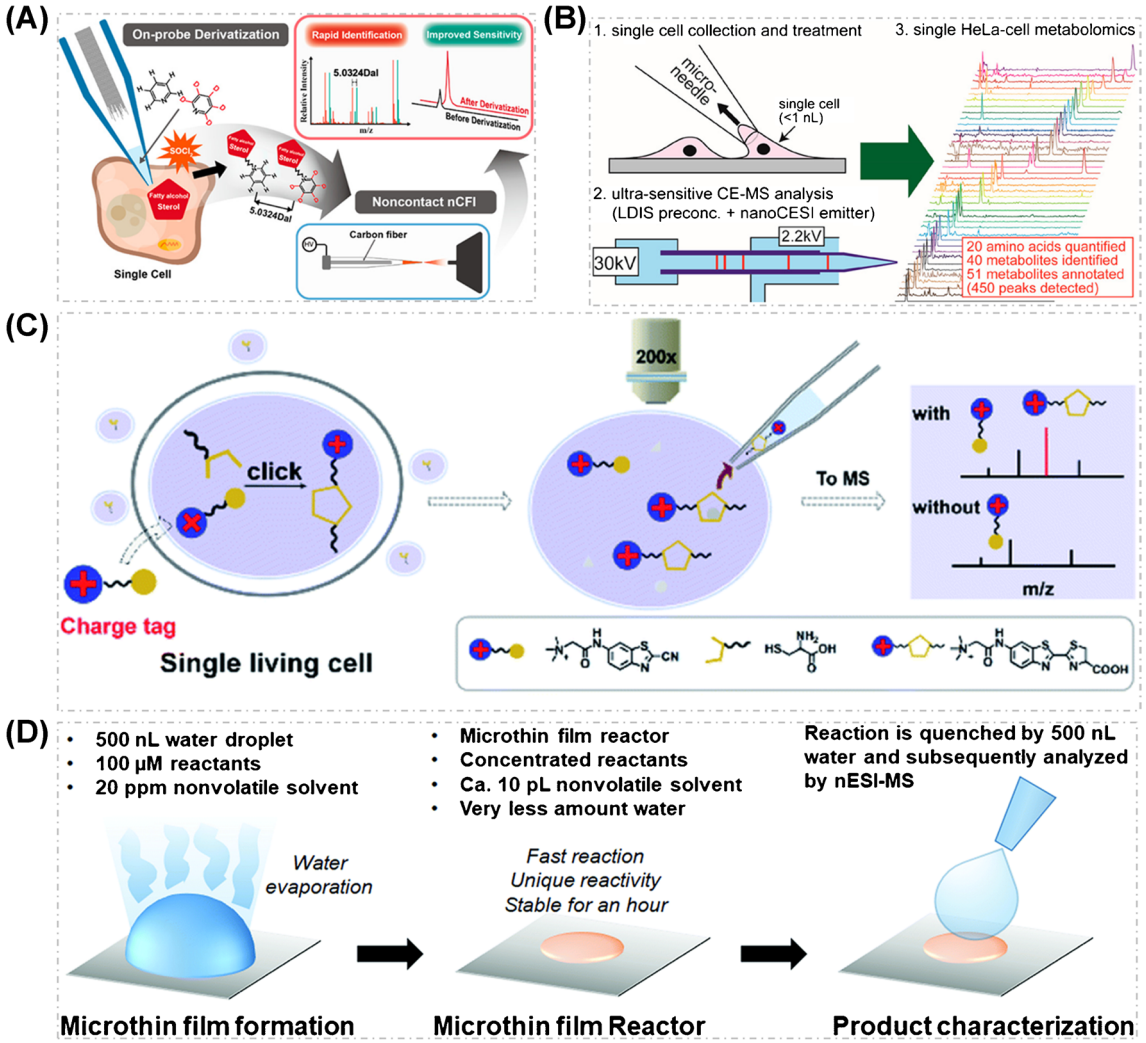

Fig. 2 Typical application examples of chemical derivatization strategies in SC metabolomics. **A** SC on-probe derivatization-noncontact nanocarbon fiber ionization. Reprinted with permission from ref. [31]. Copyright {2020} American Chemical Society. **B** Ultrasensitive single-cell metabolomics by CE-MS with a thin-walled tapered emitter and large-volume dual preconcentration by isotachophoresis and stacking (LDIS). Reprinted with permission from ref. [50]. Copyright {2019} American Chemical Society. **C** Charge tag was designed to improve the ionization efficiency of cysteine during induced nanoESI-MS. Reproduced from ref. [14] with permission from the Royal Society of Chemistry. **D** Construction of a micro thin film reactor for fast derivatization of sugars using an amine as reagents. Reproduced from ref. [34] with permission from the Royal Society of Chemistry

methodologies to precisely elucidate the structure of lipids at the single-cell level is highly desirable and urgently needed to advance SC lipidomics.

Chemical derivatization also plays a critical role in SC lipidomics. As an example, a SCMS analysis device (Fig. 3A) was introduced, in which the micropipette needle can accommodate the Paternò-Büchi (PB) reaction to determine C=C positions in unsaturated lipids at the SC level [15]. With 2-acetylpyridine (2-AP) derivatization reagent, electro-migration combined with droplet-assisted electrospray ionization (DAESI) enabled the relative quantitation of lipid C=C location isomers (Fig. 3B) to successfully discriminate gefitinib-resistant single cells in wild-type non-small-cell lung cancer (NSCLC) cell population, demonstrating the potential value of the chemical derivatization strategy in precision medicine [37]. Also, the peracetic acid (PAA)-induced epoxidation approach was employed for the structural characterization of fatty acids (FAs) C=C isomers from human cell line, with high sensitivity, simplicity, and reaction efficiency [53]. In short, the chemical derivatization of lipid C=C either by PB reaction or PAA epoxidation mainly targets C=C for the precise structural analysis and accurate quantification of lipid isomers. Recently, a novel aziridine-based isobaric tag labeling strategy was demonstrated, allowing the determination of lipid C=C positional isomers, accurate relative quantification of unsaturated

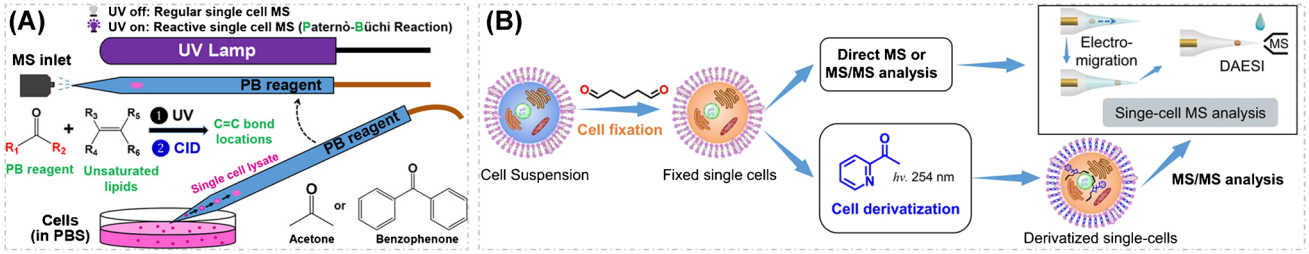

Fig. 3 Typical application examples of chemical derivatization strategies in SC structural lipidomics. **A** PB reaction to determine C=C positions in lipids at the SC level. Reprinted with permission from ref. [15]. Copyright {2020} American Chemical Society. **B** Workflow of SC fixation and optional derivatization for subsequent MS analysis. Insert indicated workflow of SCMS analysis method that allowed multi-round sampling and data acquisition. Reproduced from ref. [37] with permission from Springer Nature

lipids, and improvement of ionization efficiencies of nonpolar lipids from serum samples collected from a mouse model for Alzheimer's disease (AD) [54]. Though this method has not yet been employed for the lipid structure characterization in SC, it offers excellent potential for relatively high-throughput and deep profiling of lipids at the single-cell level. Ion mobility spectrometry-mass spectrometry (IMS-MS) is a powerful tool for the elucidation and differentiation of lipid isomers [55]; we believe that integrating IMS-MS in the separation and structural characterization of lipid isomers will lead to a major breakthrough in SC structural lipidomics. However, we should always keep in mind that the chemical derivatization of cellular lipids needs to be performed nondestructively to avoid cross-cell contamination to ensure precise lipid C=C location assignment [56]. Taken together, these advances further prove that with the right derivatization reagent and careful sample preparation, qualitative and quantitative MS analysis of low-abundance metabolites can be achieved in SC metabolomics. Table 1 presents several derivatization reagents used for the SCMS metabolomics analysis.

Although SC metabolomics has obtained great success via the ingenious use of chemical derivatization strategies, the next generation of SC metabolomics should be focused on multiple aspects: (1) one of the biggest difficulties is preparing SC samples for high-throughput metabolomics analysis [21]; (2) the other is to establish a standardized single-cell metabolomics data analysis pipeline for the cross-validation of experimental data obtained through different laboratories; (3) furthermore, it is necessary to develop the standardized data analysis methods that can correlate the metabolomics data with important biological function [4]. With the development of artificial intelligence and hardware instrument automation, we believe that SC metabolomics will be more broadly utilized by both professionals and laypeople engaged in fundamental studies and clinical applications.

Proteomics

MS-based proteomics measurements have the potential to identify post-translational modifications, providing unique insights that are unavailable through transcriptomics measurements or bulk proteomics [57, 58]. Particularly, SC proteomics can yield essential biological information indicative of cellular heterogeneity that may be obscured by bulk-scale analysis [59], which has promise to revolutionize our understanding of functional crosstalk between

Table 1 A summary of derivatization reagents used for the SCMS metabolomics analysis

Number	Sample	Analytes	Derivatization reagents	Ref.
1	L-02 and HepG2 cells	Fatty alcohols and sterol metabolites	$SOCl_2$ and pyridine	[31]
2	A549 and Beas-2B cells	Lactate	Girard's P	[46]
3	Erythrocyte	Amino acids	Naphthalene-2,3-dicarboxaldehyde	[51]
4	HeLa cell	Cysteine	[(2-Cyano-benzothiazol-6-ylcarbamoyl)-methyl]-trimethylammonium (NCBT)	[14]
5	The intracellular fluid of a single *Allium cepa* cell	Reducing sugars	N, N-Dibutyl-1,3-propanediamine	[34]
6	Human colon cancer cell line (HCT-116)	Unsaturated lipids	Acetone or benzophenone	[15]
7	Wild-type human lung cancer cells (HCC827)	Lipid C=C location and sn-position isomers	2-Acetylpyridine	[37]

cells that drive tumor development [60]. The MS-based SC proteomics method can be divided into label-free and multiplexed isobaric labeling analysis. Although label-free analysis is the most straightforward approach to quantify proteins from a single cell and toward automated sample preparation, a major challenge is throughput. To achieve higher throughput, the multiplexed isobaric labeling strategy was very attractive, with the advantages of increased analytical throughput, improved quantitation accuracy, and reduced run-to-run variability within biological or technical replicates. In multiplexed isobaric labeling analysis, each single-cell proteome is individually labeled by a unique mass tag; the same peptide from a different single-cell sample produces a single peak in full MS^1 scans. Upon tandem mass spectrometry fragmentation, each labeled sample gives rise to a distinctive reporter ion along with sequence-specific backbone cleavage for identification. The relative abundance of each reporter ion reflects protein abundances in its corresponding sample. This approach enables multiple single-cell samples to be analyzed in parallel with increased throughput and information content [38, 61].

The throughput for SC proteomics can be addressed by the multiplexed isobaric labeling strategy, while the sensitivity of proteome analysis can be improved by minimizing sample preparation. For example, Single Cell ProtEomics (SCoPE) (Fig. 4A), as a pioneering method for SC proteomics study, was facilitated by minimizing protein losses and using a carrier channel to identify and quantify peptides from SC samples simultaneously [62]. To enable inexpensive and robust SC proteomics, Single Cell ProtEomics (SCoPE2) was developed based on the key concepts of SCoPE and introduced technical and analytical improvements. In SCoPE2, single cells are isolated by fluorescence-activated cell sorting (FACS) or CellenONE into multiwell plates. The lysis, digestion of isolated cells, and the resulting peptide labeling with tandem mass tags (TMTs) can be automated and performed by either Minimal Proteomic sample Preparation (mPOP) or nano-ProteOmic sample Preparation (nPOP). SCoPE2 is applicable to any sample that can be processed to a suspension of single cells and allows analyzing ~200 single cells per 24 h [63]. Another major progress in SCMS proteomics was obtained by downscaling processing volumes to < 200 nL to minimize surface losses through using nanodroplet processing in one pot for trace samples (nanoPOTS) platform (Fig. 4B). In nanoPOTS, the chip consisted of a patterned glass slide and a glass spacer, which was sealed to a membrane-coated glass slide to minimize evaporation of the nanowell contents during the various incubation steps. The RapiGest surfactant-based one-pot protocol was employed for proteomic sample preparation [64]. The combination of nanoPOTS with TMT isobaric labeling greatly enhances proteome coverage and analysis throughput for single cells. For instance, TMT labeling facilitated multiple analysis of single cell-sized protein quantities to ~ 1600 proteins with a median coefficient of variation of 10.9% and correlation coefficient of 0.98 [65]. To address the compatibility of nanoPOTS with automated LC-MS systems, a nanoPOTS autosampler was developed to allow fully automated sample injection from nanowells to LC-MS systems. By coupling of the nanoPOTS autosampler with a TMT-11plex labeling approach, ~ 77 single cells could be analyzed per day [59].

Compared with other classical SC proteomics approaches employing either affinity reagents (such as antibodies) or fluorescent proteins [66], the coupling of nanoPOTS and SCoPE/SCoPE2 with multiplexed isobaric labeling holds great potential to facilitate the rapid development of SC proteomics in increasing throughput without compromising the extent of coverage. However, the difficulty in finding a compatible chemical structure for isobaric tag design continues to restrict the maximum number of single-cell samples that can be simultaneously analyzed [58]. With the continuing development of depth and throughput in SC proteomics, there is no doubt that SC proteomics will play a critical role in elucidating the basic mechanism of cell development and disease. In particular, together with DNA and RNA sequencing, SC proteome profiling will be highly valuable for characterizing the central dogma of biology (DNA to RNA to protein) [67].

MS imaging at the single-cell level

Increasing evidence suggests that spatial distribution of the biomolecules within an individual cell is a significant piece in solving the puzzle of SC molecular heterogeneity and chemical diversity [8]. Mass spectrometry imaging (MSI) is a powerful, label-free analytical technique [68, 69], while matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is an excellent tool for obtaining molecular weights of numerous biomolecules in cells and tissues [33, 70–73]. The coupling of MSI and MALDI-MS has enabled spatial mapping and visualization of metabolites, lipids, peptides, and proteins within a single cell. For example, MALDI-MSI of individual cells of maize leaves revealed the genetic programming and developmental alterations in thylakoid membrane lipid distribution [74]. Also, MALDI-MSI has been applied to visualize the three-dimensional spatial distribution of phospholipid classes, including phosphatidylcholine, phosphatidylethanolamines, and phosphatidylinositol, in newly fertilized individual zebrafish embryos [75]. Recently, an open-source SC metabolomics method, SpaceM, which integrates MALDI-MSI with light microscopy followed by image segmentation and registration, enabled the detection of > 100 metabolites from > 1000 individual cells per hour [76].

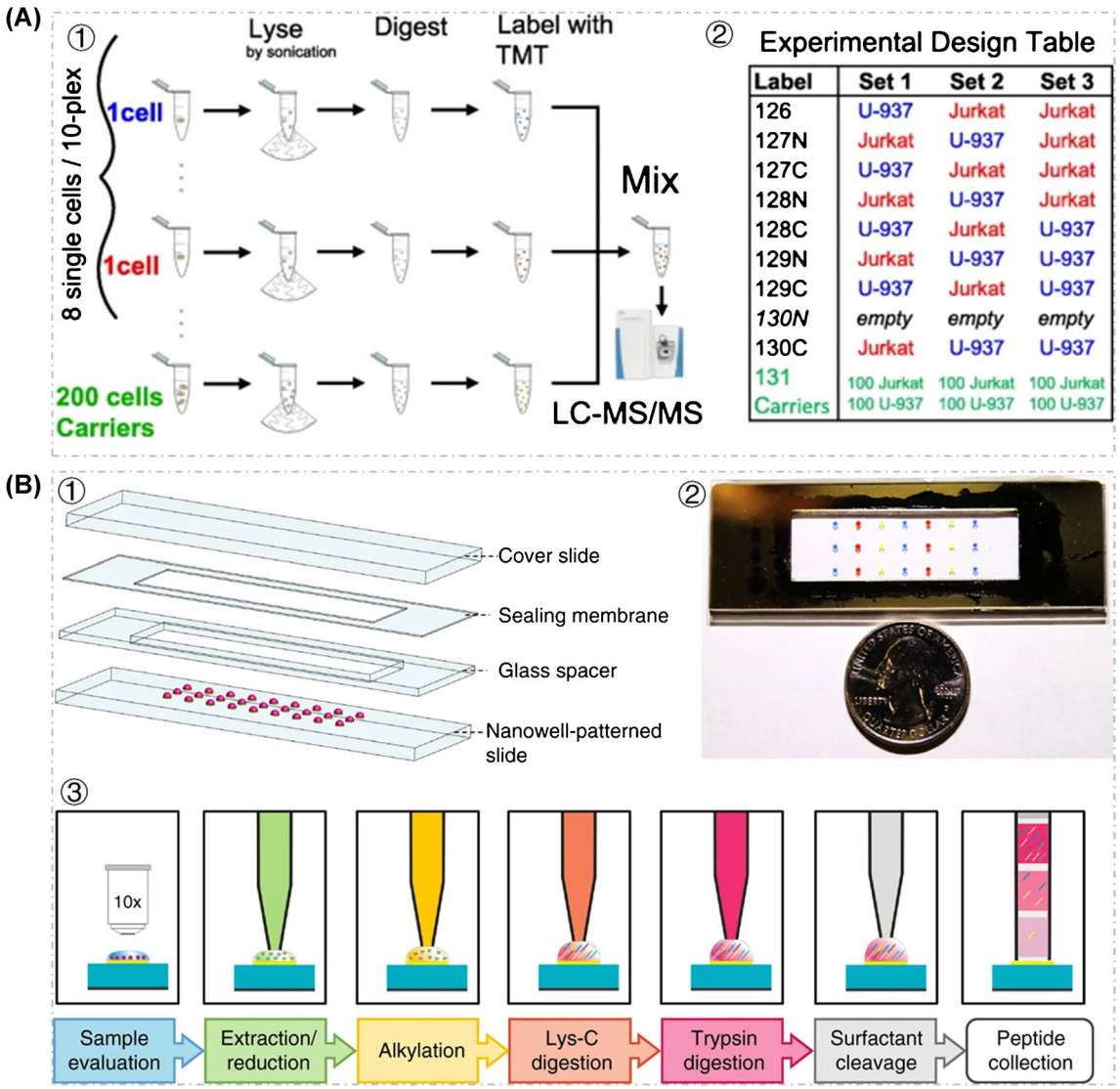

Fig. 4 Typical application examples of chemical labeling in SCMS proteomics. **A** ① The workflow of SCoPE-MS. Individually picked live cells were lysed by sonication; the proteins were digested; the resulting peptides were labeled with TMT isobaric tags, combined, and analyzed by LC-MS/MS. ② Design of control experiments used to test the ability of SCoPE-MS to distinguish U-937 cells from Jurkat cells. Reproduced from ref. [62] with permission from Springer Nature. **B** The workflow of nanoPOTS for proteomic sample preparation. ① Schematic drawing and ② photograph showing the nanoPOTS chip with each nanowell filled with 200 nL of colored dye. ③ One-pot protocol for proteomic sample preparation and capillary-based sample collection. Reproduced from ref. [64] with permission from Springer Nature

With goals to address the visualization problems for certain types of molecules caused by the ionization suppression, low concentration, and methodological interferences, emerging evidence showed that chemical derivatization is an effective solution in MSI [10]. For instance, Girard's Reagent P was used in MALDI-MSI to enhance N-glycan signals from formalin-fixed paraffin-embedded (FFPE) tissue sections. This study provides a promising approach to better understand aberrant glycosylation in the pathogenesis of cancers [70]. Similarly, MALDI-MSI enabled sensitive imaging of sialylated N-glycans from FFPE tissue sections via on-tissue amidation of sialic acid with aniline as amidation reagent. The results showed that higher glycome coverage could be achieved following chemical derivatization, demonstrating that the detection sensitivity for sialylated N-glycans is notably improved by amidation derivatization [33]. In another study, 2-picolylamine was chosen as derivative reagent; on-tissue derivatization of endogenous fatty acids in rat brain tissues has been validated via MALDI-MSI [77]. Also, a covalent charge-tagging approach, using on-tissue chemical derivatization of primary and secondary amines and phenolic hydroxyls, was presented to enable

spatial visualization of comprehensive brain neurotransmitter systems and neuroactive substances [78]. Overall, these examples further demonstrate that the usage of chemical derivatization in MSI significantly expedites the spatial analysis of previously inaccessible biologically relevant molecules in complex tissue sections, providing valuable reference for the application of chemical tagging strategy in MS imaging at the single-cell level.

Compared with traditional proteomics with the need for cell lysis and at the expense of protein location information, spatial proteomics provides a means for the visualization of proteins under their native cellular environment [79]. Consequently, diverse SCMS imaging methods have been expanded to map the spatial distribution of proteins at the subcellular level. Based on the sample preparation strategy, SCMS imaging approaches can be broadly categorized into multiplexed antibody-based MS imaging and label-free MS imaging. Multiplexed antibody-based technologies capable of capturing multiplexed protein biomarkers at the SC resolution have advanced remarkably in recent years, with widespread applications in cancer research [80–82], autoimmune disease [83], and infectious disease studies [84]. Multiplexed antibody-based imaging methods can be carried out under different antibody tagging modes (e.g., metal tag, fluorophore, DNA oligonucleotide barcode, or enzyme) [85]. In multiplexed antibody-based MS imaging, proteins or proteoforms within cells or tissue are prelabeled with specific antibodies. These antibodies contain unique mass tags that can be used to generate distinct reporter ions when sampled by laser or ion beam [8].

The two most common technologies of multiplexed antibody-based MS imaging are imaging mass cytometry (IMC) (Fig. 5A) and multiplex ion beam imaging (MIBI) (Fig. 5B). The workflows of IMC and MIBI can be summarized into three parts, including tissue staining, ionization imaging, and data analysis (Fig. 5C). IMC is a method capable of mapping protein distributions with 1-μm spatial resolution [86], in which rare earth metals are conjugated to antibodies for simultaneous imaging of ~ 40 proteins. IMC was applied to analyze human breast cancer tissue samples with subcellular resolution, enabling delineation of cell subpopulations and cell-to-cell interactions and highlighting tumor heterogeneity [87]. Like IMC, MIBI incorporates ionizable metal mass tags, using mass-tagged antibodies to analyze highly

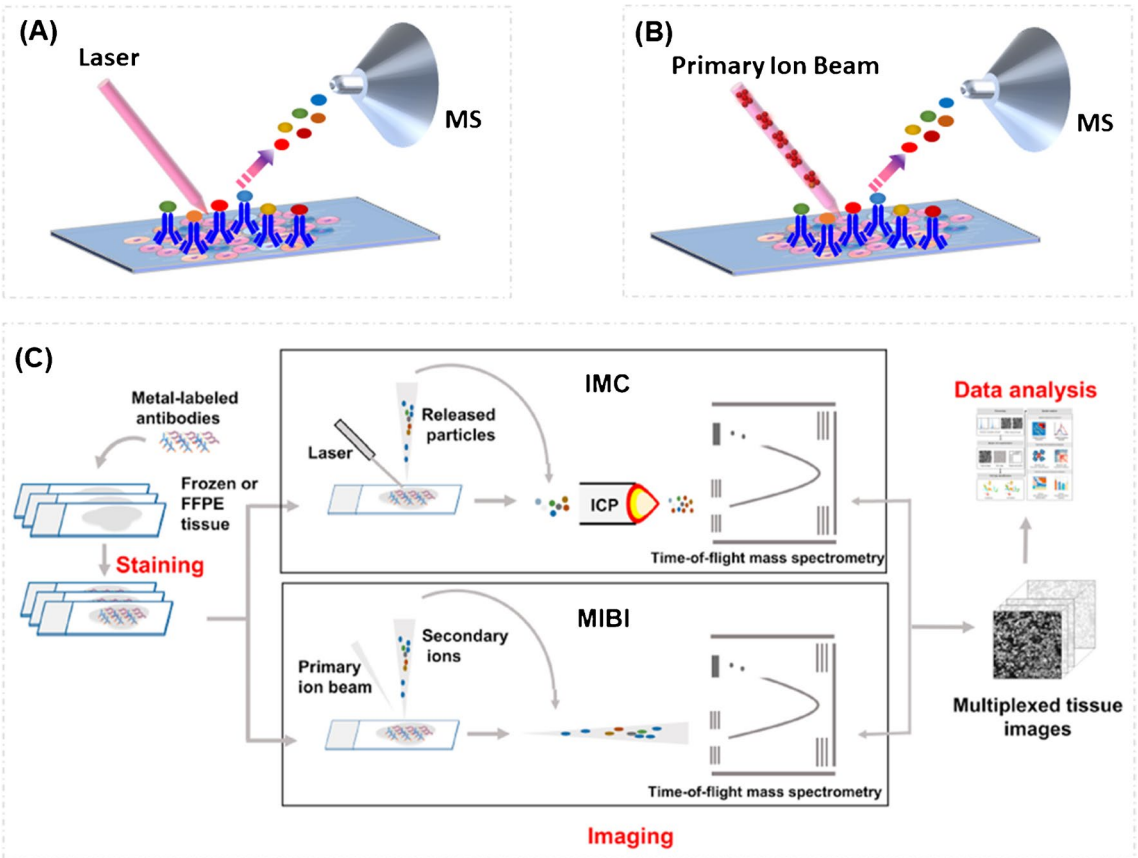

Fig. 5 Graphical representation of techniques for multiplexed antibody-based SCMS imaging. **A** IMC and **B** MIBI (inverted Y-shaped symbols indicate antibodies, colored balls indicate tags on antibodies or analyte ions). **C** The workflows of IMC and MIBI; three parts are highlighted, including tissue staining, ionization imaging, and data analysis. Reproduced from ref. [91] with permission from Elsevier

multiplexed protein expression patterns with morphological context. Instead of using a laser for tag ionization in IMC, MIBI uses an ion beam for tag ionization. MIBI can analyze up to 100 protein targets simultaneously over a five-log dynamic range. The resulting data, from FFPE human breast tumor tissue sections stained with ten labels simultaneously, indicated that MIBI can bring new opportunities for disease diagnosis and better understanding the mechanisms underlying tumor pathogenesis [88]. In addition, to characterize the metabolic regulome of single cells together with their phenotypic identities, a robust approach combining single-cell metabolic regulome profiling (scMEP) with MIBI was reported to reveal the spatial organization of metabolic programs in human tissues [89]. All in all, these examples manifested that both MIBI and IMC demonstrate great potentials in promoting the transition of basic research toward translational research and facilitating individualized molecularly targeted diagnosis and therapies. However, both MIBI and IMC take longer imaging times in addition to being expensive. The sampling area is also limited by the time required to convert MS data obtained from each pixel to an image [86]. More details of IMC and MIBI techniques have been reported previously in the literatures [90, 91]. In the upcoming years, we anticipate that advances in SCMS spatial proteomics could reveal the localization of key pathway components that drive alterations in cellular states during the early stages of various diseases such as cancer and Alzheimer's disease and other neurodegenerative conditions [92].

Conclusions and future perspectives

In summary, the coupling of MS with chemical tagging/derivatization strategy has greatly enhanced capabilities and information content offered by SC omics studies, and offers new perspectives to deeply explore the mysteries underneath single cells. Significant advancements have enabled the following improvements in SC omics experiments: (1) greatly improved molecular information coverage, including but not limited to high-abundance compounds; (2) precisely characterized lipid structure; (3) achieving high-throughput analysis by multiplexed isobaric labeling strategy; and (4) spatially resolved SC omics.

Although great strides have been made and dedicated efforts from multiple groups are rapidly advancing SC omics analyses by the application of chemical tagging strategy, remaining challenges and limitations need to be recognized to allow further development and improvement. Sample loss is inevitable in most current chemical tagging/derivatization methods; optimization of each step is necessary to enable SC omics with higher information coverage. With the rapid development of in situ, nondestructive single-cell sampling techniques and novel ionization techniques, we anticipate that chemical tagging/derivatization can be tailored to maximize derivatization efficiency with enhanced sensitivity while minimizing sample loss. Multiple functional tagging/derivatization agents need to be developed for simultaneous characterization of multi-omics from a single-cell sample, because this is imperative to minimize systematic errors in precision molecular diagnosis and systems biology studies. Also, great efforts should be made on establishing open-access data analysis platforms and analytical informatic pipelines that are suitable for different data formats collected by different laboratories. Additionally, it is necessary to develop new computational methods for integrated analysis of multimodal SC data and correctly co-registering SCMS images. Moreover, we expect the ongoing developments of SC analysis will be beneficial to accelerate fundamental biology research such as single-cell phenotyping and biomarker discovery, in addition to related biomedical applications including clinical diagnostics, and therapeutic strategy development in personalized and precision medicine.

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Declarations

Conflict of interest The authors declare no competing interests.

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