Advanced technologies of single-cell metabolomics unveiling cellular metabolic heterogeneity for biological and biomedical research

Shang-Che Kuo [a,1], Ting-Wei Tsao [b,1], I-Fan Wang [a], Ying-Lan Chen [a,b,c,d,*]

[a] Department of Biotechnology and Bioindustry Sciences, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan
[b] Graduate Program in Translational Agricultural Sciences, National Cheng Kung University and Academia Sinica, Tainan, Taiwan
[c] Program in Specialty Crops and Metabolomics, Academy of Circular Economy, National Chung Hsing University, Nantou, Taiwan
[d] Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan

Abstract

Metabolomics provides direct insights into cellular physiology, yet it faces greater analytical challenges compared to genomics and transcriptomics due to the chemical diversity, instability, and environmental sensitivity of metabolites. Conventional bulk metabolomics averages signals across cell populations, thereby masking cellular heterogeneity critical for understanding disease mechanisms. Recent breakthroughs in single-cell metabolomics (SCM), driven by advances in mass spectrometry, microfluidics, isotope tracing, and spatial omics, have enabled the detection of metabolic diversity at unprecedented resolution. SCM has uncovered cell-type-specific biomarkers, revealed metabolic reprogramming in cancer and immunity and revealed disease progression. These studies highlight SCM's transformative potential in biomarker discovery, clinical diagnostics, and precision medicine. Despite rapid progress, SCM remains limited by low metabolite abundance, instability during cell handling, lack of standardized quantification methods, and challenges in integrative multi-omics analysis. Future developments will require innovations to improve sensitivity and spatial resolution, establish cross-laboratory quality control frameworks, and apply artificial intelligence for data interpretation. With continued technological convergence, SCM is poised to evolve from a niche research tool into a cornerstone platform for biological and biomedical research.

Keywords: Laser capture microdissection, Mass spectrometry, Metabolic heterogenrity, Single-cell metabolomics

1. Differences between metabolomics and other omics research

Compared to genomics, transcriptomics and proteomics, analysis in metabolomics presents far greater challenges due to the intrinsic complexity and dynamic nature of metabolites [1]. Metabolites are highly diverse and heterogeneous, exhibiting vastly different molecular properties such as polarity, acidity/alkalinity, and volatility [2,3]. Although there are many studies on all-in-one profiling methods with multiple molecular extraction, this diversity makes it impossible to comprehensively extract and detect all metabolites using a single preparation or detection method [4–7]. Therefore, comprehensive coverage of metabolic pathways typically requires the use of multiple analytical approaches and relies heavily on the availability of standard compounds for accurate identification and quantification [8]. Furthermore, metabolites are inherently unstable and can easily degrade or undergo new biochemical reactions

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* Corresponding author at: Rm. 89902, 9 F., No. 112, Dongfeng Rd., North Dist., Tainan City 704016, Taiwan.
E-mail address: cyl0828@dragon.nchu.edu.tw (Y.-L. Chen).
[1] These authors contributed equally to this work.

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during sample collection, storage, and processing, introducing bias and variability into measurement results [9]. It is difficult to define metabolic signatures existing in certain tissues because metabolites are also distributed across multiple tissues or blood [10–13]. In addition, they are highly sensitive to external environmental factors such as chemical exposure, nutrition stimuli, and environmental stressors [14,15], which further complicates accurate detection and interpretation.

Unlike genomics, which benefits from well-established reference sequences and standardized global databases, metabolomics lacks fully populated metabolite databases and widely adoptable quantitative standards [16], resulting in poor reproducibility across laboratories [17–21]. Moreover, while genomics and transcriptomics can utilize amplification techniques (e.g. PCR) to increase signal strength, metabolomics has no analogous amplification step to boost metabolite concentrations [22,23]. This fundamental limitation exacerbates sensitivity challenges and makes metabolomics inherently more complex in both technical and analytical dimensions.

Although mass spectrometry (MS) technologies have advanced significantly, current methods still face limitations in sensitivity and resolution, making it difficult to detect low-abundance metabolites and to distinguish structural isomers with similar masses [23]. Adding to this complexity, changes in metabolite levels often arise from multiple interconnected biochemical pathways, making it challenging to directly attribute variations to a single biological or pathological event [24]. Furthermore, data generated by different platforms such as liquid chromatography tandem mass spectrometry (LC-MS/MS), gas chromatography (GC)-MS, and nuclear magnetic resonance (NMR) are difficult to integrate into a unified analytical framework [25,26], while metabolomics datasets themselves are inherently high-dimensional and noisy, with statistical analysis, machine learning modeling, and pathway analysis tools still in a stage of rapid development [27–29].

Given the critical role of metabolites as direct indicators of cellular function and physiological state, there is a growing global effort to develop high-sensitivity MS technologies [30]. These advancements aim to improve detection limits [31,32], enhance resolution [33], and enable more comprehensive and accurate profiling of the metabolome, ultimately driving forward our understanding of health, disease mechanisms, and precision medicine [30,34,35].

In addition, studying multi-omics at the single-cell level is essential because it reveals biological heterogeneity that is completely masked in bulk measurements [23,36–39]. Even though bulk metabolomics with the same genetic background provides an average metabolic profile of a tissue or cell population, it cannot capture the differences between individual cells, which are often critical for understanding disease mechanisms, developmental processes, and treatment responses [23,24].

2. Advances in single-cell metabolomics

Single-cell metabolomics (SCM) has experienced rapid growth in recent years, driven by breakthroughs in mass spectrometry, microfluidics, and spatial omics technologies [22,40–44] (Table 1). Traditional metabolomics captures averaged signals from bulk samples, concealing the variability among individual cells. In contrast, SCM enables the characterization of metabolic diversity at the single-cell level, providing revolutionary insights into cancer, immunology, development, and plant biology fields [22,45,46] (Tables 2–7). Technological innovations such as mass spectrometry imaging (MSI), including Matrix-Assisted Laser Desorption/

Table 1. Current technologies for single-cell metabolomics.

Technology Type	Techniques/Instruments	Key Features	Reference
Direct Mass Spectrometry Analysis	DESI, LAESI, ELDI	No derivatization or extensive pre-processing required	[40]
Mass Spectrometry Imaging	MALDI-MSI, SIMS-MSI, LA-ICP-MSI	Provides spatial distribution of metabolites; resolution can reach subcellular level	[41]
Microprobe-based Extraction + MS	Microsampling integrated with MS	Detect the metabolomic content in a live single cell	[42]
Mass Cytometry	Flow cytometry integrated with MS	Flow cytometry integrated with MS	[43]
Droplet-Based Microfluidics + MS	Microfluidics integrated with MS	Microfluidics integrated with MS	[44]

Abbreviations: DESI: desorption electrospray ionization; ELDI: electrospray-assisted laser desorption/ionization; LA-ICP: laser ablation inductively coupled plasma; LAESI: laser ablation electrospray ionization; MALDI: matrix-assisted laser desorption/ionization; MSI: mass spectrometry imaging; SIMS: secondary ion mass spectrometry.

Table 2. Overview of biological samples and identified features from single-cell metabolomic researches focusing on cell line samples.

Ref.	Species	Tissue/Cell type	Cell type annotation method	Total cell number	Max feature count across experiments
[3]	*H. sapiens*	MCF7 cel 97H cell PC3 cell DU145 cell CWR-22Rv1 cell	Cell types are known. No further annotation.	252 cells	A single MCF7 cell: 254 metabolites
[32]	*H. sapiens*	U2OS GBM	Cell types are known. No further annotation.	12 cells	At least 272 ion species in total U2OS cells: 15 lipids GBM cells: 17 lipids
[47]	*H. sapiens* *M. musculus*	HeLa cell NIH3T3 cell Nine NCI-60 cell lines: BT-549 cell HS 578T cell HT29 cell NCI-H460 cell HOP-62 cell IGR-OV1 cell OVCAR-5 cell A498 cell MALME-3M cell	Cell types are known. For co-culture, cell types are distinguished using native GFP expression and morphology.	140763 cells	202 small-molecule ions
[53]	*H. sapiens* *M. musculus*	HEK293T cell Mouse embryonal fibroblast	Cell types are known. Subtypes/states (oxidative stress levels) are identified by live-cell fluorescent staining.	2804 cells	More than 500 ion signals 162 metabolites (with MS) 83 metabolites (with MS/MS)
[54]	*N. tabacum*	*N. tabacum* cell	Cell types are known. No further annotation. The mitotic phase of each cell is identified by fluorescent microscopy using a histone 2B-RFP marker.	A minimum of three single cells in each sub-phase	Phase-specific metabolites: Prophase: 230 (+), 74 (−) Metaphase: 284 (+), 40 (−) Anaphase: 158 (+), 128 (−) Telophase: 43 (+), 43 (−)
[55]	*H. sapiens*	HeLa cell	Cell types are known. No further annotation.	173 cells	23 control cells: 340 metabolites
[63]	*M. musculus*	Peritoneal primary macrophages	Cell types are known. No further annotation.	37 cells	More than 1000 ion peaks About 100 intracellular metabolites
[90]	*H. sapiens*	HeLa cell	Cell types are known. No further annotation.	A single HeLa cell	20 amino acids using TQ/MS 40 metabolites using QTOF/MS
[91]	*H. sapiens*	HeLa cell	Cell types are known. No further annotation.	NA	13 metabolites (−) 19 lipid species (+)

Abbreviations: *H. sapiens*: *Homo sapiens*; *M. musculus*: *Mus musculus*; *N. tabacum*: *Nicotiana tabacum*; U2OS: human bone osteosarcoma; GBM: glioblastoma; NA: not available.

Ionization (MALDI)-MSI and Desorption Electrospray Ionization (DESI)-MSI, now allow mapping of metabolite distributions at subcellular resolution [47,48]. Microfluidics integrated with MS enables high-throughput single-cell analysis [49,50], while stable isotope tracing (e.g. ^{13}C) allows dynamic observation of metabolic pathway activities [51,52].

Beyond methodological refinement, SCM studies are fundamentally aimed at unraveling cellular heterogeneity and differentiation [3,47,53]. By analyzing individual cells, researchers can identify distinct metabolic states within seemingly homogenous cell populations, shedding light on how cells diverge in their metabolic activities during developmental processes [54], in response to stimuli [3,47,53–55], or within disease contexts [47,56]. This granularity is critical for understanding why some cells react differently to treatments or adopt unique functions. With this in mind, SCM is a key tool for exploring phenotypic/metabolic dynamics and stress response, allowing for the real-time tracking of metabolic shifts as cells undergo changes in their environment or experience stress [47,53,57]. For instance, studies have observed metabolic dynamics during plant mitosis [54] and in the developmental relationships of plant structures, such as cannabis trichomes [45]. SCM also reveals how metabolism response to environmental stressors, including salt stress in plants [45], or to nutrient changes, such as glucose starvation or

Table 3. Overview of biological samples and identified features from single-cell metabolomic researches focusing on tissue samples.

Ref.	Species	Tissue/Cell type	Cell type annotation method	Total cell number	Max feature count across experiments
[56]	*M. musculus*	(1) Result 1: glutamertagic neurons (acute phase of spared nerve injury) (2) Result 2: glutamertagic neurons (subchronic phase of spared nerve injury)	Cell types are known. No further annotation. Glutamatergic neurons are identified by fluorescent labeling.	At least 61 samples	1205 ion signals 616 metabolites
[59]	*C. roseus*	Epidermal cells and idioblasts in leaves	Cell types are unknown. (1) Idioblasts: serpentine and vindoline (2) Epidermal cells: secoiridoid secologanin	586 cells	8268 representative features
[60]	*C. roseus*	SA leaf SA root SA petal LBE petal ABH petal	Cell sources are known. Specific cell types are identified. (1) IPAP cells: loganic acid (2) Idioblasts: serpentine	1106 cells	Features with formula/MS2 data: SA leaf: 557/295 SA root: 869/438 SA petal: 765/321 LBE petal: 1373/567 ABH petal: 513/239 Total: Approximately 1000/NA
[92]	*R. sativus*	Pith Cortical tissue Mesophyll tissue Epidermal tissue	Cell sources are known. No further annotation.	Three single cells in each tissue	Tissue-specific peaks: Pith: 98 (+), 98 (−) Cortical tissue: 246 (+), 75 (−) Mesophyll tissue: 102 (+), 54 (−) Epidermal tissue: 151 (+), 105 (−)
[93]	*A. cepa* *H. sapiens*	Epidermal cell of onion bulbs PANC-1 cell	Cell types are known. No further annotation.	Dozens of cells per at least three replicates	Onion: 86 metabolites Human: 111 metabolites

Abbreviations: *M. musculus*: *Mus musculus*; *C. roseus*: *Catharanthus roseus*; *R. sativus*: *Raphanus sativus*; *A. cepa*: *Allium cepa*; SA: Sunstorm Apricot; LBE: Little Bright Eyes; ABH: Atlantis Burgundy Halo; IPAP: internal phloem-associated parenchyma.

nutrient deprivation in humans [3] and microalgal cells [58]. The elucidation of metabolic pathways and localization is another key objective, helping to map out the intricate networks of metabolic reactions at the single-cell level and understand where specific metabolites are produced or consumed within tissues [45,47,59,60].

The utility of SCM extends to biomarker discovery and phenotypic prediction, where unique metabolic signatures from individual cells can serve as indicators of specific physiological states [32,53,57] or disease progression [3,47,56,61]. In oncology, SCM significantly enhances the understanding of cancer metabolism and tumor heterogeneity, aiding in drug assessment and design, and disease diagnosis [45]. Furthermore, SCM has been employed to identify metabolic adaptations of immune cell populations *in vivo*, revealing distinct metabolic states of early-activated T cells during infection and CAR T cells in lymphoma patients [62]. These studies not only deepen our understanding of disease mechanisms but also highlight SCM's potential in precise clinical diagnostics and precision medicine. Moreover, the integration of

Table 4. Overview of biological samples and identified features from single-cell metabolomic researches focusing on unicellular samples.

Ref.	Species	Tissue/Cell type	Cell type annotation method	Total cell number	Max feature count across experiments
[57]	*C. granii* (Helg2016 and SCCAP-K1834) *H. pluvialis* (SAG 192.80)	*C. granii* (Helg2016 and SCCAP-K1834) *H. pluvialis* (SAG 192.80)	Cell types are known. No further annotation.	At least 20 cells in each species/strain	*C. granii* (Helg2016): 326 peaks *C. granii* (SCCAP-K1834): 400 peaks
[58]	*S. cerevisiae* *D. salina* *S. obliquus* *E. viridis* *C. reinhardtii*	*S. cerevisiae* *D. salina* *S. obliquus* *E. viridis* *C. reinhardtii*	Cell types are known. No further annotation.	At least 66 cells	Metabolite ions: 95 (−), 19 (+) Metabolite compounds: 71 (−), 17 (+)

Abbreviations: *S. cerevisiae*: *Saccharomyces cerevisiae*; *D. salina*: *Dunaliella salina*; *S. obliquus*: *Scenedesmus obliquus*; *E. viridis*: *Euglena viridis*; *C. reinhardtii*: *Chlamydomonas reinhardtii*; *C. granii*: *Coscinodiscus granii*; *H. pluvialis*: *Haematococcus pluvialis*.

Table 5. Overview of experimental and analytical techniques from single-cell metabolomic researches focusing on cell line samples.

Ref.	Extraction method	Instrument type	Analytical method	Raw data processing software/method	Identification software/method
[3]	Capillary microsampling	Orbitrap	Full-scan MS (positive mode)	Raw data processing, peak alignment, stable feature ion screening: Python	Python based on HMDB, METLIN, mzCloud, MassBank of North America and home-made database OSI-SMMS
[32]	Extracting with buffer	Orbitrap	top10 data-dependent acquisition	Peak alignment: Compound Discoverer	Lipid database establishment: LipidSearch with a 500-cell extract Metabolite identification: based on the homemade lipid database Candidate molecular prediction: MetFrag based on HMDB, KEGG, and LIPID MAPS
[47]	Laser desorption/ionization	Orbitrap	Full-scan MS (negative mode)	Raw data conversion: imzMLConverter	METASPACE based on HMDB and CoreMetabolome database
[53]	Capillary microsampling	Orbitrap	Full-scan MS (negative mode)	Raw data conversion: ProteoWizard Peak calling, peak alignment, quality control: R (XCMS package)	Annotation based on HMDB
[54]	Capillary microsampling	Orbitrap	Full-scan MS (positive and negative mode)	Peak alignment: MarkerView	R based on candidates obtained from literature (Bach et al., 2011)
[55]	Capillary microsampling	Orbitrap	Full-scan MS (positive mode)	Metabolomic peak list generation: Xcalibur MS background removal, peak alignment: Geena 2	MetaboAnalyst
[63]	Capillary microsampling	Q-TOF	Full-scan MS (negative mode)		MassHunter Quantitative Analysis Software
[90]	Extracting with buffer	Triple quadrupole	MRM mode for TQ/MS DIA mode for QTOF/MS		DIA: MS-DIAL based on MS Bank database
[91]	Capillary microsampling	Orbitrap	Full-scan MS (positive and negative mode)		

Abbreviations: HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Table 6. Overview of experimental and analytical techniques from single-cell metabolomic researches focusing on tissue samples.

Ref.	Extraction method	Instrument type	Analytical method	Raw data processing software/method	Identification software/method
[56]	Capillary microsampling	Orbitrap	Full-scan MS (positive mode)	Raw data conversion: ProtroWizard Quality control, peak detection, filtering, correspondence: XCMS	Match accurate masses based on HMDB
[60]	Extracting with buffer	Orbitrap	Full-scan MS (positive mode) and data-dependent MS/MS mode	Formula assigment: Compound Discoverer	Compound Discoverer
[59]	Extracting with buffer	Orbitrap	Full-scan MS (positive mode) and data-dependent MS/MS mode	Peak detection: XCMS centWave Group redundant features: CAMERA	XCMS
[92]	Capillary microsampling	Orbitrap	Full-scan MS (positive and negative mode)	Peak table extraction: Thermo Xcalibur Qual Browser Peak alignment: MarkerView or MZmine2	Annotation based on KEGG database
[93]	Capillary microsampling	Orbitrap	Full-scan MS (positive mode)	Data conversion: MSConvert Preprocessing: Matlab	Annotation based on PMDB, HMDB and METLIN

Abbreviations: HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes; PMDB: Plant Metabolome Database.

Table 7. Overview of experimental and analytical techniques from single-cell metabolomic researches focusing on unicellular samples.

Ref.	Extraction method	Instrument type	Analytical method	Raw data processing software/method	Identification software/method
[57]	Laser desorption/ionization	Orbitrap	Full-scan MS (positive and negative mode)	Raw data conversion: Thermo File Converter Noise removal, peak alignment: R (packages MALDIquant and MALDIquantForeign)	
[58]	Electro-migration and electroporation	TIMS-TOF	Full-scan MS (positive and negative mode)		Annotation based on YMDB, HMDB and PMPD

Abbreviations: YMDB: Yeast Metabolome Database; HMDB: Human Metabolome Database; PMDB: Plant Metabolome Database; PMPD: plant metabolic pathway databases.

metabolomics with other omics data, referred to as multi-omics integration, is a rapidly evolving area [46]. SCM can be combined with single-cell transcriptomics and proteomics to link metabolic states with gene expression patterns [63,64]. By correlating metabolic profiles with transcriptomic data, researchers can construct comprehensive molecular networks, revealing the intricate interplay between genes, and metabolites that govern cellular functions and dysfunctions [63].

3. Core technologies or workflow of single-cell metabolomics

SCM relies on ultra-sensitive technologies that enable metabolic profiling from individual cells across a wide range of biological and medical systems. The workflow typically begins with precise cell isolation by cell sorter or *in-situ* tissue sampling [36,47].

In biomedical research, FACS and microfluidic sorting are widely used to isolate circulating tumor cells (CTCs), drug-resistant cancer subclones, and rare immune populations such as activated $CD8^+$ T cells from patient blood samples [49,62]. In clinical pathology, laser-capture microdissection (LCM) enables precise isolation of defined regions within biopsies, including paracancerous normal tissues, fibroblast tissues and tumor tissues from human hepatocellular carcinoma tissue [3]. Microcapillary sampling has also been applied to large mammalian cells such as hepatocytes [65], while imaging mass spectrometry techniques, such as MALDI and DESI, enable direct metabolic profiling of tumor tissue sections, providing valuable insights in cancer research [41]. These techniques offer high sensitivity and spatial resolution, making them suitable for detailed molecular analysis of complex biological samples. In plant biology, single-cell transcriptomics is increasingly used to dissect developmental trajectories [37,38]. Single cells are typically obtained through protoplast isolation [66], which removes the cell wall to release individual cells. Findings from single-cell transcriptomics can be further strengthened and complemented by additional layers of evidence, such as MS−based spatial multi-omics. Recent studies of tension wood formation in poplar under mechanical stress have combined single-cell transcriptomic analyses with MALDI imaging and LCM-based metabolomics to validate developmental pathways [46]. Cross-species investigations, such as those comparing reductive evolution in gymnosperms and angiosperms, integrate single-cell transcriptomics with MALDI imaging and LCM proteomics to provide deeper insights and robust support for evolutionary conclusions [67].

Metabolite detection is then performed using platforms adapted to sample type and clinical questions. MALDI-MS imaging provides spatially resolved metabolite maps in cancer biopsies, enabling visualization of metabolic gradients between tumor, stroma, and immune infiltrates [68]. Nano-ESI coupled with high-resolution MS is commonly used for cancer cells to quantify key metabolites such as sterol lipids, eicosanoids, or oncometabolites (e.g., 2-hydroxyglutarate in IDH-mutant gliomas) [69,70]. CE-MS excels in profiling highly polar metabolites in primary human cells, including GABAergic and glutamatergic neuron cells or pancreatic islet cells in diabetes research [71,72]. SIMS, with its subcellular resolution, has been used to probe drug and cellular metabolites distribution within individual macrophages [72], as well as metabolic anomalies in specific neuronal compartments and organelles [73].

Because single cells contain only femto- to picogram levels of metabolites and cannot be amplified like DNA or RNA, the resulting datasets are sparse and noisy, making high sensitivity and carefully

optimized preprocessing essential [36]. Downstream analysis includes robust feature detection, rigorous metabolite annotation, which is especially important in complex medical samples containing drugs, metabolites, and lipids, and normalization strategies that account for large differences in cell size, from small lymphocytes to enlarged cancer cells [74,75]. Dimensionality reduction and clustering reveal metabolic heterogeneity underlying tumor drug resistance [76], immune cell in the tumor microenvironment [77], or pathogen metabolic adaptation [78]. Finally, pathway analysis or spatial mapping connects these metabolic signatures to disease mechanisms, such as immunometabolic heterogeneity in chronic infection or autoimmune diseases [79], metabolic rewiring in aggressive tumors [80], altered glucose and energy metabolism in neurodegeneration [81], or cell-type-specific vulnerabilities that can guide precision medicine [82].

4. Current limitations and challenges

Despite its rapid progress, SCM still faces significant bottlenecks. The abundance of metabolites in a single cell is extremely low, making detection highly sensitive to background noise [3,55,58,83]. Sample handling also poses major challenges, as metabolites can rapidly degrade or interconvert during cell isolation [84,85], requiring the development of ultra-low-loss, rapid-freezing, or microfluidic-based real-time capture techniques [86]. Quantification remains another limitation, as most SCM studies rely on relative quantification and lack robust internal standards or standardized quality control frameworks [57,83], hindering reproducibility across laboratories. Additionally, SCM data are chemically diverse and difficult to align with single-cell or spatial transcriptomics data for integrative analyses. A truly holistic multi-omics study of a single cell requires a comprehensive analytical workflow that captures all molecular types, enabling deeper insights into the relationships between its genotype and phenotype [83]. Lastly, clinical translation is still in its infancy due to the high cost and complexity of instrumentation, making it challenging to implement SCM in large-scale clinical or population studies.

5. Future directions and outlook

SCM is rapidly evolving, and future research will focus on three main areas: **(1) Enhancing sensitivity and spatial resolution.** Due to the extremely low abundance of metabolites in individual cells and the lack of amplification strategies, traditional MS faces significant challenges. To improve sensitivity and spatial resolution, researchers are developing zero-dead-volume, ultra-low-flow nano electrospray ionization (nanoESI) systems and integrating spatial omics technologies to achieve three-dimensional subcellular metabolite mapping. These technological advances will facilitate a deeper understanding of intracellular metabolic processes [3,32]. **(2) Improving metabolite identification accuracy and reliability.** Accurate metabolite identification is a critical challenge due to the low concentrations of metabolites in single cells. To enhance reliability, the establishment of stable internal standard systems and cross-laboratory quality control protocols is essential. High-throughput and reproducible analytical workflows are also needed to improve confidence in SCM results and facilitate potential clinical applications [87]. **(3) Artificial intelligence (AI)-assisted data analysis and clinical translation.** Given the high dimensionality and complexity of single-cell metabolomics data, conventional data analysis methods are often insufficient. AI and machine learning techniques will increasingly be employed to automatically extract biologically meaningful signals from complex single-cell metabolic landscapes, supporting data interpretation and clinical translation [88,89].

With the convergence of these technological advancements, standardized workflows, and AI-assisted analyses, SCM is poised to evolve from a research tool for exploring cellular heterogeneity into a cornerstone platform for precision medicine and early disease diagnosis.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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