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Current Opinion in Biotechnology

Single-cell metabolomics by mass spectrometry: ready for primetime?

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Single-cell metabolomics (SCMs) is a powerful tool for studying cellular heterogeneity by providing insight into the differences between individual cells. With the development of a set of promising SCMs pipelines, this maturing technology is expected to be widely used in biomedical research. However, before SCMs is ready for primetime, there are some challenges to overcome. In this review, we summarize the trends and challenges in the development of SCMs. We also highlight the latest methodologies, applications, and sketch the perspective for integration with other omics and imaging approaches.

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Current Opinion in Biotechnology 2023, **82**:102963

This review comes from a themed issue on **Analytical Biotechnology**

Edited by **Alexander Gruenberger, Janina Stephanie Bahnemann** and **Christian Dusny**

Available online xxxx

https://doi.org/10.1016/j.copbio.2023.102963

Introduction

Traditionally, knowledge on cellular biology has been obtained by analyzing large populations of cells. While population-level studies are essential in answering a multitude of biological questions. They are not without their limitations. Mainly, information from rare cell populations that might be relevant is averaged out, as well as the differences between individual cells, that is, cellular heterogeneity. Two genetically identical cells can have very different metabolomes [1]. Therefore, accounting for this intercellular heterogeneity in model systems is crucial for a more accurate understanding of the mechanisms of cellular biology [2–4]. Furthermore, the hidden cellular phenotypes play a key role in the diagnosis and treatment of diseases. For example, differences in single-cell metabolism can classify breast cancer cells into different subtypes, which can provide new insights for early diagnosis and cancer treatment and predict therapeutic outcomes [5–10]. Therefore, to address these gaps in knowledge, single-cell metabolomics (SCM) approaches have emerged [1,11,12].

Metabolomics is a field of research that involves identifying metabolites, measuring their abundance, and ultimately elucidating metabolism-related mechanisms that govern biological systems' behavior. Metabolomic studies are traditionally done using mass spectrometry (MS) or nuclear magnetic resonance [13,14]. Owing to the small volume of a mammalian cell, the sensitivity of the detection method is critical to the success of any SCM experiment. MS is therefore the method of choice in SCM because of its high sensitivity and the ability to identify metabolites by structure elucidation [13]. The current single-cell-based MS methods can be broadly divided into two types: imaging MS and live single-cell MS (LSC-MS). Single-cell imaging MS is usually using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) or laser ablation electrospray ionization mass spectrometry (LAESI-MS), which allows the mapping of the cellular metabolome in 3D. Secondary ion mass spectrometry allows for even higher spatial resolution, however, this comes at a cost of throughput, and increased fragmentation of measured analytes, which complicates downstream analysis. Imaging MS methods often requires extensive sample preparation that can interfere with the cells' microenvironment and by extension, its metabolome. LSC-MS attempts to address this by sampling and measuring live cells directly into the MS, with minimal disruption to their microenvironment. Imaging MS allows only manipulation of the sample and molecules in the gas phase, whereas live-cell SCMs allows manipulation of the sample in the liquid phase before MS analysis that allows for more quantitative results or increasing the sensitivity via derivatization for example [15–17].

Despite the promise of these single-cell MS methods, there are several challenges to overcome before their

wide-scale adoption in biological studies. First, the small sample volume and fast metabolite transition rate place high demands on the sensitivity, and the ability of the method to characterize the metabolome with minimal perturbations; second, traditional metabolomics methods, such as liquid chromatography–mass spectrometry (LC–MS), are wholly unsuitable for single-cell analysis due to the limited sample size. Third, achieving high-throughput single-cell sampling is a major technical challenge. Finally, quantitation and data analysis, especially in untargeted metabolomics, are still maturing [18]. In this review, new approaches and challenges in SCM metabolomics will be critically discussed, as well as what is needed to bring SCMs to primetime.

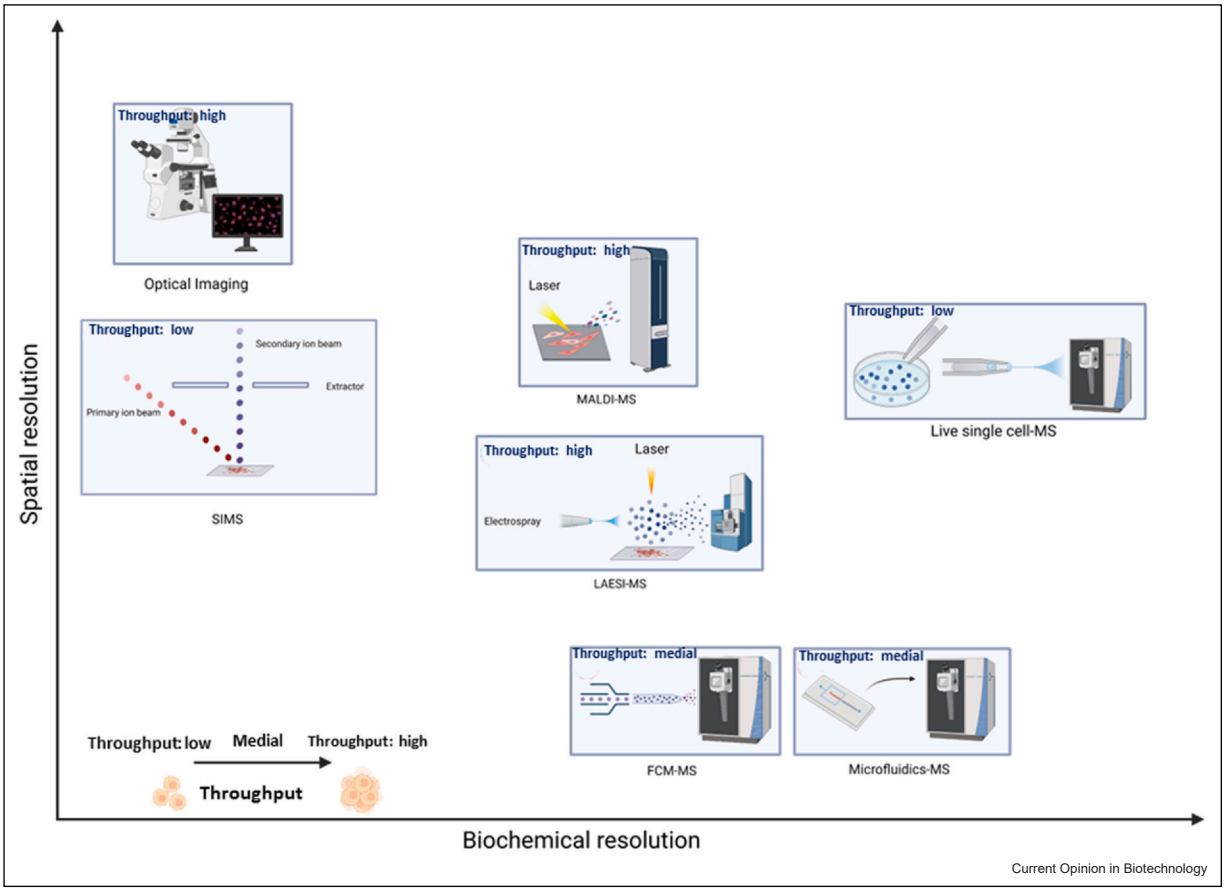
Single-cell metabolomics with mass spectrometry

In contrast to other omics, signals obtained from metabolic measurements cannot be amplified due to the great structural diversity of metabolites and the wide range of concentrations that can change rapidly over time. Furthermore, SCM requires precise single-cell isolation methods and sensitive instrumentation to detect metabolites in minimal volumes [13]. SCM by MS can be characterized broadly into imaging single-cell MS and LSC-MS. The recent developments and challenges in both approaches will be discussed in the following section (Figure 1).

Imaging single-cell mass spectrometry

The most common method in single-cell imaging MS is MALDI-MS. Typically, MALDI-MS measurements begin by mixing the desired sample with a matrix and then irradiating it with a UV laser beam under vacuum conditions. Subsequently, the analytes are ionized and accelerated to the MS [14]. Recently, some studies have also combined MALDI and optical microscopy to provide not only metabolic profiles but also more accurate spatial information and morphological properties of the measured cells. For instance, SpaceM, a platform developed by Luca Rappez et al [19••], combines

Figure 1



Schematics of different methods for SCM. Created with BioRender.com, accessed on EW24YEX58A, 31 January, 2023.

MALDI-MS and light microscopy to perform single-cell measurements. SpaceM is based on the precise estimation of which cell fractions are locally ablated by the MALDI laser, which is achieved by comatching microscopy images of cells with subcellular precision with MALDI laser ablation-labeled microscopy images. Ultimately, SpaceM generates a spatial molecular matrix for each cell containing normalized metabolic profiles and microscope-derived phenotypic properties such as cellular fluorescence intensity and morphospatial characteristics. MALDI-MS methods' main advantage is their high throughput, which is exemplified in SpaceM's ability to detect 100 metabolites and lipids per cell with a 1000-cell/hour throughput [19••]. The main limitation of MALDI-MS is the need to treat the cells before measurements, which increases the chances of perturbations that might lead to metabolic changes. LAESI-MS attempts to circumvent this by sampling the cells while they are still alive in their cellular environment. A promising technique by Michael J. Taylor et al. reported the integration of a microscope into the optical column of a LAESI source to allow visually informative ambient in situ single-cell analysis, and in combination with a drift tube, ion mobility mass spectrometery (IM-MS) can enable the system to identify structures with high confidence and their spatial position in tissue to analyze the metabolite content of onion epidermal cells with 25 annotated metabolites reported. While LAESI-MS offers less perturbations/disruptions, it possesses relatively less throughput and coverage than the aforementioned MALDI-MS method [20•]. Furthermore, with the current spot size of ± 50 μm, the method is not well suited for studying typical mammalian cells, which has an average diameter of ± 10 μm. In summary, imaging single-cell MS methods can have very high throughput, and can give more spatial information, but with some exceptions, it usually requires pretreatment of the sample before analysis, which can have an impact on the true metabolic profile. Furthermore, they are often only qualitative methods. This is largely because the sample such as cells, is manipulated in the gas phase, with no easy method to add an internal standard for quantitation.

Live single-cell mass spectrometry

LSC-MS methods are designed to sample cells with minimal perturbation to their microenvironment, ideally, they also aim for more quantitative performance than imaging MS methods. A typical setup consists of a micropipette attached to a 3D micromanipulator that is connected to a microscope. The cells are sampled under microscopic observation and then transferred directly to the MS via direct infusion (DI–MS) or after separation by nano-LC–MS. Masujima et al. reported a DI-based LSC-MS method, which is among the simplest in terms of sample manipulation, where cells are collected and introduced to the MS using a coated capillary that is also a nanospray emitter [21]. This method only brings limited perturbations to the cells. It can also achieve relative quantitation by adding internal standards to the ionization solvent [22]. However, because there is no metabolite isolation and enrichment step, its biochemical resolution is low. To counter this, Hsiao-WeiLiao et al. used field-amplified sample injection capillary electrophoresis electrospray ionization mass spectrometry (FASI CE-ESI-MS) to detect 37 intracellular metabolites in individual neurons [23]. Except combined with CE, nano-LC is also a good method to separate metabolites to reach a high biochemical resolution. For example, Kohta Nakatani et al developed a SCMs analysis system based on highly sensitive nano-liquid chromatography–tandem mass spectrometry (nano-LC–MS/MS). Using a combination of single-cell in vivo sampling and nano-LC–MS/MS, they successfully detected 18 relatively abundant hydrophilic metabolites (16 amino acids and 2 nucleic acid-related metabolites) from single Henrietta Lacks (HeLa) cells (n = 22) and classified these 22 HeLa species into three different subclasses of cells, indicating differences in metabolic functions of cultured HeLa cell populations [24••].

Although the combination with isolation methods can have a higher biochemical sensitivity, a series of steps are required during the sample collection and its processing and they all can have a significant impact on the intracellular metabolism. Yan Zheng et al. established a high-performance functional probe electrospray mass spectrometry (FPESI-MS) to address this issue. This method uses a homemade linear manual manipulator for direct live-cell sampling, followed by desorption of the analytes using a nitrogen aggregation/gas heating system and a reduced graphene oxide-functional copper probe to aggregate the product ions into ion clusters, which improves ion transport and detection efficiency under conditions that restore the living cell growth environment as much as possible. They successfully analyzed 7 Alzheimer's disease (AD)-related neurotransmitters, 16 biomarkers, and 12 serum metabolites in pheochromocytoma of the rat adrenal medulla (PC12) single cells of AD rats using FPESI-MS, and the method is expected to be used for the rapid detection of biological samples and metabolites in vivo [25••].

LSC-MS methods are not without their limitations. One of them is that obtaining a large number of cells to gain statistically significant data is challenging due to the difficulty of sampling single cells. This can be addressed by automation, with several automated single-cell sampling platforms already commercially available. Another limitation is the lack of sample preparation, which makes quantitation difficult, and detection of certain difficult-to- ionize compounds where derivatization is needed.

Is single-cell metabolomic by mass spectrometry ready for primetime?

As previously mentioned, SCM succeeded in gaining information about cellular metabolism at the single-cell

level, which provided valuable biomedical insights [26–29]. The field of SCM is now approaching maturity, however, SCM still faces several challenges due to the complex chemical composition of metabolites in the metabolome and the very small size of the individual cells analyzed. Furthermore, since all methods are still complex and mainly focus on achieving high sensitivity and dynamic range, there is less emphasis on robustness and on reproducibility and data sharing and integration [30–33]. This exacerbated further by the lack of a standardized model to follow in reporting experimental and biological parameters for each method, an example of such model is standardized initiative for metabolomics, which can be adapted and applied to single-cell experiments [34].

Based on these challenges, the whole field is moving toward higher throughput to overcome technical differences, data analysis, and higher data quality, and to integrate with other histological techniques to obtain a more comprehensive picture that describes the cell behavior in biochemical terms. In the following section, these techniques that will help SCM reach primetime are highlighted.

High-throughput single-cell metabolomic

The hyphenation of high-throughput techniques such as microfluidics and SCMs is critical for improving throughput of SCM analyses. On this front, Leicheng Zhang et al. developed a microfluidics-based approach where combining spiral inertial microfluidics and IM-MS resulted in a high- throughput SCM method, with an increased biochemical resolving power. This was achieved by initially focusing cells suspended in methanol into a single stream in a helical microchannel. The separated cell streams are transferred to nanoelectrospray needles for lysis and ionization and subsequently analyzed by IM-MS in real time. The analysis system collects 6–8 single-cell metabolic fingerprints per minute and includes gas-phase collision cross-section measurements as additional molecular descriptors, improving the confidence of metabolite identification [35•]. Another technique that can be used to improve the throughput of SCMs is flow cytometry. Huan Yao et al. proposed a general strategy to reveal leukocyte heterogeneity and screen differentiated metabolites as biomarker candidates for leukocyte subtypes using label-free mass spectrometry (cytometry electrospray ionization mass spectrometry, CyESI-MS) combined with a homemade data processing workflow. The method had a throughput of up to 40 cells/min, annotated 36 significantly different metabolites, and clearly distinguished five leukemic subtypes [36•]. Techniques such as these are essential to improve the statistical power of any SCM experiment, however, the large datasets generated from the aforementioned methods require special consideration so that the statistical findings can translate to biological insights. Furthermore, the manipulation of the cells' microenvironment that typically occurs in such methods might result in changes in the cellular metabolome such as an altered redox state. Depending on the biological question to be answered, this might impact the data generated significantly, especially without proper controls in place such as measuring the possible metabolic shift in cells undergoing microfluidic treatment versus direct microsampling for example [37]. A summary highlighting the key differences between all the methods discussed earlier is shown in Table 1.

Data analysis and sharing

The data analysis and sharing of MS-based SCMs represent another important step in the maturity of the field. A typical readout of a metabolomics MS-based measurement is a large matrix of detected mass-to-charge ratio (m/z) features and their abundance. The number of features detected in untargeted experiments is usually in the tens of thousands, while the number of samples is usually less than that by orders of magnitude. Owing to its inherent complexity, multiple (pre)processing steps are often implemented to transform and reduce the data to a more manageable size, and to filter out the noise and false positives [38–43]. Therefore, a transparent processing pipeline is important to connect the raw data to biological interpretation. Furthermore, unlike genome, transcriptome, and so on, single-cell metabolome does not have its own database for researchers to refer to, and the lack of a secure and open data sharing and storage environment has hindered information exchange and collaboration between different laboratories. The findability, accessibility, interoperability, and reuse initiative was proposed to address these limitations and to improve data sharing across different laboratories [44,45]. Finally, the only way to integrate experimental data from multiple omics to get more accurate information is to obtain more quantitative data. There are two ways to achieve quantitative SCMs, the first is to add internal standards to the measurement process, the second is to make data processing more standardized to reduce technical differences and produce as much quantitative data as possible using mathematical normalization techniques.

Metabolomics in context

Despite the innovations highlighted above, it remains unclear how close single-cell MS data are to the biological truth. The only way to know this is to look at the data generated in SCM by MS experiments in context. This can be achieved by integrating other techniques such as microscopic imaging with an MS-based single-cell method. This integration will allow the measurement of the deviation in single-cell MS data compared with reporter-based imaging data, for example, which are arguably closer to the biological truth. This will also have

Table 1

A summary of the discussed methods, the pretreatment steps in each, the degree of undesired perturbations, the throughput, and coverage in terms of molecules detected.

Method	Pretreatment	Disruption	Throughput	Coverage
MALDI-MS [19]	Fixation, fluorescence staining, and drying	High (estimate)	> 1000 cells/hour	More than 100 metabolites
LAESI-MS [20]	Direct measurement on a Peltier-cooled stage (16 °C)	Low (estimate)	Tens/hour (estimate)	171 spectral features, 25 annotated species
FASI CE-ESI-MS [23]	Isolation, washing, – 20 °C freezing, drying, and reconstitution	High (estimate)	< 10 cells/hour (estimate)	37 metabolites
Nano-LC-MS/MS [24]	Trypsin-EDTA treatment and PBS wash	High (estimate)	< 10 cells/hour (estimate)	18 metabolites
FPESI-MS [25]	Wash with PBS, sampled in native environment	Low (estimate)	< 10 cells/hour	7 neurotransmitters and 16 metabolites
IM-MS (inertial microfluidics) [35]	Trypsinization and PBS wash	High	360–480 cells/hour	19 lipid species
Flow cytometry [36]	Trypsinization, centrifugation, and DPBS wash	High	Up to 40 cells/min	36 metabolites

the added benefit of quantifying the degree of undesired perturbations caused by the sample preparation in each single-cell MS method, and possibly highlights the metabolic pathways that are resistant to such perturbations. Another method of looking at the single-cell MS data in context is to combine the data obtained with other omics information to capture the full biological picture. There are multiple cross-omics initiatives that attempt to do just so [44–47]. These cross-omics initiatives are crucial for gaining biological insights into cellular behavior at the single-cell level [48–54]. Without in-context information, SCM data translatability into clinical or drug discovery context will be limited.

Conclusion

SCM is developing rapidly in various fields, but there are still many challenges to be solved, such as how to improve the sensitivity of assays, achieve high throughput, integrate with other omics, and optimize methods for standardized data processing and data sharing. Despite these challenges, we can expect that in the near future, single-cell MS metabolomics will be widely adopted in basic research and clinical applications.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare no conflicts of interests in this paper.

Acknowledgements

This work was supported by the Chinese Scholarship Council Grant [grant number: csc202006210037], Dutch Research Council Grant (NWO) [Grant number: IMMUNMET-16249], Dutch Research Council Grant (NWO) [Grant number: Expososome-NL-024004017], ZonMw TKI-LSH Neuromet project [grant number: LSHM18092], and NWO Exposome-scan project [grant number: 175.2019.032].

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