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# Single-cell metabolomics: where are we and where are we going?

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Single-cell metabolomics with mass spectrometry enables a large variety of metabolites to be simultaneously detected from individual cells, without any preselection or labelling, to map phenotypes on the single cell level. Although the field is relatively young, it is steadily progressing with an increasing number of active research groups, techniques for cell sampling and ionization, tools for data analysis, and applications to answer important biomedical and environmental questions. In addition, the community shows great creativity in overcoming challenges associated with low sample volumes, a wide range of metabolite species, and large datasets. Here, we briefly discuss publications since 2019 and aim to provide the unfamiliar reader with an insight into the field and the expert reader with an update on the current status of the field.

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## Introduction

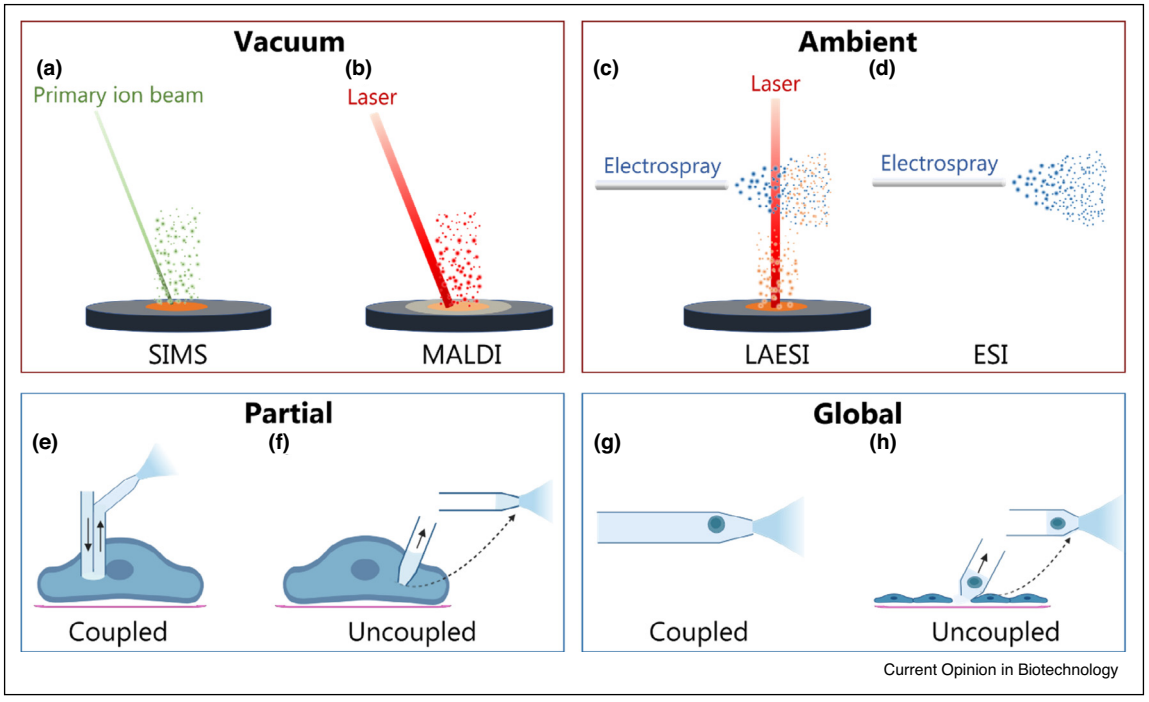
Individual cells govern the function of biological systems and are inherently heterogeneous to promote resilience. The cellular genotype constitutes the complete set of genes, however, the cell's characteristics are depicted in its phenotype, which can be observed by proteins and metabolites. Metabolites are precursors, intermediates, and end products of cellular processes, which makes them instrumental in signaling, sensing, and modulating the phenotype [1]. The cell's metabolome is dynamic and shifts rapidly upon cellular activity and environmental alterations. Thus, profiling the cell's metabolome with single-cell metabolomics provides a direct insight into its phenotype [1,2]. Although bulk analysis of a large amount of cells has more material available for analysis, single-cell metabolomics is required to identify phenotypic heterogeneity among individual cells and find subpopulations of seemingly similar cells to decipher the origin of drug resistance, differentiation, and disease progression.

Challenges in single-cell metabolomics include the low picoliter volume of material available for analysis, the rapid metabolome shift, and the large diversity in metabolite structures, properties, and concentrations, which range from nanomolar to millimolar [2,3]. Mass spectrometry (MS) enables detection of all ionizable metabolites in the cell sample without labelling or preselection. However, limitations include co-detection of isomeric and isobaric species, quantification, throughput, and high limits of detection due to low ionization efficiencies of some metabolites and signal division into several mass channels from natural adduct formation.

Despite the challenges, single-cell metabolomics is successfully achieved with MS using a collection of sampling strategies and ionization techniques (Figure 1a–h, Table 1, Box 1). Metabolites from individual cells can be sampled and ionized from a solid surface with secondary ion mass spectrometry (SIMS), matrix assisted laser desorption (MALDI), and laser ablation electrospray ionization (LAESI) (Figure 1a–c). Alternatively, metabolites can be sampled from the cell in liquid phase and ionized with electrospray ionization (ESI) (Figure 1d–h), either by directly infusing the sample or after separating it with capillary electrophoresis (CE) or liquid chromatography (LC).

10 years ago Heinemann and Zenobi summarized the field of single-cell metabolomics and declared that hardly any new biological insights had been generated. They also stated that techniques probing the metabolome at a single cell level to realize phenotypical differences were largely lacking although it possible with giant cells [4]. Since then the analytical community has successfully generated new technologies, but the challenges of sensitivity and quantification of metabolites in a 500 fL mammalian cell volume beyond readily ionizable metabolites, including lipids, remain. This review briefly summarizes strategies that are published since 2019 for achieving single-cell metabolomics and highlights some applications providing biological insights. The discussed strategies include cell handling, sampling, ionization, detection, annotation, quantification, throughput, and data analysis. Note that publications focusing on cells embedded in sectioned tissue or drug analysis are excluded. The interested reader is encouraged to explore additional and complementary reviews on metabolomics [1], spatial

**Figure 1**




Schematics of ionization and sampling techniques for single-cell metabolomics. The plumes represent ionized metabolites about to enter the mass spectrometer, see Box 1 for descriptions of each technique. **(a)** Secondary ion mass spectrometry (SIMS) **(b)** Matrix assisted laser desorption ionization (MALDI) **(c)** Laser ablation electrospray ionization (LAESI) **(d)** Electrospray ionization (ESI) **(e)** Partial sampling directly coupled to ESI **(f)** Partial sampling uncoupled to ESI **(g)** Global sampling directly coupled to ESI **(h)** Global sampling uncoupled to ESI. Note that schematics in d–h represent examples of the different sampling types for ESI.

metabolomics [3], single-cell metabolomics [2], chemical analysis of single cells [5], microfluidics [6], and data analysis [7].

## Cell handling and metabolite sampling

Generation of excellent data requires exceptional samples, which for single-cell metabolomics is a direct result of how the cells are handled before and during sampling. Most eukaryotic cells are only 10−20 μm in diameter and therefore challenging to handle individually. For single-cell metabolomics there are three main strategies for handling and sampling metabolites from individual cells reported: culture cells on a substrate; keep cells in suspension; allow individual cells in suspension to settle on a substrate. The optimal strategy depends both on the cell type and the analytical procedure of metabolite sampling and ionization that will follow.

For sampling and ionizing cell samples in vacuum, most studies use dried cultured cells on substrates (Table 1). Important considerations for sample preparation are ensuring cell integrity, with conserved metabolite localizations for subcellular imaging with SIMS [8,9,10•], and high quality of the chemical matrix applied on top of the sample for MALDI (Figure 1a and b) [11••,12,13]. For LAESI, individual cell handling is minimal when individual cells are targeted in intact plant tissue under ambient conditions (Figure 1c) [14,15]. In contrast to SIMS, MALDI, and LAESI, where metabolites are sampled and ionized in gas phase directly from the solid cell surface; ESI requires metabolites to be sampled into a liquid (Figure 1d). ESI is the most commonly used ionization technique for single-cell metabolomics and is also combined with the most cell handling and sampling strategies (Figure 1e–h, Table 1).

Strategies for sampling metabolites from individual cells for ESI can be divided into two main categories: partial sampling where a portion of the cytoplasm is physically removed with a probe or thin capillary, and global sampling where metabolites from the entire cell are desorbed into solution. After partial or global sampling the metabolites are transferred for ESI either directly using a coupled approach (Figure 1e and g) or in a two-step fashion using an uncoupled approach (Figure 1f and h) (Table 1). In the partial coupled strategy, a probe with a continuously flowing solvent is inserted into an adherent cell and the material desorbed into the solvent is directly transported for ESI (Figure 1e) [16,17]. In comparison, in the partial uncoupled strategy a thin capillary is inserted

**Table 1**

**Ionization techniques employed for cells in different milieus before sampling, during metabolite extraction, and analysis. ESI* represents strategies using coupled ESI, ESI** uncoupled ESI, and ESI*** separation before ESI**

| | | SIMS | MALDI/LDI | LAESI | ESI* | ESI** | ESI*** | No. papers |
|---|---|---|---|---|---|---|---|---|
| Cell milieu | Cultured on substrate | [8,9,10•] | [11••,13] | | [17,27] | [18•,20,31,32,47] | [35,40,41] | 15 |
| | Settled on substrate | | [12,48–50] | | | [19,30,33] | [36] | 8 |
| | In suspension | | | | [16,22–24,25•, 26,27,29••] | [34] | [42•] | 10 |
| | Intact in tissue | | | [14,15,43] | | | [37,38,39•,51] | 7 |
| Cell sampling state | Lysed | | [50] | | | [47] | [35,36,40,41] | 6 |
| | Frozen | [8,9,10•] | [13] | | | | | 4 |
| | Fixed | | [11••,12] | | [28] | | | 3 |
| | Dried (alive) | | | | | [30–33] | | 4 |
| | None (alive) | | [48,49] | [14,15,43] | [16,17,22–24,25•, 26,27,29••] | [18•,19,20,34] | [37,38,39•,42•,51] | 23 |
| Analysis | Global | | [11••,12,48, 49,50] | [14,15,43] | [16,22–24,25•, 26,27,29••] | [30–34,47] | [35,36,40,41,42•] | 28 |
| | Partial | | [13] | | [17] | [18•,19,20] | [37,38,39•,51] | 9 |
| | Imaging | [8,9,10•] | | | | | | 3 |
| No. papers | | 3 | 6 | 3 | 10 | 9 | 9 | |

into an adherent cell to withdraw a small volume of the cytoplasm. Subsequently, the capillary is moved to the MS inlet for ESI (Figure 1f) [16,17,18•,19–21]. The partial sampling strategy can provide specific chemical information from organelles and the cytosol to target metabolites inside the cell and molecule transported through the plasma membrane. Limitations of partial sampling include the time needed to precisely insert the tip into the cell and the restricted reproducibility due to sampling the chemically heterogeneous cellular interior.

**Box 1 Mass spectrometry and ionization**

Mass spectrometry requires molecules to become gas phase ions before separating them and detecting them in the mass analyzer according to their mass-to-charge ratio ($m/z$). The intensity of the detected signal depends both on the molecule's concentration and on how well it ionizes. There are several approaches to ionization and the four briefly described below have been recently used in single-cell metabolomics. In SIMS, the solid surface in vacuum is bombarded by a highly focused beam of primary ions. As the primary ions hit the surface, material is sputtered from the surface and ionized. The choice of primary ions govern the size of the beam and the extent of molecular fragmentation during ionization. In MALDI, the sample surface is first covered with a chemical matrix capable of absorbing energy from a laser. Following that, the sample is placed in vacuum and irradiated with a laser beam. This causes ablation of material from the surface into a plume where molecules are ionized from the charged chemical matrix. The choice of laser determines the size of the beam and needs to be matched with the choice of matrix, which also influences the ionization of molecular species. In LAESI, the solid sample surface is irradiated by a laser under ambient conditions and the ablated molecules are ionized in gas phase by charged droplets from ESI. In ESI, the molecules are ionized in liquid phase under high voltage and transferred to gas phase ions from fine droplets. The choice of voltage, solvent and flow rate influences ionization of molecular species. See Figure 1 for schematics.

These limitations are avoided using global sampling, which is the most commonly used sampling strategy (Table 1). Global sampling using a coupled strategy is achieved by directly infusing a diluted suspension of cells (Figure 1g) [22–24], or separating a cell in a droplet [25•], in front of [26] or inside a capillary for ESI [27,28,29••]. A solvent with high organic content is generally used to simultaneously lyse the cell and desorb the cellular metabolites for subsequent ESI. In the global uncoupled sampling strategy the cell can be adherent on a substrate [30–32] or trapped in a microwell [33]. Metabolites are then desorbed into a dispensed solvent droplet that is withdrawn into a capillary and moved for ESI, or the entire cell is sucked into the capillary for ESI (Figure 1h) [34]. Global sampling for ESI of suspended cells enables the entire metabolome of the cell to be analyzed in a high throughput analysis, but the limitations include morphological and phenotypical alterations of adherent cells forced into suspension.

Considerations for cell handling and sampling strategies in future strategies should ideally include: ensuring cell integrity to avoid analyzing the metabolome of stressed or dying cells, which is a risk when living cells are dried or fixed and adherent cells are in suspension; and facilitate high throughput sampling and analysis to enable statistical separation of individual cells and subpopulations of cells. As the field of single-cell metabolomics continues to advance, we anticipate that detected artifacts due to cell

handling and suggestions for best practices will be increasingly explored and reported.

## Detection, annotation and quantification of metabolites

To realize the impact of metabolites and metabolic pathways on the cell or cellular system, metabolite species first need to be ionized (Box 1). The ionization efficiency for selected metabolites can be increased by charge tagging, separation before ionization, or instrumental settings. Charge tagging refers to adding a permanently charged functional group, such as a quaternary amine, to the metabolite [18•,19,20]. This has, for example, decreased the limit of detection 100 times for cholesterol [19] and over 100 times for ribonucleotides [35]. The addition of a separation step, such as CE [36–38,39•,40] or LC [35,41], before ionization pre-concentrates metabolites into bands that elute separately and ionize one at a time, which increases analysis time but lowers detection limits. Enhancing detectability of metabolites is an important topic that has the possibility to generate data beyond the most ionizable species.

Metabolites are mostly composed of the same basic elements C, O, N, S, P, and H, which results in numerous isomeric and isobaric structures that overlap in the mass spectrum and challenges annotation. Instruments with high mass resolving power minimize overlap with isobars; however, the isomers cannot be distinguished. A common strategy is to use tandem MS to detect unique fragmentation ions corresponding to each isomer. Additional tools for metabolite annotation include chemical derivatization [18•,20] that can be combined with tandem MS [19,28,34] or LC [35,41], ion mobility spectrometry (IMS) [15,24], and separation using CE [36–38,39•,40] or LC [42•]. Derivatization can pinpoint double bond positions in isomeric lipids [28,34], and IMS can separate isomeric species by their collisional cross sections [15]. Furthermore, CE enables separation and annotation of the isomeric amino acids leucine and isoleucine in an individual HeLa cell [40]. Because of their different actions, the ability to separate and annotate isomeric species is essential to realize their impact on the biological system.

Another point for realizing the metabolite's impact on the biological system is determining to what extent its concentration is different between individual cells. Although MS enables untargeted detection of metabolites, concentrations are not directly correlated to their detected intensities due to differences in ionization efficiency. Since 2019, studies using CE have reported using external calibration curves for quantifying metabolites [36,40], and quantification with ESI have used one point calibrations achieved by including internal standards of known concentration into the solvent [25•,33]. With quantitative data the fold change can be calculated and used to describe the extent of phenotypical alterations associated with the metabolic state of the cell. We foresee that quantification will be additionally explored together with enhancing detectability and annotation in future studies.

## Throughput and data analysis

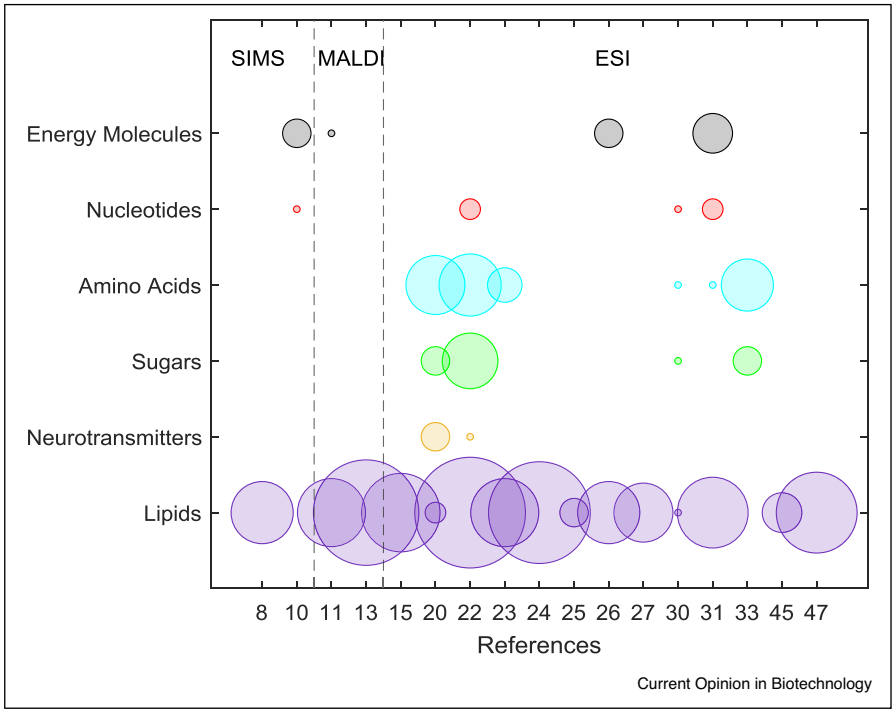
Deciphering metabolite heterogeneity and identification of unique subpopulations of cells may be key to understanding disease onset, progression, and recovery. However, this requires the ability to analyze a large amount of cells in a reasonable period of time. Although up to 1000 individual cells have been analyzed with ESI [25•] and LAESI [14,43], the highest numbers of 5000 [12] and 30 000 [11••] cells have been reported using MALDI. Data for the 30 000 cells were collected in MALDI imaging mode and to ensure that only signals obtained from the cell body were used for further evaluation, data was correlated with fluorescent microscopy and 10 000 of the 30 000 cells were excluded from further evaluation [11••]. Phenotypical classification of cells has been achieved with 800 cells using MALDI [44], and 100–200 using ESI [22,23,45]. Overall, these results indicate that the absolute number of cells necessary for making conclusions may depend both on cell type, treatment, and repeatability of analysis. In addition, depending on the analytical technique chosen for single cell metabolomics the inherent uncertainty will be different. Therefore, conducting a power analysis that includes all sources of error can assist to realize the number of cells required for the planned experiment.

The large amount of data generated from hundreds to thousands of cells requires specialized strategies for data processing. These strategies include using in-house developed scripts [23,25•] and new software based on machine learning methods [11••,23,37,44–46]. For visualization and multi-dimensional data analyses approaches such as t-distributed stochastic neighbor embedding [11••,16,22,27,30], principal component analysis [17,18•,21,23,25•,31,47], linear discriminant analysis [26], and partial least square discriminant analysis [15,43] are generally used. Software tools for data handling are anticipated to keep developing as more metabolomics data of a large amount of individual cells are generated.

## Selected applications

The field of single-cell metabolomics is continuously evolving. Although many studies focus on either sampling, detecting metabolites from individual cells, or data analysis, the number of papers with biological applications are increasing. The majority of detected metabolite species are phospholipids, but several studies using ESI also report detecting metabolites such as neurotransmitters, sugars, amino acids, nucleotides, and energy transferring molecules from individual mammalian cells (Figure 2). For example, single-cell metabolomics using ESI has revealed phenotypical differences between

**Figure 2**




Reported metabolites detected from mammalian cells (grouped on the y-axis) using different ionization techniques (individual papers on the x-axis). The size of the bubble represents the number of species reported, from 1 to 38. Metabolites involved in intracellular energy transfer such as ATP are grouped as energy metabolites. Nucleotides represent non-energy transferring nucleotides, nucleosides and nucleic acids. Sugars consist of mostly monosaccharides, neurotransmitters mainly gamma-aminobutyric acid and acetylcholine, and lipids mainly of fatty acids, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, lysophosphatidylcholine, monoacylglycerol, diacylglycerol, and triacylglycerol. Strategies using separation are excluded.

individual wild type and chemo resistant cells. [22] Specifically, differential metabolites included arginine, glutamine, glutamic acid, and individual sphingomyelin and phosphatidylcholine species. Interestingly, a subpopulation of wild type cells showed similar phenotype as the chemo resistant cells, suggesting their progression towards resistance [22]. Another study found that human leukemia cells reacted differently to treatment with an anticancer drug. In particular, bimodal distributions of metabolites involved in the glycolysis pathway and cellular energy state, including 2-deoxy-D-glucose-phosphate and adenosine di-phosphate, indicated a difference in drug uptake based on the phenotype [33]. In addition, identification of lipid isomers by derivatization enabled differentiation of four breast cancer subtypes, showing the importance of specificity in metabolite detection [28].

The ability for subcellular imaging using SIMS has revealed local biosynthetic hotspots of *de novo* purine synthesis by detecting cytoplasmic loci of $^{15}$N-enriched 5-aminoimidazol-5-carboxamide ribonucleotide and adenosine triphosphate [8]. SIMS has also detected increased plasticity of the plasma membrane lipid composition resulting from repeated exocytosis events [9]. Specifically, an increase of the high curvature phospholipids phosphatidylethanolamine and phosphatidylinositol and decreased phosphatidylcholine were reported. Furthermore, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol have all been identified as markers for homeostatic state of hepatocytes with MALDI, while neutral lipids, including triacylglycerol and diacylglycerol, were markers for the steatotic state [11••]. Phosphatidylethanolamine was also found with higher intensity in primary isolated astrocytes while phosphatidylcholine and sphingomyeline were higher in neurons, reflecting the metabolic importance of cellular shape and function [12].

## Conclusions

Single-cell metabolomics is progressing rapidly and the challenges are accepted and creatively explored by an increasing community. Nevertheless, there is still work to be done in cell and sample handling to ensure cell integrity, in ionization to increasing metabolite coverage, and in quantification, throughput, and data handling to establish the metabolic status and phenotype of

individual cells. Overall, we anticipate that single-cell metabolomics will continue to achieve novel insights in individual cells or cellular subpopulations with different phenotypes that are essential to cellular differentiation, disease onset and progression, and drug treatment.

## Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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