Single-Cell Metabolomics

Hamidun Bunawan and Syarul Nataqain Baharum

Abstract Metabolomics, in conjunction with more conventional practises of genomics, transcriptomics and proteomics, provides a significant and holistic approach for a better understanding of the behaviour of individual cells. In a single cell, the transcription process produces mRNA which is translated into proteins, and these proteins act as biocatalysts to control metabolite biosynthesis. The study of metabolites in cellular processes is considered as a bridge that closes the gap between genotype and phenotype, and it provides a complete view on the "functionality" of each individual cell. Metabolomics is more reliable in comparison to other single-cell omics studies, as it offers a big picture on the dynamic functionality of a cell. Nevertheless, this technique is also the most problematic to quantify as the metabolome changes rapidly. The metabolome at the level of single cells is a perfect indicator of phenotypic heterogeneity, however, the techniques required to study the metabolome are relatively new. Further research is required to enhance the technique to improve the coverage of the metabolome, faster and accurate identification of metabolites, and to develop rapid non-destructive measurements. Analysis of the metabolome has to contend with the diversity of biomolecules and the grouping of different analytical platforms for complete metabolomics studies especially in single-cells metabolomics. In this chapter, the recent improvements in analytical tools to unravel single-cell metabolomics, as well as their specificity, will be described. This includes the exciting development and expansion of analytical tool technologies in metabolite analysis. These remarkable technological improvements applied to single cells have encountered several intrinsic limitations and challenges, and these major challenges will be discussed alongside the future prospects of single-cell metabolomics in systems biology.

Keywords Metabolomics · Single cell · Omics · Analytical · Systems biology

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1 Introduction

Metabolomics is a developing technology that is used to assist in the biochemical analysis of the metabolome: small intracellular and extracellular molecules within a biological sample [1–3]. These molecules are involved in multiple basic intracellular processes and the physiology of the cell can be indicated by the metabolome. Metabolomics also includes the technology for sampling cells, the experimental methods used, the detection of metabolites, measuring concentrations of metabolites and interpreting the metabolomics data generated [4, 5]. Being able to detect and characterise cellular metabolites at the single-cell level, as well as to quantify their amounts, permits a variety of interesting researches including the analysis of functional heterogeneity between cells, even those which appear to have a homogeneous cell population [6, 7].

The analysis of single cells is an emergent field of research that captivates researchers from many disciplines because it provides a glimpse into the fundamental biological aspects such as evolution, cell adaptation and cell differentiation [8–10]. Metabolomics can assist in deciphering key mechanisms of cellular behaviour and contributes significantly to our understanding of metabolism. With the genetic information present in every cell and as genomics is a well-defined discipline, using DNA sequencing and bioinformatics, gene structure and function can be analysed. In cells, mRNA transcripts are produced and translated into proteins, which subsequently act as biocatalysts to control metabolite biosynthesis (Fig. 1). Understanding the final products, or metabolites, produced by this process is fundamental for gaining an insight into the metabolic functions of a particular cell. The swift development of metabolomics supplements genomic, transcriptomic and proteomic data to enable systematical research into biological systems and networks of single cells [11, 12]. Metabolomics has been demonstrated as a significant tool in

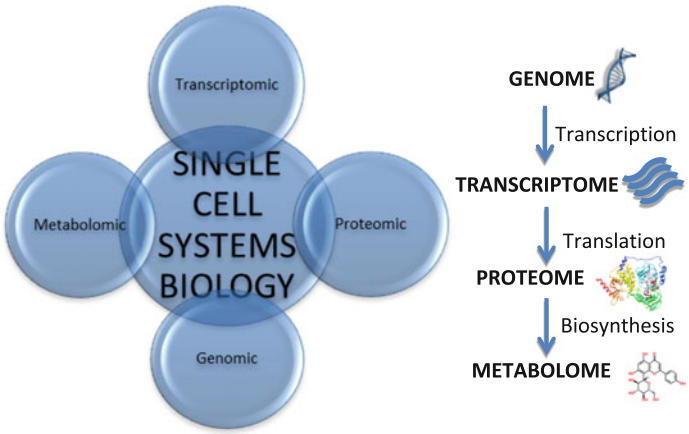

Fig. 1 Interconnection of the genome, transcriptome, proteome and metabolome within cells

anticipating and elucidating complex phenotypes in varied biological systems and has been shown as a vital method in functional genomics, illuminating individual gene functions from the comprehensive analysis of the metabolome.

Single-cell metabolomics analysis aims to help further understanding of cellular functions and to elucidate differences in single cells relative to cell populations [12–14]. One key factor is that the coordinated activities among individual cells contribute to the physiology and behaviour of multicellular organisms, and also to the organisation of ecological communities in unicellular organisms [15, 16]. Cellular variability, as well as pathological and functional heterogeneities, are integral to the development of individual biological and behavioural traits, and have important roles in the aetiology of many diseases [17, 18].

Metabolites are varied and have fundamental roles in key cellular processes. The detection, characterisation and quantification of a diverse assortment of metabolites in a single, multistage experiment are important technological objectives in single-cell metabolomics. Multiple recent advances in bioanalytical technologies for individual cell metabolome studies are available [12].

Since every single cell contributes to the product yield of the fermentation process in industrial biotechnology, it is important that lifeless, inactive or weakly active cells can be identified, as they reduce the productivity [19]. This heterogeneity is due to distinct intrinsic cell features such as age, cell cycle stage, position of the division plane, gene transfer or loss, mutations or epigenetic inheritance [19]. Therefore, single-cell related analytical techniques are required to assist in evaluating and controlling these processes. Similarly, external parameters influence cellular features due to numerous microenvironmental variations, such as the availability of carbon or other energy sources and the prevalence of stress conditions. Furthermore, in many research areas including haematology, stem cell biology, tissue engineering and cancer biology, the interpretation of data from the analysis of multiple cells can be problematic. The heterogeneity of populations and variation in dynamics within the sample is responsible for some of the difficulties in analysis and the generation of ambiguous measurements of the cell population. These emphasise the need for molecular biology methods that work at the level of the single cell [13, 20].

In this chapter, we aim to provide an overview of methodological advances in single-cell metabolomics to enable the metabolomes of every single cell in a population to be studied individually, and to elucidate information that is not obtainable from studies at the population level. Significant advances in single-cell sampling using microfluidics and nanoscale devices are discussed. The recent enhancements in sensitivity and specificity for analysis at the single-cell level using mass spectrometry, mass spectrometry imaging, capillary electrophoresis (CE) and nuclear magnetic resonance (NMR) are highlighted. The limitations and challenges of single-cell metabolomics are also discussed, as well as emerging developments within different fields aimed to illuminate the immense knowledge available upon analysis of the metabolomes of single cells.

2 Metabolomics Approach in Single-Cell Study

Single-cell metabolomics is an emerging research field with the development of new and sophisticated analytical platforms with high sensitivity and the ability to perform quantitative analyses. Advancements in mass spectrometry (MS) metabolomics for example, have made the study of metabolites at a cellular level a reality, hence increasing the unbiased characterisation of metabolites at the cellular level of biological systems [21]. Single-cell analysis mass spectrometry has been used widely and a significant numbers of other analytical approaches were developed to target very low metabolites in a cell [22]. Misra et al. [23] has summarised the recent studies in plant single cell and single-cell-type metabolomics as presented in Table 1. The studies were based on different metabolomics platforms such as GC-MS, UPLC-FT-MS, LC-ESI-MS and NMR. We have also added more information about single-cell studies on other type of samples such as microbes, algae and animals.

The first key step for single-cell metabolomics is to isolate the appropriate cells from an organism of interest. In this chapter, we highlight two small-volume separations techniques for single-cell sampling and/or manipulating of the particular cell for the metabolites analysis. Microfluidics and nanoscale devices are two common methods to retrieve single cells in a high-throughput fashion. Subsequently, analytical approaches for detecting metabolites in single cell also are discussed. In particular, mass spectrometry, MS imaging, capillary electrophoresis and nuclear magnetic resonance (NMR).

3 Technologies in Sampling Single Cells

3.1 Microfluidics

Experimental mechanisms to measure the dynamics of single cells using manual pipetting and conventional cell culture methods have restricted output and reproducibility. In addition, these are not always able to accurately alter the cellular environment in real time. Furthermore, the large volume of media required contributes to background fluorescence, inconsistency in concentration and a decrease in cell-to-cell paracrine signalling as a result of the dilution of secreted molecules [59, 60].

Microfluidic devices have significantly influenced the field of analytical chemistry since their introduction in the early 1990s. These devices provide numerous advantages over comparable bench-top instruments [61, 62]. For example, the reduction in sample size and reagent volume is a crucial advantage. It is now possible to use and manipulate volumes that are orders of magnitude lower than what was feasible a few decades ago. Another advantage specific to microfluidic devices includes the integration of multiple analytical processes onto single platforms with very little dilution, increasing the overall sensitivity of the assay (Fig. 2)

Table 1 Recent studies highlighting the analytical tools used and results obtained in single-cell and single-cell-type metabolomics

Species	Material	Analytical approach[a]	Metabolites identififed	Class of metabolites	References
Subcellular metabolomics					
Hordeum vulgare	Vacuoles from mesophyll cell protoplast	GC-MS, UPLC-FT-MS	259	Amino acids, organic acids, sugars, specialised metabolites	[24]
Single-cell metabolomics					
Torenia hybrid	Petal cell	Nano-HPLC-MS	5	Anthocyanins	[25]
Closterium acerosum	Single cell	MALDI-MS	4	Central metabolites	[26]
Pelargonium zonale	Single leaf, stem, petal cell	Nano-ESI-MS	22	Monoterpenoids	[27]
Allium cepa, Narcissus pseudonarcissus	Epidermal single cell of bulbs	AP-ESI-MS	32, 22	Specialised metabolites, oligosaccharides	[28]
Arabidopsis thaliana, Hypericum perforatum, Hypericum reflexum	Individual dark glands from petals, leaves; glandular trichomes	LDI-ToF-MS	15	Naphthodianthrones, flavonoids	[29]
Chara. australis	Vacuole and cytoplasm	CE-MS	125	Sugar phosphate, coenzyme, organic acids	[30]
Aplysia. californica *Bacillus atrophaeus* *Xenopus. laevis* *Rattus. norvegicus*	Neurons Single cells Oocyte Peripheral nervous systems	CE-ESI-MS BAMS GC-MS CE-ESI-MS	31 15 26 300	Nucleotides Amino acids, organic acids Carbohydrates, organic acids, fatty acids Amino acids, carbohydrates	[31] [32] [33] [34]
Single-cell-type metabolomics					
Dilatris pillansii	Leaf and flower secretory cavities	Cryogenic, NMR, HPLC	7	Methoxyphenylphenalenones	[35]
Eucalyptus spp.	Leaf subdermal secretory cavities	GC, GC-MS	24	Mono- and sesqui-terpenoids	[36]

(continued)

Table 1 (continued)

Species	Material	Analytical approach[a]	Metabolites identififed	Class of metabolites	References
Catharanthus roseus	Leaf epidermome	LC-ESI-MS	2	Oleanolic and ursolic acid	[37]
Picea abies	Phloem parenchyma cells	Cryogenic NMR	2	Stilbene glucosides, flavonoids	[38]
P. abies	Stone cells/sclereids	Cryogenic NMR, MS	2	Phenolic glycosides	[39]
Cucurbita maxima	Phloem latex (sieve tubes)	GC-ToF-MS, HPLC, FIE-MS	80	Amino acids, sugars	[40]
Glycine max	Root hairs	GC-MS, UPLC-QToF-MS	634	Amino acids, sugars, sugar alcohols, fatty acids, flavonoids, organic acids, nucleosides, phenolic, glucosinolates, saponins, alkaloids	[41]
Lilium longiflorum	Pollen grains	GC-ToF-MS	252	Sugars, organic acids, amino acids	[42]
Gossypium hirsutum	Fibre cells	GC-MS	86	Non-polar(sterols, alkanes) and polar (sugars, sugar alcohols, amino acids)	[43]
G. hirsutum	Fibre cells	GC-MS	27	Organic acids, amino acids, sugars	[44]
Citrus paradise	Epithelial and parenchyma cells	GC, GC-MS, UPLC-QToF-MS	28	Terpenoid, sterols, fatty acids, carotenoids, oxygen heterocyclics	[45]
A. thaliana	Guard cell and mesophyll cell protoplasts	LC-(MRM)-MS/MS	85	Phytohormones, signalling molecules, phenolics, flavonoids, amino acids	[46]
Lycopersicon hirsutum	Glandular trichomes	GC-MS	7	Terpenoids	[47]
Solanum spp	Trichomes	LC-MS	119	Terpenoid, flavonoids, fatty acids, alkaloids, acyl sugars	[48]
Solanum lycopersicum	Glandular trichomes	LC-ToF-MS	32	Acylated molecules, flavonoids, glycosides	[49]
Artemisia annua	Glandular trichomes	GC-MS	12	Mono- and sesqui-terpenoids	[50]
Cannabis sativa	Trichomes types	LC-MS, NMR	9	Cannabinoids	[51]

(continued)

Table 1 (continued)

Species	Material	Analytical approach[a]	Metabolites identififed	Class of metabolites	References
A. thaliana	Epidermal trichomes, basal/pavement cells	GC-ToF-MS	117	Amino acids, fatty acids and alcohols, alkanes, lipids N-compounds, organic acids, polyhydroxy acids, polyols, sugars, sugar conjugates, phenylpropanoids	[52]
A. thaliana	Glandular trichomes	UPLC-ESI-QToF-MS	13	Glucosinolates	[53]
Colquhounia coccinea	Peltate glandular trichomes	UPLC-MS/MS, X-ray diffraction	3	Sesqui-terpenoids (colquhounoids)	[54]
Nicotiana attenuate	Glandular trichomes	^{1}H-NMR, LC-QToF-MS	>2	Nicotine, phaseoloidin, acyl sugars, fatty acids	[55]
Ocimum basilicum	Peltate and capitate glandular trichomes	GC-MS, HPLC	15	Terpenoids, phenylpropenes	[56]
A. thaliana	Epidermal cell layer, palisade mesophyll cells, vascular bundle	MALDI-ToF-MS	18	Cell wall polysaccharides	[57]
A. thaliana	Endodermis, epidermis, columella, cortex, stele	GFP-FACS, UPLC-QToF-MS	50	Glucosinolates, phenylpropanoids, dipeptides	[58]

[a]Abbreviations: AP, atmospheric pressure; ESI, electrospray ionization; FACS, fluorescence-activated cell sorting; FIE, flow injection electrospray; FT, Fourier transform; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LDI, matrix-free laser desorption/ionization; MALDI, matrix-assisted laser desorption/ionization; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; QToF, quadrupole time-of-flight; ToF, time-of-flight; UPLC, ultra-performance liquid chromatography. CE, capillary electrophoresis, BAMS, bioaerosol mass spectrometry. Adapted from Ref. [23], Copyright 2014, with permission from Elsevier

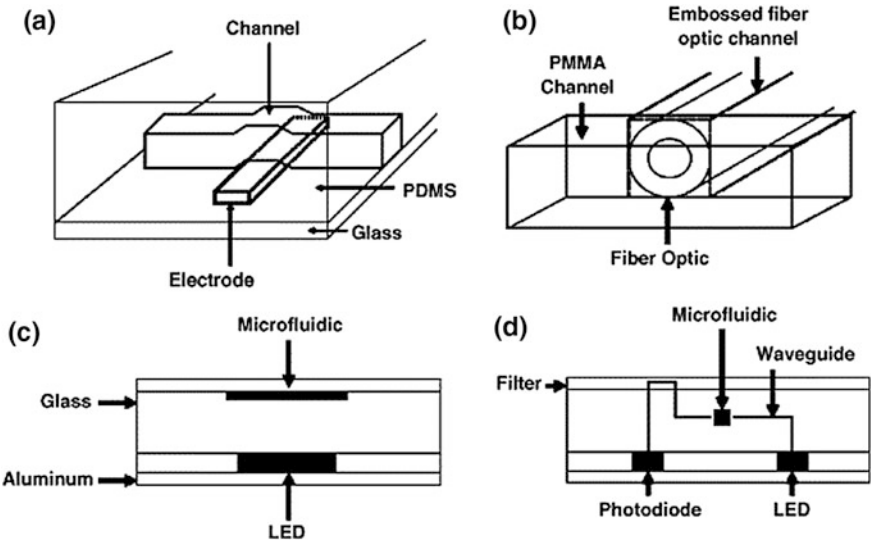

Fig. 2 Different techniques are integrated on microfluidics devices. **a** Soft polymer lithography, **b** Poly(Methylmethacrylate), **c** Complementary metal–oxide–semiconductor (CMOS) processing and **d** two-photon three-dimensional lithography. Reprinted from Ref. [64], Copyright 2007, with permission from Elsevier

[63, 64]. Furthermore, microfluidics is also useful for the observation and classification of individual cells, their stimulation within the microfluidics device, and their rapid and accurate identification.

The analysis of microbial single cells using microfluidics has shown potential in several fields including growth, strain characterisation and morphological analysis, population heterogeneity, analysis of cellular response at defined constant environmental conditions and cell-to-cell heterogeneity at specific concentration gradients [65, 66].

Microbial single cells have been analysed by four different types of simple perfusion microfluidics [65]. These can be classified by the spatial directions in which single cells can propagate, specifically 3D, 2D, 1D and 0D. Fewer cells per cultivation volume mean that the environment surrounding the single cell can be controlled more accurately. Larger 3D as well as 2D planar populations are more likely to have environmental inhomogeneity and gradients across the micro-colonies than 1D and 0D systems. In addition/as a result, the replenishment of the cultivation medium is more inefficient.

Several microfluidics technologies are available for cell culture applications, including automated antibody labelling for determining signal transduction across multiple time points in fixed cells [67–69], the study of single-cell dynamic under microfluidic gradients [70]; microfluidic perfusion of cell culture arrays [71], single-cell trap assays [72] and the isolation of single cells in microwells [73]. Recently, Kellogs et al. [74] developed and described a useful method for

single-cell analysis using microfluidics. The protocol improves accuracy and greatly increases the output, whilst enhancing existing abilities in cell and fluid manipulation. This is a significant advancement in the analysis of single cells.

3.2 Nanoscale Devices

In the past 10 years, nanobiodevice techniques have focused on establishing four main fields in biomedical applications including disease diagnostics, in vivo imaging, regenerative medicine and nanotherapy [75]. This rapid progression in nanotechnology has developed outstanding nanotools such as near-field scanning optical microscopy (NSOM), optical fiber nanosensors, nanowire-based field effect transistors (FETs), scanning ion conductance microscopy (SICM) and atomic force microscopy (AFM) at the nanoscale level [76]. Recent new advances in these technologies have promised new discoveries that could help to reveal the nanostructure of cellular organelles, spatial biomolecules organisation and biochemical reactions at nanodomains. Two different types of nanofabrication technologies have been established: (1) top–down nanotechnology, (2) bottom-up nanotechnologies. Top-down nanotechnologies involve a combination of electron-beam lithography and plasma dry etching. In contrast, bottom-up technologies involve vapour–liquid–solid nanowire growth techniques [75].

Analysis of single cells at a high resolution for a nanoscale sample of the previously undetectable cell organelles is a tedious work. In addition, determining various cellular components and their three-dimensional organisation, unravelling the nanodomains for biochemical reactions and profiling cell-to-cell variations at the cellular level are also not easy tasks. Recently, a great effort has been made in analytical methods for observing, manipulating and exploring single cells at the nanoscale level [76]. Nevertheless, it is extremely exciting to accomplish a high spatial resolution for detecting the structure at the nanoscale. It is a must to have high sensitivity and specificity with high signal-to-noise (S/N) ratio for small amounts of a compound in individual organelles. An efficient set of tools must be established in order to analyse nanometrics organelles.

Pan et al. [77] developed the single-probe mass spectrometry (MS) technology, for real-time analysis of in situ metabolomics study of single living cells. The researchers have used the single-probe to detect several cellular metabolites and the anticancer small molecules paclitaxel, doxorubicin and OSW-1 in individual cervical cancer cells (HeLa) (Fig. 3).

In the past ten years, the progress in nanotechnology has accelerated the development of nanotools in single-cell studies at the nanoscale level for small structures and compound compositions. Nanoprobes are a type of nanoscale devices designed to probe single cells with minimum intrusion [76]. These techniques have huge potentials in important cellular processes. Developments in this technology

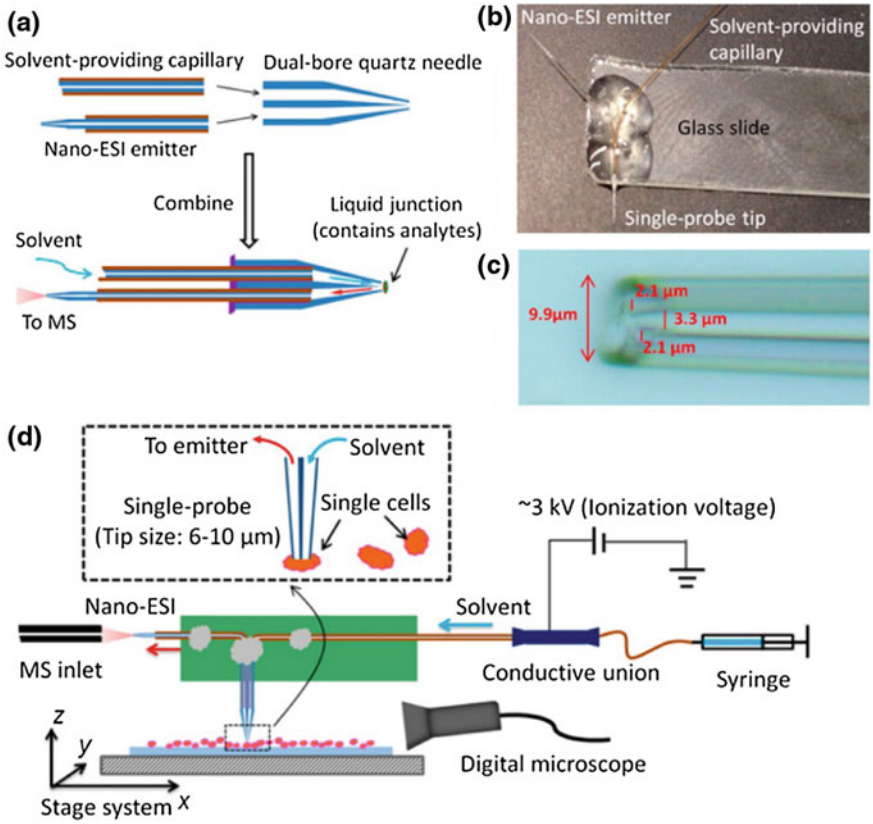

Fig. 3 Single-probe MS system from fabrication to utilisation. **a** Fabrication steps of the single-probe; **b** photograph of a working Single-probe; **c** 40× magnification of the single-probe tip with measurements obtained from the calibrated digital microscope; **d** setup schematic of a single-cell MS analysis. Reprinted with the permission from Ref. [77]. Copyright 2014 American Chemical Society

have helped in the observation and manipulation of single cells at the nanoscale level while elucidating their functions. The extensive use of high-resolution techniques can give insight in single-cell analysis at the nanoscale.

4 Recent Improvement of Sensitivity and Specificity

4.1 Mass Spectrometry (MS)

Mass spectrometry is both sensitive and fast, and therefore has a principal place in metabolomics. The requirement for simple protocols for diagnosis of human

diseases has pushed forward the development of mass spectrometry and improvements in equipment, methodology and software and databases. This has enabled metabolomics to move away from the quantitative approaches generally used. As a result, methods such as isotope labelling and tracing are being frequently used. Moreover, ambient ionization techniques such as desorption ionization and rapid evaporative ionization have permitted new MS imaging methods. Direct, real-time MS analysis has also proved as being useful [12].

Analytical chemistry has also been instrumental in the development of MS-based single-cell metabolomics, particularly those in which labelling of targeted molecules is not required and the methods are suitable for the detection of unknown molecules [12, 78, 79]. Several analytical techniques have been established that enable single-cell analyses: matrix-assisted laser desorption ionization (MALDI), desorption electrospray ionization, secondary ion MS (SIMS), laser desorption ionization, laser ablation electrospray, electrospray ionization (ESI), inductively coupled plasma (ICP) and nanostructure-initiator [12, 23, 80, 81].

Matrix-assisted laser desorption ionization (MALDI) and secondary ion mass spectrometry (SIMS) are commonly conducted in a vacuum and live cell analysis is therefore not possible. In addition to MALDI and SIMS, laser ablation electrospray ionization mass spectrometry (LAESI-MS) can also be used to analyse single cells [82]. Subcellular detection of metabolites has also been possible with LAESI-MS [83]. The use of a nanospray tip to obtain a small volume of cellular content prior to analysis by MS has also been used to analyse live cells.

Other methodologies which are useful for the analysis of single-cell metabolites include microarrays for mass spectrometry (MAMS) [84]. Whilst there were initially issues with the quality of the microarrays, meaning that direct microarrays' comparisons were difficult and required many normalisation steps to reduce experimental noise in the data, an improved MAMS fabrication process, implemented by Schmidt et al. [85] was able to enhance the quality of the data produced. This also enabled data from measurements of single cells to be amalgamated to facilitate analysis of diverse metabolic phenotypes within a population.

The investigation of cellular populations using MAMS can also be improved using Raman and fluorescence techniques. This can reveal alterations in the metabolite profile during cellular transitions, for example from a motile to a dormant state in the alga *Haematococcus pluvialis* [86]. This process also identified a metabolically unique cell, illustrating the capability to discover rare cell types using these multi-method high-throughput techniques.

4.2 MS Imaging

Mass spectrometry imaging is another useful technique to analyse the metabolome of individual cells. This technique not only provides morphological data but it also can reveal metabolites within subcellular compartments, identify them and track their spatial distribution within the cell. To produce the images, the data from mass

spectrometry is used to construct mass spectrum charts to indicate the location of various molecules within the cells. Matrix-assisted laser desorption ionization (MALDI), desorption electrospray ionization (DESI) or secondary ion mass spectrometry (SIMS) are often used to produce the images and imaging mass spectrometry has proved useful in analysing drug effects, drug screening and medicinal diagnosis [87].

The most established mass spectrometry techniques in single-cell metabolomics that can provide information on cellular morphology coupled with chemical data are probably SIMS and MALDI (Fig. 4) [88]. SIMS is a surface analysis method that can obtain chemical information from the first few nanometres of the sample surface, so it is useful in visualising membrane-localised molecules including phospholipids and other small molecules. SIMS is the most effective method to analyse samples at the sub-micron level and it also provides valuable quantitative data.

There are two SIMS methods: dynamic and static. Dynamic SIMS is generally combined with other techniques such as electron, atomic force and fluorescence microscopy, which provide high-resolution imaging [89]. It provides high sensitivity with good lateral resolution and has been used to visualise the location of protein and nucleic acids within cells. In addition, it can be used for compounds labelled with rare elements, for example in determining the cellular localisation of cancer drugs and the distribution of iron in diseased cells of Alzheimer sufferers [89]. Static SIMS is often used with other techniques such as a time-of-flight (TOF) analyser, which obtains mass spectra for each pixel. Softer ionization and higher-yield cluster ion sources have recently enhanced the utility of SIMS, increasing the availability of molecular data and molecular depth profiling. As a result, this technique is enhancing the subcellular mapping of the location of unlabelled biomolecules of interest [90, 91].

Matrix-assisted laser desorption ionization (MALDI) MS is the most versatile technique for imaging single cells, and it is well established and easy to use. This technique is highly sensitive and is able to detect analytes over a large mass range, as well as from within complex mixtures. Furthermore, it has been successfully

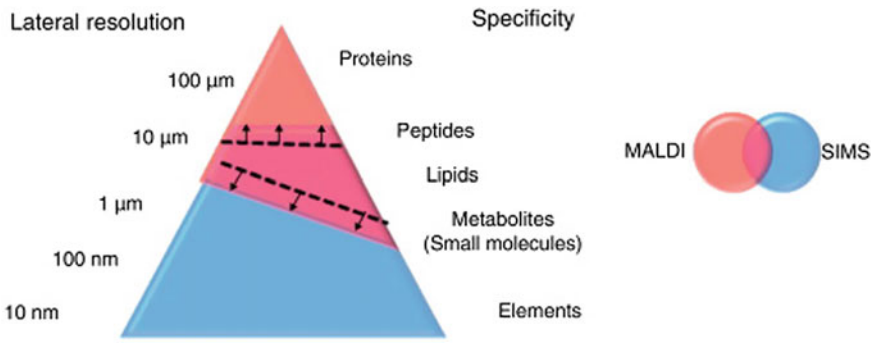

Fig. 4 The overlap of MALDI and SIMS technologies. Reprinted from Ref. [88], Copyright 2013, with permission from Elsevier

utilised in single cell and organelle profiling. Some of the challenges of MALDI-MS, including the poor spatial resolution for cellular and subcellular investigations, have been overcome in recent years to achieve a resolution between 4 and 7 μm. The use of scanning microprobe MALDI (SMALDI) enabled Spengler and Hubert [92] to obtain a resolution between 0.6 and 1.5 μm.

4.3 Capillary Electrophoresis

In the emerging field of metabolomics, CE-MS is now considered as a useful analytical technique for the polar ionogenic metabolites. Over the decades, significant contribution has been reported in metabolic profiling study using CE-MS [93]. Capillary electrophoresis (CE) mass spectrometry (MS) is one of many analytical techniques that have been used in metabolomics. CE is greater in the separation efficiency of ionic metabolites, and MS can offer detection at very high sensitivity. Therefore, the platform of CE with MS can help to improve the performance of analytical tools with high resolution, sensitivity and efficient separation of metabolites. CE-MS has been demonstrated as a superior technique for profiling polar metabolites in bacterial, plant, urine, plasma and other biological samples. CE-MS has also been used to detect ionic metabolites mostly from primary groups of metabolites. For example: amino acids, organic acids, nucleotides and sugar phosphates.

Among the separation methods, capillary electrophoresis (CE) has proven to be an efficient method for single-cells separation. Single-cell studies involve low detection limits and highly effective separation. CE with nano or picoliter sample volumes and high separation effectiveness has been shown to enhance the coverage of analyte in metabolites profiling and quantification in single-cell studies [94, 95]. A study demonstrated that CE can be hyphenated with MS for single-cell metabolomics profiling of *Aplysia californicas* the neurons. Their study served as a starting point for the advancement of single-cell analysis in terms of anionic metabolites as a complementary with cationic metabolites [31]. CE compromises the ability to separate a wide range of biomolecules from different types of samples. Lapainis et al. [94] developed a single-cell metabolomic by utilising the CE-MS method. Most of the reported works described about neuron cells. Recent trends suggested that CE analyses are being developed to help to increase the quality of compounds detected in minuscule structures [94].

Nemes et al. [34] demonstrated the capability of CE-ESI-MS in profiling and quantifying the metabolites in single cells from model organisms in neuroscience and systems biology [34]. The experimental setup designed is shown in Fig. 5. There searchers have proved that CE-ESI-MS is capable of profiling and quantifying the metabolic content of single cells from model organisms in neuroscience and systems biology. They have also successfully established the single-cell procedure to detect and characterise multiple metabolites in individual neurons collected from the *A. californica* central nervous system (CNS) and the *Rattus*

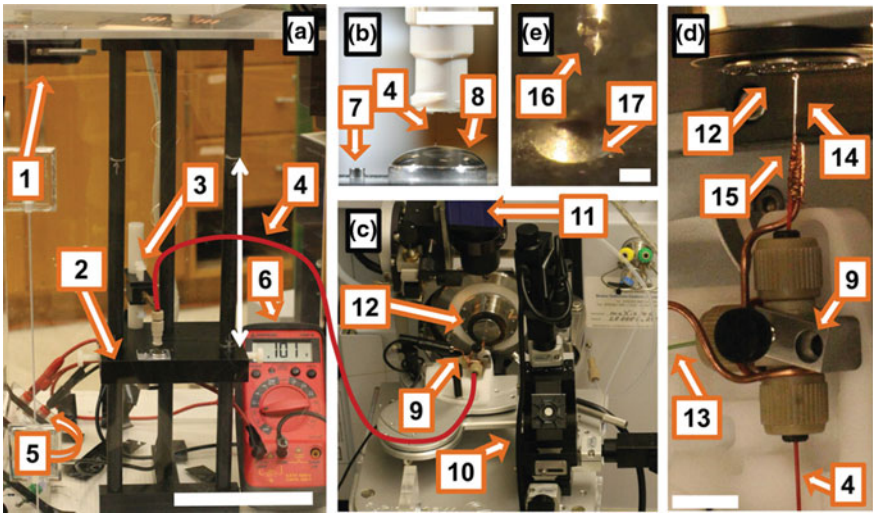

Fig. 5 Experimental setup of the single-cell CE-ESI-MS system. **a** Front view of the CE platform highlighting (1) the enclosure equipped with a safety-interlock-enabled door, (2) platform for sample loading, which can be rapidly elevated by 15 cm (see *arrow*), (3) holder allowing manual positioning of the separation capillary in three degrees of freedom, (4) separation capillary (*solid line* in *red*), (5) resistor connected in series to a stable HVPS (Figure S2) and the CE platform, and (6) digital multimeter connected in parallel measuring voltage drop on the resistor. Scale = 10 cm. **b** Magnified view of the sample-loading platform consisting of (7) the sample-loading vial and (8) electrolyte-containing vial with the separation capillary positioned 2 mm below the electrolyte meniscus. Scale = 1 cm. **c** Distant view of the CEESI-MS ion source consisting of (9) the CE-ESI interface mounted on (10) the three-axis translation stage of a PicoView nanospray source, (11) CCD camera equipped with light collimating and focusing lenses to record ES performance, and (12) a mass spectrometer equipped with a nanospray sampling plate. **d** Close-up view of the CE-ESI-MS ion source highlighting a T-union that houses fused silica capillaries for (4) CE separation and (13) ES sheath solution delivery as well as (14) a metal emitter grounded (earth) through a (15) thin copper wire. Scale = 1 cm. **e** Magnified view of (16) the stable Taylor cone formed upon operating the ES in the cone-jet spraying mode (see reflected image) in front of (17) the orifice of the mass spectrometer sampling plate. Scale = 500 µm. Reprinted by permission from Macmillan Publishers Ltd: Ref. [34], copyright 2013

norvegicus peripheral nervous system. The analytical workflow started with sample preparation, then CE-MS separation detection, and finally data analysis and quantitation. The developed protocol can be implemented in characterising the metabolome of smaller cells and/or subcellular domains.

The approaches could be implemented *in planta* by improving the experimental conditions of single-cell isolation [96]. A few studies have reported on the plant single-cells metabolite analysis. Due to the benefits and prospective of single-cell *in planta* studies, the analysis in plant single cell will be speedup. Oikawa et al. [30] demonstrated the metabolomics approach using CE-MS to explore the metabolomics of a single organelle by looking at the giant internodal cell of the algae *Chara australis* [30]. In the study, they utilised this unique cell to define the single

vacuole and cytoplasm metabolome, thus leading to the elucidation of the metabolite dissemination in a single cell.

In plant studies, single-cell analysis could be implemented to understand the changes between each de-differentiated cell to understand plant development mechanisms. Analysis of exudates from the cells could provide an insight into the chemical communication between cells [96].

4.4 Nuclear Magnetic Resonance (NMR)

NMR has been widely used for metabolomic studies in biological samples, such as in animals, plants and microbes. NMR-based metabolomics have a potential to deliver a 'complete view' of the metabolites under different treatments. NMR spectroscopy is a highly useful tool for the study of metabolites in individual bigger cells, for example; *Xenopus laevis* oocytes and *A. californica* neurons. However, NMR spectroscopy is hindered by low coverage and sensitivity. Therefore, improvements in NMR sensitivity have been accomplished with new technologies. These include small-scale NMR probes which have improved the detection limits of NMR and enable the characterisation of single-cell samples. It is possible to propose a system that could exploit NMR with fluorescent probes to observe different amount of metabolites. NMR spectroscopy has also been applied widely for the quantitative and non-invasive detection of metabolites [97]. Currently, NMR has been utilised efficiently for single-cell metabolite studies. Grant et al. [98] proved the potential of NMR spectroscopy for single-cells studies [98]. This increased the application of NMR spectroscopy from entire living organisms, isolated tissues and even down to single cells.

A study by Lee et al. [99] was intended to assess the possibility of NMR spectroscopy to explore subcellular phenomena. They have successfully recorded the first compartment-selective in vivo NMR spectra from oocytes of the frog *X. laevis* using a high magnetic field and a home-built microscopy probe. This study shows that the two cytoplasmic regions differed in their lipid contents. Their study demonstrates that NMR may be used as a tool in the study of cell biology.

Despite limitations in resolutions, this study has clearly shown metabolite localization in plant tissue by NMR signals. The study proved that NMR technique is promising for single-cells study. NMR was expected to deliver low coverage of metabolite detection due to the low sensitivity. However, as demonstrated by Krojanski et al. [100], this limitation has been improved by using a small volume of probes. This technique permits single-cell-sized detection and quantification.

5 Limitations and Challenges

In comparison to other single cell "omics", metabolomics is most challenging to measure. Whilst there have been substantial developments in metabolome coverage, there is still no analytical protocol that can investigate the entire cellular metabolome in a single measurement [12].

One of the key challenges in metabolomics is the fact that the metabolome can dynamically respond to the environment very rapidly. Therefore, the cell's metabolism needs to be paused immediately in order for it to be accurately measured. To overcome this, sample preparations need to be carefully managed, as this will also affect the information obtained. At this time, isolation of single cells and direct sampling from individual cells are still technically difficult.

Another key challenge in metabolomics is the large range of metabolites in a cell, relative to the molecules analysed in genomics and transcriptomics. These diverse unidentified molecules in the metabolome may confound the observation of other metabolites. Similarly, another issue that is not experienced with the analysis of nucleic acids is the lack of a technique to amplify the small amount of metabolites present to facilitate detection. Furthermore, the molecules in the medium in which cells are grown can be very similar to the metabolites produced by the cells, making separation of the metabolic products difficult. In all these cases, metabolic precursor concentrations may be increased experimentally and may allow the identification of formerly indiscernible metabolites. In addition, instruments to increase sensitivity of detection may need to be developed. The most common methods to measure the untargeted metabolome are mass spectrometry and nuclear magnetic resonance spectroscopy (NMR), although so far the utility of NMR for single-cell metabolomics is limited.

Other challenges of single-cell metabolomics include inadequate databases. The NIST electron ionization mass spectral library is incomplete and inconsistent and as such database comparison can be problematic. Finally, high-throughput methodologies should be progressed in order to make accurate conclusions from the data obtained from the analysis of multiple individual single cells simultaneously.

6 Future Prospects and Conclusion

Over the past few years, metabolomics methodologies have progressed swiftly and there are several valuable databases that store, manage and analyse metabolomic data. These technologies that are used to analyse the metabolites from single cells can provide valuable insights into biological interactions, which the fields of genomics and transcriptomics cannot deliver. Metabolomics at a single-cell level remains technically challenging owing to numerous fundamental limitations, including the rapid changing of the metabolome, small sampling volumes, low

quantities of metabolites, diverse range of metabolites present in the cell and inadequate sensitivity of analytical instruments.

Metabolomics at the single-cell level, however, is in its infancy. Enhancements allowing increased coverage of the metabolome, the improvement and more rapid identification of metabolites and the implementation of non-destructive measurements are expected. This will enable us to start to comprehend how biological systems interact with one another and the environment. This will therefore enable us to understand the unique properties of cells, cell–cell communications and cell-environment interactions. Large-scale single-cell metabolomics data will provide insights to permit the formation and testing of hypotheses to further understand the fundamental biological mechanisms and to address clinical issues in diagnostics and diseases. Further challenges need to be addressed pertaining to the integration of genomics, transcriptomics, proteomics and metabolomics data before a complete understanding of cellular physiology and development can be obtained. Nevertheless, current developments signpost an impending archetypal shift from analysis of tissue-scale metabolomics to the study of single-cell metabolomics, which will supplement other "omics" approaches on the path towards amalgamated systems biology of single cells.

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