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Single-cell metabolomics by mass spectrometry: Advances, challenges, and future applications

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ABSTRACT

Cellular heterogeneity is a phenomenon that is often observed but poorly understood. Single-cell metabolomics can provide insights into the phenotypical variations between individual cells. Recent advances in microfluidics, micromanipulation, image analysis, and automation allowed for high-throughput isolation of single cells in a minimally disruptive manner as to not affect the cell metabolism. Coupled with new innovations in mass spectrometry-based analytical techniques, single cell metabolomics stands at the cusp of becoming an established field. In this review, some of the recent single cell isolation platforms that are especially suited for metabolomics will be highlighted, as well as the recent trends in mass spectrometry-based single cell platforms. Additionally, some of the limitations of single-cell metabolomics and its recent applications will be briefly discussed.

1. Introduction

Cellular heterogeneity is ubiquitous in biology but poorly understood due to technical challenges. Various genetic, epigenetic and environmental factors contribute to cellular heterogeneity [1–3]. Cells can show heterogeneity in morphology, physiology and pathology, thus highlighting the necessity to study the biochemical and physiological characteristics of individual cells and their environment. However, most of the current technologies focus on obtaining information about the cellular processes and functions from average cell populations, ignoring the unique behavior resulting from cell-to-cell variations [1]. Recent advances have enabled studying single-cell genome [4], transcriptome [5], morphology and mechanics [6]. The link between the genotype and phenotype of a single cell can be understood by complete molecular profiling of the cell, especially its metabolome since it offers the most accurate depiction of the cellular reaction network.

Metabolomics is the study of small molecular weight compounds, typically less than 1500 Da [7]. It offers a comprehensive profiling of the full complement of small molecules. In comparison to other omics studies, characterization of the metabolome is difficult to achieve mainly due to complex diversity of the metabolites. Contrary to genomics, the inability to amplify metabolites, coupled with their high degree of dynamical changes, present a unique challenge in metabolomic studies. Notwithstanding, the large diversity of metabolic classes which makes developing a universal platform for metabolomic analysis a challenging task [2,8]. In addition, single-cell metabolomics exhibit its unique set of challenges. First, depending on the cell size, sample volume can go

Abbreviations: Da, Dalton; FACS, fluorescence assisted cell sorting; CTC, circulating tumor cell; PDMS, polydimethylsiloxane; MS, mass spectrometry; MALDI, matrix assisted laser desorption/ionization; TOF, time-of-flight; MAMS, microarrays for mass spectrometry; LDI, laser desorption ionization; SIMS, secondary ion mass spectrometry; ESI, electrospray ionization; FT-ICR, Fourier transform ion cyclotron resonance; FWHM, full width at half maximum; CE, capillary electrophoresis; D1L, left dorsal-animal; V1L, left ventral animal; SPE, solid phase extraction; MS/MS, tandem mass spectrometry.

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as low as few femtoliters or less which complicates cell sampling, preparation, and analysis. Furthermore, the wide dynamic range of metabolite amounts per cell stands in the way of any given analytical technique to cover the whole metabolome of the cell. Overall, the small sample volume combined with the inability of amplification makes it exceedingly difficult to analyze metabolites on the single cell level in an accurate, reliable, and reproducible manner.

However, significant progress has been made in the development of sample preparation and bioanalytical tools for single-cell metabolomics, revealing the unexploited potential of single cell metabolomics in several fields of applications. This review focuses on the recent developments in single-cell isolation and analytical techniques that are especially suited for single-cell metabolomics, with special focus on mass spectrometry-based analytical platforms. Additionally, some of the recent applications and current challenges are briefly highlighted.

2. Discussion

2.1. Sample preparation

As in any analysis, unravelling the metabolome on a single cell level usually starts with sample preparation. The main goal of sample preparation in single cell metabolomics is not only to isolate and prepare single cells for further downstream analysis, but to do so in a minimally disruptive manner to the cell and its microenvironment. This is due to the rapid changes of metabolite concentrations depending on the cell condition and its surrounding environment. It is also worth mentioning that conventional cell pretreatment approaches such as trypsinization and cell scraping are wholly unsuitable for metabolome analysis, doubly so on the single cell level due to metabolite leakage [9]. Thus, it is recommended to halt the enzymatic and metabolic activity of the cell directly after sampling, while preserving the integrity of the cell membrane to reduce metabolite leakage. To this end, in adherent mammalian cells, a washing step with PBS, followed by quenching with cold (−50°C) mixture of 60% methanol and 70 mM HEPES buffer resulted in minimum intracellular metabolites leakage [10].

Several cell isolation and manipulation approaches have been used to prepare single cells for analysis while causing minimal disruption to their microenvironment. Some of these methods are: fluorescence activated cell sorting (FACS), microfluidic arrays and pipette-based micromanipulation [11–13]. FACS excels at sorting fluorescently labeled cell populations into two or more subpopulations which can be analyzed by other methods. However, FACS measurements are largely unrelated to the metabolome of cells, therefore, it is mostly used, in conjunction with other sampling techniques, in isolating rare cell populations such as circulating tumor cells (CTCs) followed by their subsequent mass spectrometry analysis [14]. On the other hand, pipette-based micromanipulation, and microfluidic arrays are more readily integrated into single-cell metabolomics' analysis platforms, however, each comes with its own set of advantages, limitations and possible applications which will be briefly mentioned in this section.

2.1.1. Pipette-based micromanipulation

Microscale pipettes have been increasingly used to isolate and sample live single cells in their native environment [11]. Traditionally, single-cell sampling using pipette-based micromanipulation starts by observing and choosing target cells suspended in a normal culture dish or 96-well plate by the aid of microscopy. Then, a motorized stage or micromanipulator is used to move a tapered glass capillary to the proximity of the cell in question. Finally, aspiration is done by applying negative pressure to the micropipette where the whole cell, or its cytoplasm can be sampled, the aspirated sample can then be dispensed into a collection vessel by applying positive pressure on the capillary as shown in Fig. 1(A) [15–18]. Typically, this process is done manually at the expense of throughput, which is a critical limitation in large-scale single-cell studies.

Despite its utility in visualizing and sampling live single cells, single-cell micromanipulation is often a laborious affair involving skilled personnel and prolonged periods of time. Thus, to adapt this method to wide scale use, several improvements must be made in terms of throughput, high skill requirements and overall convenience. To this end, several automated methods have been developed that are capable of single-cell manipulation [19–21]. One recent system that is suitable for suspended and adherent cells works by automatic selection of cells of interest depending on their visual characteristics by image analysis [22]. Then, a robot aspirates the desired cell by using a vacuum system attached to a tapered glass capillary. Finally, the cell is dispensed in a 3D printed microwells on a petri dish for subsequent analysis by the analytical method of choice. By automating target selection and sampling, this system was able to improve upon the throughput of manual micromanipulation by several orders of magnitude (from dozens of cells per day to over a thousand cells per day), while maintaining the inherent advantages of single cell micromanipulation, e.g., minimal disruption the cells' microenvironment, label-free selection, and simultaneous visual observation of cells.

While automated systems improve upon throughput and ease of use, they do so by increasing instrumental complexity and cost. On the other hand, manual systems are relatively simple and cost-effective, but their throughput is largely limited by human factors, not to mention the high skill requirements for efficient sampling. As always, it is best to select a platform depending on the research goal. For large-scale systems biology and cell heterogeneity studies, automated micromanipulation systems would be a good choice. But, in the case of small-scale experiments, where a few dozen cells are needed, a simple setup involving a microscope with a manual micromanipulator might be sufficient.

2.1.2. Microfluidics and lab-on-a-chip systems

Contrary to the serial method of isolation in the previous methods, microfluidics or lab-on-a-chip systems aim to isolate cells in parallel by passing cells through microfluidic channels, which results in their separation into individual cells that can subsequently be collected in wells or storage vessels as shown in Fig. 1(B) [23]. Due to their miniaturized and parallel architecture, they have several inherent advantages over traditional cell separation methods. Among these advantages are their scalability, high automation potential, reduced reagent costs, and the possibility of performing multiple analytical assays simultaneously [24].

Recently, there were many innovations that incorporated microfluidics in single cell isolation and subsequent analysis. One example is a droplet-based microfluidics platform that divides blood samples into small nanoliter aqueous droplets that are embedded in oil, each droplet contains a single cell and its secretions [25]. This platform was subsequently used to detect rare circulating tumor cells in blood, based on their altered metabolic profile, due to their excessive lactic acid secretion in comparison to normal blood cells. Another technique incorporates a hydrodynamic trap in a pipette tip that allows for single-cell isolation from low cell-population suspensions (5–10 cells/μL) [26]. Additionally, an array of nano wells that are capable of trapping single cells by utilizing passive hydrodynamic flow and the least flow resistance path principles. This system has the advantage of having greater capture rates of single cells in comparison to others (almost 100% capture rates of single cells) [27].

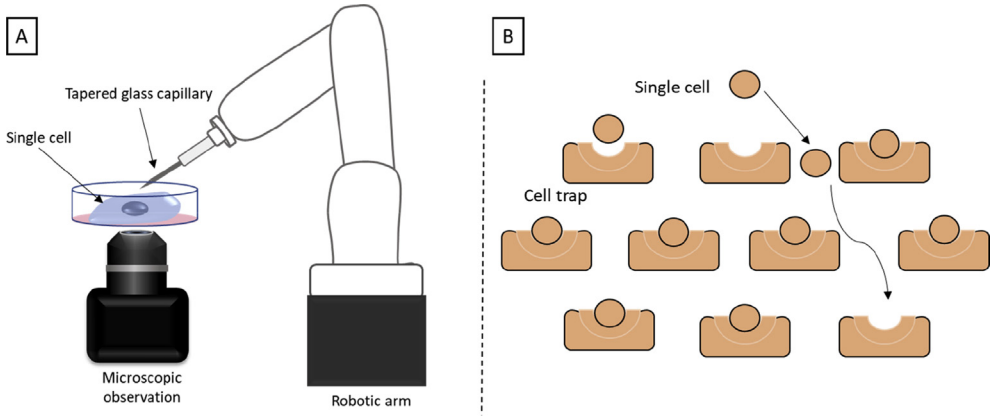

Fig. 1. Schematic showing several examples of single cell isolation and sampling techniques. A) Automatic single cell micromanipulator system, B) Microfluidic array cell trapping system.

Microfluidic-based systems have been used extensively in systems biology [28,29], however, they are known to be difficult to couple with techniques used in metabolomics studies such as mass spectrometry. This is largely due to the inherent difficulty of sampling picolitre volumes from the microfluidic device with minimal sample loss, as well as using of polydimethylsiloxane (PDMS) as a construction material which has a significant background signal [30]. The latter issue could be resolved by using glass as a construction material, but this often increases manufacturing complexity and cost [31]. Despite these pitfalls, several techniques succeeded in coupling microfluidic systems with MS systems, highlighting their potential as a comprehensive single cell isolation and analysis platforms. Another issue that arises from using microfluidic platforms in single cell metabolomics studies is their sensitivity to variations in cell size, thus limiting their use in heterogenous cell cultures. Finally, the isolation process could cause perturbations in the metabolome, due to the interruption in cell–cell communication [32], possible reduction in cell growth, and restriction on cell shape imposed by the dimensions of the traps [33,34].

2.2. Analytical techniques

Despite the presence of multiple analytical techniques suited for analyzing organic molecules, there are several issues to keep in mind when choosing the optimal method for single cell metabolomic analysis. First, unlike nucleic acids, metabolites' signal cannot be amplified, which presents a significant challenge, especially when dealing with the small volumes associated with single cells (usually in the picolitre scale). Thus, the sensitivity of the analytical technique is of utmost importance in single cell metabolome studies. Moreover, to gain a complete picture of the metabolome, the analytical technique of choice must possess sufficient selectivity for molecular characterization of individual unlabeled metabolites, as well as being exhaustive enough to cover a wide range of metabolites. Keeping these considerations in mind, mass spectrometry has gained prominence as the method of choice in single cell metabolomic studies since it possesses the necessary sensitivity to reliably detect molecules in the single cell scale, as well as its molecular profiling capability which is perfectly suited for detection of unlabeled metabolites.

Accordingly, there are multiple techniques which utilize MS that possess the necessary sensitivity, accuracy and resolution for single cell metabolomics. Some of these methods, along with their uses, limitations and possible improvements are highlighted in this section.

2.2.1. Matrix-assisted laser desorption/ionization

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is one of the main techniques used for studying large molecules while causing minimal fragmentation. Typically, MALDI-MS measurements start with mixing the desired sample with a matrix, followed by irradiation with a UV laser beam under vacuum condition. Subsequently, analytes are ionized and accelerated to a mass spectrometer analyzer (traditionally, a time-of flight (TOF-MS)) [35]. Owing to the recent developments in optics and the incorporation of nano-particle matrices, MADLI-MS has been reliably used for traditional single-cell analyses as well as 2D and 3D imaging of metabolites in cellular and sub-cellular space [36–39]. However, as with most single cell analysis techniques, achieving high throughput is always a challenge. An interesting platform that attempts to alleviate this issue was proposed by Ong et al., in which, the process of optical imaging of single cells followed by MALDI-TOF analysis, was automated [40]. Accordingly, this platform was successfully used in phenotypic classification of rat cells according to their metabolome. Another promising high-throughput platform utilized microarrays for mass spectrometry (MAMS) to automatically isolate single cells by using hydrophilic reservoirs [41]. Then, cells were co-crystallized within the confines of the reservoirs with 9-aminoacridine, which led to increased sensitivity in comparison to traditional MALDI-MS methods. Finally, the metabolomic differences between environmentally and genetically perturbed *Saccharomyces cerevisiae* cells on the single cell scale were observed utilizing the same platform [42].

There are some limitations when MALDI-MS is employed in single cell metabolomics. First, traditional MALDI-MS methods usually sample the cell in vacuum after extensive sample preparation, which goes against the ethos of metabolomic studies, i.e., sampling the cell in its native environment to minimize metabolic changes. Consequently, several techniques tried to utilize ambient conditions for ionization followed by traditional MS measurements (albeit at the possible expense of sensitivity) [43]. Moreover, due to its use of low molecular weight organic compounds in the matrix preparation, reliably measuring most of low molecular weight metabolites (less than 800 Da) is often a difficult task, due to interference from the matrix signals [44]. In addition, the usage of UV laser might be detrimental to the cell condition, especially in the case of fragile cells that might disintegrate easily upon mixing with the matrix and its subsequent laser bombardment [45]. However, multiple techniques are proposed to address these limitations, among these techniques is an approach that utilizes visible-wave MALDI coupled with a low background signal matrix that can protect, to some extent, cells from absorbing excessive laser energy.

This method was used to successfully analyze single cells from several cell lines, highlighting the specific phenotype variations among them [46].

An interesting approach that also attempts to circumvent the limitations of traditional MALDI-based methods is matrix-free laser desorption/ionization (matrix-free LDI). Among the many strategies of achieving matrix-free LDI is replacing the matrix with nanostructures [47]. The nanostructures replace the function of matrices by acting as nanoantennae that harvest light from the laser followed by subsequent ionization of the sample, a process termed nanophotonic ionization-LDI [48,49]. An enhanced nanophotonic-ionization LDI technique was proposed by Walker et al. [50]. They utilized silicon nano post arrays in a matrix-free LDI-MS system in which, *S. cerevisiae* single cells were analyzed. Changes in the metabolome, owing to oxidative stress, as well as intra and inter-population differences were discerned. Due to its superior performance in analyzing small molecular weight compounds, matrix-free LDI was also utilized in several imaging mass spectrometry platforms [51,52]. However, the relatively large spot size (few micrometers) makes MALDI and LDI single cell imaging techniques suitable for relatively large cells only. Wang et al. attempted to circumvent this limitation by utilizing vacuum ultraviolet laser coupled to a TOF mass analyzer that could achieve sub-micron resolution with the added benefit of acquiring fluorescent signals during the mass spectrometry imaging process [53], in which, they succeeded in imaging several metabolites in single-HeLa cells, with minimal fragmentation and higher sensitivity than traditional imaging mass spectrometry approaches. However, if a higher resolution is needed, secondary ion mass spectrometry-based methods are traditionally used instead.

2.2.2. Secondary ion mass spectrometry imaging techniques

Secondary ion mass spectrometry (SIMS) recently emerged as a promising MS imaging technique that is capable of single cell and sub-cellular resolutions [8]. Fundamentally, SIMS works by directing a primary pulsed ion beam on the sample surface and measuring the secondary ions ejected using a mass spectrometer as shown in Fig. 2 [54]. Typically, time-of-flight MS (TOF-MS) is used due to its exceptional acquisition rates and dynamic range. By contrast to MALDI-MS and matrix-free LDI imaging, SIMS imaging methods can achieve submicron lateral resolutions, combined with its nanometer depth resolution. Therefore, SIMS-based techniques are uniquely suited for studying the distribution of endogenous and exogenous species in the sub-cellular space. This was demonstrated by visualizing the sub-cellular, 3D distribution of the drug amiodarone and cellular metabolites in single macrophage cells by using TOF-SIMS, showing the high affinity of the drug to the surface and subsurface regions of microphages [55]. Another study succeeded in measuring the drug induced changes in metabolites in single HeLa cells, the analysis was done in a high throughput manner by integrating single cell microarrays with automated TOF-SIMS acquisition [56]. Furthermore, the 3D depth profiling accuracy of TOF-SIMS was validated by comparing the spatial distribution of polymeric nanoparticles measured by SIMS within HeLa cells with 3D optical data obtained by fluorescence labeling of polymeric nanoparticles [57].

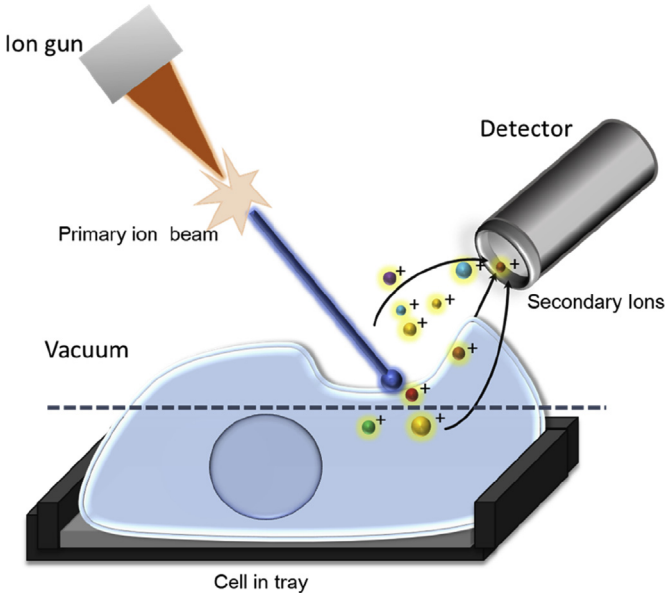

Fig. 2. Schematic showing the mechanism of secondary ion mass spectrometry (SIMS), in which, a primary ion beam is directed at the sample surface resulting in secondary ions which are subsequently measured by mass spectrometry.

Despite SIMS utility at 3D label-free imaging of biological samples [58], there are several limitations that should be kept in mind. First, typical SIM-based techniques are incapable of carrying out simultaneous full scan and MS/MS analyses, an issue that is exacerbated by the possible isobaric interferences caused by the primary beam. A possible solution to this issue was presented by Fisher et al., in which a modified SIM-TOF design allowed for parallel full scan measurements of a desired mass range and MS/MS target identification that improves upon the identification power of traditional SIM methods significantly [59]. Moreover, since most of SIM-based techniques utilize TOF-MS as the MS analyzer, they share some of its limitations, such as the lower mass resolution relative to other MS analyzers. Finally, despite SIMS methods' high performance in 3D imaging, it comes at the price of throughput since the methods can either be optimized for high throughput with less lateral and depth resolution, or high resolution with low throughput. An interesting approach that intends to address some of the previously mentioned limitations involves coupling a TOF-MS with an orbitrap, which allowed for fast 3D acquisition of single cells by using the TOF analyzer, followed by high mass accuracy and resolution measurement of an area of interest using the orbitrap analyzer, with an optional MS/MS fragmentation [60].

2.2.3. Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) is considered as a “soft” ionization technique like MALDI. However, in contrast to most MALDI and SIMS-based methods, ESI-MS methods ionize the sample in ambient conditions with minimal pretreatment, thus minimizing the disruption of the cell and its metabolome. The utility of ESI-MS techniques in metabolomics has increased by the incorporation of high resolution, accurate mass instruments such as Fourier transform ion cyclotron resonance-MS (FT-ICR-MS) and orbitrap-based instruments [61]. FT-ICR-MS and orbitrap MS are powerful tools in metabolomic studies that can achieve ultrahigh resolution (>1,000,000 FWHM and 240,000 FWHM, respectively) and accurate mass (<1 ppm) at the possible expense of acquisition time.

One of the prevalent techniques that employs ESI-MS is known as “live single cell mass spectrometry” (LSC-MS), in which a metal coated capillary is used to sample live single cells or single organelles, followed by nano-ESI-MS measurements of the capillary contents after the addition of a suitable organic solvent as shown in Fig. 3 [62]. This method has been successful in discerning the metabolic differences and identifying specific metabolites of single mammalian [63] and plant cells [17]. Recently, this method was also used to distinguish the localization of terpenoid indole alkaloids in specific cell types from *Catharanthus roseus* stem tissues [64]. While it is

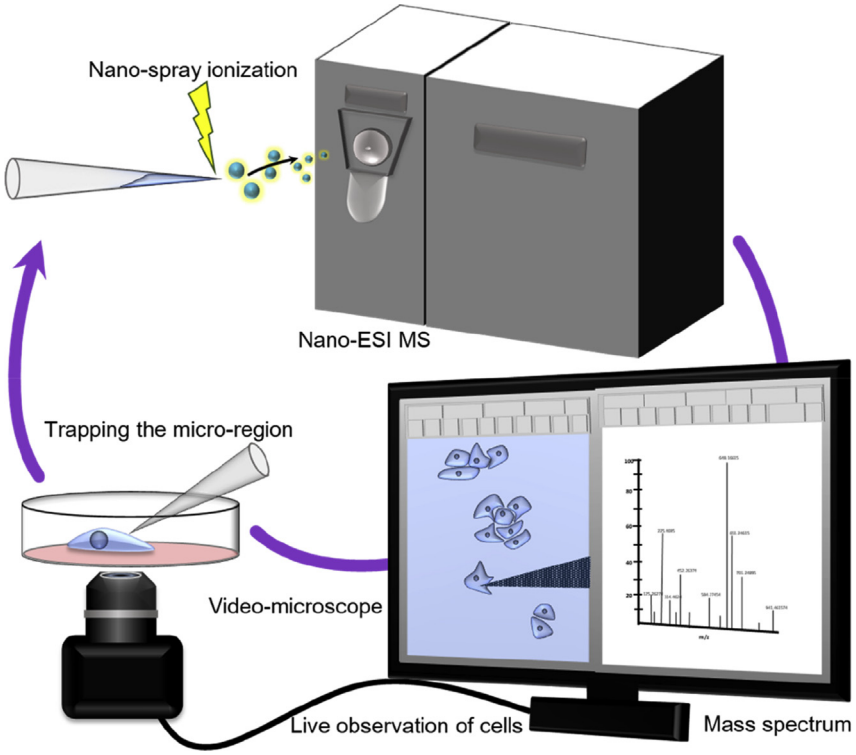

Fig. 3. Live single cell mass spectrometry system work flow. Cells are observed in their native environment using video microscopy, and the targeted cell or organelle is then sampled using a tapered glass capillary. The capillary's content is directly introduced to a nano-ESI mass spectrometer after the addition of an ionization solvent.

arguably the best in direct sampling of single cells in their native environment, this comes at the expense of throughput due to the multistep process that also limits the real-time detection. Pan et al. achieved a higher throughput as well as real-time analysis by employing a probe-based approach termed "Single-probe MS", which is capable of sampling and ionizing the intracellular components of live single cells in one step [65]. This miniaturized, multifunctional system incorporates a "Single-probe" which consists of a laser-pulled dual-bore quartz needle, a solvent-providing capillary and a nano-ESI emitter. The dual-bore needle portion is inserted into single cells to sample intracellular components, in which, the solvent flows in the first channel (the solvent-providing capillary) to the second channel (nano-ESI emitter) through a liquid junction located at the sampling site inside the cell. An ionization voltage of (~3 kV) is transmitted through the solution to allow the extraction and ionization of the intracellular components and subsequent measurement by nano-ESI-MS. This approach was used to analyze drug treated and untreated individual HeLa cells in which, several cellular metabolites and lipids were detected in both positive and negative ion mode. In drug treated cells, the anticancer compounds doxorubicin, paclitaxel and OSW-1 were successfully detected in single HeLa cells. Additionally, negatively charged cellular metabolites from single HeLa cells analysis in positive ion mode could be achieved through the addition of dicationic ion-pairing compounds [66] as well as monitoring the metabolic changes of single phytoplankton cells under different environmental conditions [67]. The same group improved upon their previous design with a simpler "T-probe" system that incorporated the junction orthogonally between the sampling channel and the nano-ESI channel (Fig. 4), this system was then used to analyze the metabolic changes of single HeLa cells upon treatment with anticancer drugs [68].

A different approach with an improved resolution combines capillary micro sampling with ESI-MS and ion mobility separation (ESI-IMS-MS) [69]. This improved platform was then used to assess the metabolic and the lipidomic profile changes of single human hepatocytes upon treating cells with rotenone drug, the changes in the distribution of the adenylate energy charge was accurately measured. Moreover, the incorporation of ion mobility separation process reduced the chemical background, which resulted in doubling the number of the detected metabolites and the tentative annotation of ~23 metabolites per single cell [70]. Another recent platform involves the integration of patch clamp sampling technique with nano-ESI-MS for the analysis of single neurons from mouse brain [71]. This unique integration allowed rapid and sensitive profiling of the metabolic and the physiological state of a single neuron, as well as the identification of more than 50 metabolites from the cytoplasm of a living neuron, highlighting its potential as a useful tool in neuroscience research.

One limitation that the previous studies shared is their inadequate quantitation performance. Admittedly, several challenges stand in the way of metabolites' quantitation on the single-cell level [72]. Controlling the exact volume sampled, precise extraction of cellular contents, and accurate and sensitive analysis of the extracted analytes followed by their subsequent quantitation are examples of the aforementioned challenges. However, a recent study succeeded in addressing some of these challenges by quantifying glucose extracted from single *Allium cepa* epidermal cells using electroosmotic extraction and nano-ESI MS [73]. Electroosmotic extraction from live cells was achieved using two electrodes and a finely pulled nanopipette containing a hydrophobic electrolyte (Fig. 5). Extracted analytes were electro-osmotically dragged from the cell into the pipette for subsequent nano-ESI MS analysis. The extracted volume can be precisely controlled by controlling the applied voltage between the two electrodes and the extraction time. Extracted glucose was successfully quantitated using an internal standard (glucose-d_2) of known concentration.

Most of the previously mentioned ESI-MS-based techniques provide direct, rapid and real-time analysis of metabolites and

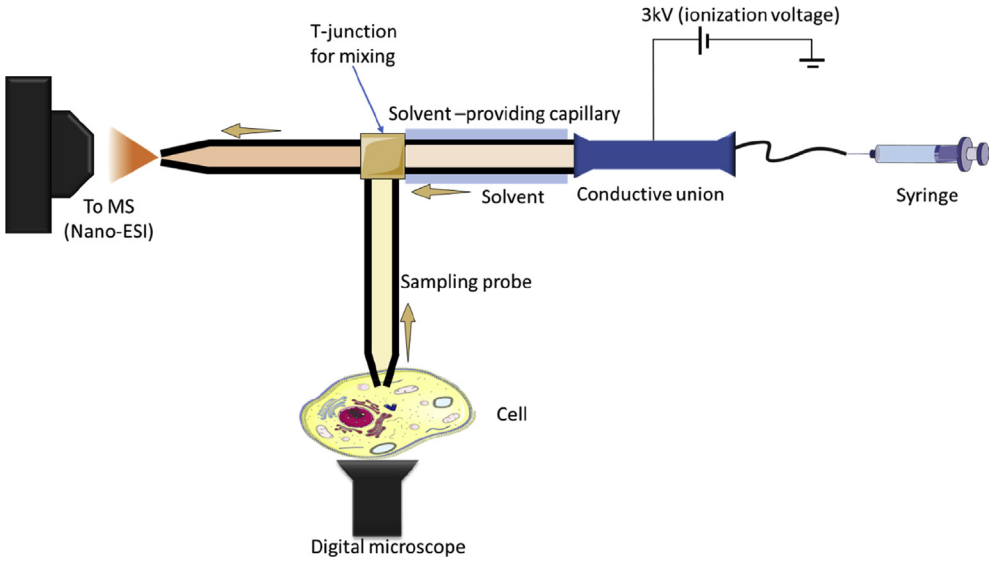

Fig. 4. Illustration of the working mechanism of the "T-probe" system used in single cell mass spectrometry. The sampling probe is first embedded into a single cell and cellular contents are then withdrawn. The cellular contents are immediately ionized and measured using nano-ESI MS since the solvent is continuously flowing through the solvent-providing capillary into the nano-ESI emitter.

lipids at the single cell scale. From a metabolomic point of view, ESI-MS-based methods are arguably best suited for analyzing cells at their native environment with minimal disruption. However, they are inherently more susceptible to matrix effects due to the absence of a separation step before MS analysis, which also results in low metabolite coverage.

Separation-based methods coupled to ESI-MS techniques such as capillary electrophoresis MS (CE-MS) attempt to address some of the limitations of ESI-MS. Over the past few years, the use of electrophoretic separation (CE) coupled with ESI-MS in metabolomic studies has increased significantly. In capillary electrophoresis, ions are typically separated based on their electrophoretic mobility, mainly dependent on the charge and the size of the molecule, which makes CE-based techniques the favored method for the analysis of highly polar and charged metabolites. The recent technological developments in CE-MS approaches resulted in achieving higher separation efficiency, and improved concentration sensitivity, allowing the identification and/or quantification of metabolites on the single cell scale [74]. CE-MS coupling benefits from the advantages of both CE and MS, providing sensitive, swift and wide range analysis of metabolites [75]. In addition, CE inherently requires small loading volumes, which is advantageous in single cell studies. Recent studies demonstrated the potential of applying single cell CE-MS in the fields of biomedical, clinical, and plant metabolomics. An interesting platform combined patch clamp electrophysiology with CE-MS to interrogate the metabolic profile of GABAergic and glutamatergic neuron cells on the sub-cellular scale [76]. The study revealed metabolic differences between the two cell types and succeeded in detecting both common and cell-type specific metabolites (i.e., GABA in GABAergic cells), highlighting the chemical heterogeneity between GABAergic and other neuron cells. Furthermore, a more recent study demonstrated metabolic heterogeneity between the 8- and 16-cell embryo of South African clawed frog (*Xenopus laevis*) by utilizing microprobe single-cell CE-ESI-MS [77]. The study illustrated different metabolic activity across the dorsal-ventral axis and successfully detected specific molecules (i.e. asparagine and glycine betaine) in the left dorsal-animal (D1L cell) which were not found in the left-ventral animal (V1L cell), showing the differences in the metabolic activity between cells derived from the same hemisphere.

Although, CE-MS-based techniques showed great potential and, to date, a promising performance, they still face several pitfalls and challenges hindering their widespread use in the field of single cell metabolomics. Among these challenges are: method robustness,

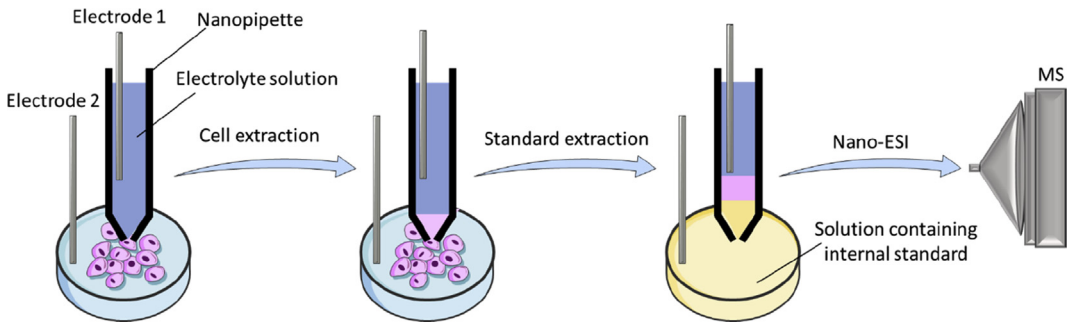

Fig. 5. The process of electroosmotic extraction for subsequent quantitative MS analysis of single cells. A nanopipette is filled with a suitable hydrophobic electrolyte solution. Electrode 1 is inserted into the nanopipette while electrode 2 is inserted into the cell culture dish. Cellular extraction takes place upon applying negative pressure between the two electrodes. Followed by the addition of a known volume of a solution containing an internal standard to the nanopipette using electroosmotic extraction. The extracted cellular contents are subsequently analyzed using nano-ESI MS.

migration time variability, poor concentration sensitivity (due to small sample volume injections) and postcapillary dilution upon the use of a coaxial sheath liquid interface [78]. However, this could possibly be mitigated with the incorporation of patch clamp or any similar sampling approach to improve specific cells' selection. Nevertheless, significant progress has been made in the development of novel interfaces for coupling CE to MS which offers new perspectives for highly sensitive and robust metabolic profiling of various samples as shown in Fig. 6 [79]. An interesting solution that combines the advantages of MALDI and CE-MS has been used lately to improve method throughput and metabolite coverage on a single cell level, in which, MALDI was used for metabolite guidance to the CE-ESI-MS instrument [80]. In addition, improvements in CE-MS detection sensitivity for metabolic profiling can also be achieved by using sample pre-concentration techniques such as solid phase extraction (SPE) systems [74]. Overall, CE-MS systems excel at analyzing highly polar metabolites which constitute a major fraction of known metabolites involved in primary metabolism [81].

2.3. Recent applications

Single cell metabolomics exhibit a great potential in the advancement of several fields, on two fronts. First, it provides a comprehensive picture about the cellular dynamic and its phenotypic fingerprint, through adding an extra dimension to the flow of information from DNA to RNA to proteins to finally metabolites. In addition, single cell resolution studies reveal crucial information about individual cells that were often unattainable by traditional population-based studies [82,83]. Hereby, we summarize some of the recent applications of single cell metabolomics in biological, pharmaceutical, and medical sciences.

2.3.1. Biological

Studies of the plant metabolome offers an insight on the complex cellular dynamic diversity and processes responsible for plant response to abiotic stress [84]. Thus, understanding cellular mechanisms and identifying stress-responsive metabolic pathways will aid significantly in developing adaptive strategies for plants' survival and growth. Single cell metabolomics demonstrated the dynamic cellular processes of the encystment of fresh water algae (*Haematococcus pluvialis*) through monitoring ATP, ADP, astaxanthin and β-carotene in single cells [85]. The study succeeded in detecting 13 metabolites on a single cell level as well as highlighting the correlation between the reduction of ATP/ADP ratio and the accumulation of astaxanthin. Another group succeeded in analyzing single cells obtained from petal, leaf and stem of wild malva (*Pelargonium zonale*) [86]. Several compounds (i.e., geraniol, terpenoid and methyl citronellate) were successfully identified together with specific metabolites to each cell type (petal, leaf and stem).

Moreover, due to the inherent chemical complexity of neurotransmitters and neuropeptides, metabolic characterization of neurons on the single cell level can help in understanding complex neural communications. Potentially aiding in the development of new approaches for understanding and treating persistent neurological disorders [87]. Single cell metabolomics revealed the metabolic differences not only between different neuron types, but also neuron cells originating from the same genotype or even having similar phenotype. This was achieved by studying the isolated neurons of *Aplysia californica*, where over 300 metabolites were successfully detected in neurons obtained from different neuronal cell types [88]. Another study incorporated single-cell mass spectrometry with isotopic labeling to detect molecular and cellular changes in single neurons upon moderate UV exposure. In the study, single-cell measurements of hippocampal (HPC) neurons in mice revealed elevated urocanic acid (UCA) levels upon moderate UV exposure [89]. Furthermore, a novel glutamate biosynthetic pathway in the brain was discovered in which, UCA was converted to glutamate that lead to neurobehavioral changes, mainly, improvements in learning and recognition memory.

2.3.2. Pharmaceutical

Metabolomics is not only limited to the study of endogenous molecules, but it also includes the study of exogenous molecules such as detecting and quantifying drugs and their metabolites for drug discovery and development purposes. In pharmaceutical industry, there is an increased interest in analyzing and quantitating the spatial distribution of drugs and their metabolites on a single cell level, which will provide a better understanding about the pharmacokinetic and dynamic characteristics of drugs that is essential for: target validation studies, improving drug efficacy and reducing failure rates, especially at the late stage of drug development [90,91]. Recently, a study was done on single yeast cells (*S. cerevisiae*) [42], where the cells were chemically perturbed with 2-deoxy-D-glucose to monitor its effect on the single cells. The study reported quantitative variations in glycolytic metabolites and ATP/ADP ratio and showed that detection of a specific drug metabolite (2-deoxy-*d*-glucose-6-phosphate) could be achieved. A more recent study [60] included the incubation of rat alveolar macrophage cells with different concentrations of amiodarone drug followed by the evaluation of the metabolic profile of individual cells. The study showed the correlation between the upregulation of phospholipids and cholesterol with the accumulation of amiodarone. It also showed high degree of variability in drug uptake and the phospholipids concentrations between single cells. Accordingly, accounting for cellular heterogeneity is crucial for gaining a comprehensive picture about drug's pharmacokinetic and pharmacodynamic properties on the single cell level.

2.3.3. Clinical

Metabolomics has a largely untapped potential in improving cancer diagnostics and prognostics through the characterization of the unique cancer metabolome as well as monitoring any

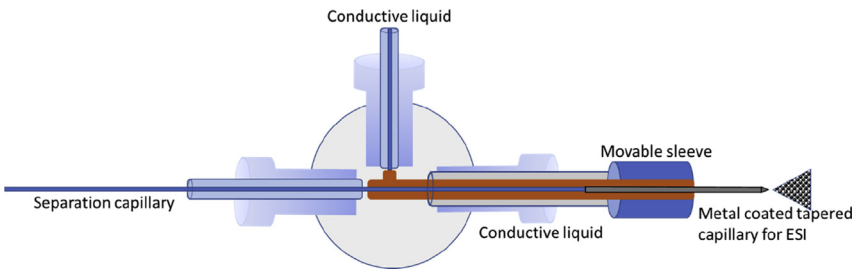

Fig. 6. Sheathless transient capillary isotachophoresis (CITP)/(CZE)-MS interface. The sheathless interface is consisted of a metal tee (T). The left arm is used to fix the separation capillary, the right arm is used to fix the metal tube, while the top arm is connected to a syringe filled with conductive liquid. ESI voltage is applied directly to the metal tube. Stable nano-ESI spray is achieved through the electric contact between the ESI voltage power supply and the CE separation liquid done through the conductive liquid.

Table 1
Open-access software tools that offer end-to-end data processing, analysis, annotation and interpretation of MS-based metabolomic studies.

Tool	Analytical technique	Compatible file formats	Software type	References
XCMS online	Chromatography-based MS	AIA/ANDI NetCDF, mzXML, mzData and mzML	Web App	[103]
MALDIquant	Direct-MS (MALDI and Infusion-based methods)	txt, csv, Bruker fid, Ciphergen XML, mzXML, mzML, imzML, Analyze 7.5, CDF, mMass MSD	R Package	[104]
Galaxy-M	Chromatography-based MS and direct infusion MS methods	DI-MS: Thermo Raw files, datLC-MS: mzML	Galaxy	[102]
Workflow4metabolomics	Chromatography-based MS methods	mzML, mzXML, Thermo Raw files	Galaxy	[105]
MetaboAnalyst 3.0	Chromatography-based MS methods	mzXML, NetCDF, mzDATA, csv, txt	Web App	[106]
MAIT	LC-MS	mzData, NetCDF	R Package	[107]

alterations in it. Moreover, metabolites of small molecular weight can provide mechanistic insights into novel biomarkers for diseases. A prime target for single cell metabolomics in cancer studies are CTCs, due to their low concentrations in the peripheral blood (1–100 cells per 7.5 ml) [92]. CTCs are cells detached from the primary tumor circulating into the blood stream and they are believed to have a great role in cancer metastasis. Thus, CTC metabolomic profiling has a tremendous potential in providing information about the tumor composition and allowing for the study of tumor relapse and resistance. However, due to CTCs' scarcity and difficulty to be isolated, enriched and analyzed, very limited studies have succeeded to investigate their metabolic profile on a single cell scale. One of the studies that depicted potential results succeeded in acquiring the metabolic profile of a single CTC obtained from a neuroblastoma patient as well as detecting specific metabolites corresponding to the same cancer type [14]. Another study succeeded in identifying single circulating tumor cells, through measuring the elevated lactic acid concentration resulted from the Warburg effect specific only in cancer cells [25], demonstrating the utility of CTC metabolic profiling in future diagnostic applications. Recently, Zhang, et al. further highlighted the potential of MS-based single-cell metabolomics in differentiating between normal cells (astrocytes), and cancer cells (glioblastoma) [93]. This was achieved by integrating their previous droplet extraction [94] and pulsed direct current electrospray ionization MS [95] methods into one platform. This platform was then able to detect glioblastoma cells' unique features, such as the unique coexistence of PC(17:1) and PE (20:1) which could only be discerned by using tandem mass spectrometry (MS/MS), a unique advantage offered by their integrated platform.

3. Challenges and future prospects

Despite the numerous techniques and applications in single-cell metabolomics, mass spectrometry-based single cell metabolomics still needs further development to become an established field. In addition to the considerations mentioned in Section 2.1 regarding sample preparation and isolation, other challenges must be addressed that make wide-scale use of single-cell metabolomics difficult. In particular, the difficulty to export single-cell metabolomic techniques to other laboratories/groups. This is largely due to low dissimilation of single-cell protocols, data, and metadata of metabolic studies by using public repositories such as MetaboLights [96], and metabolomics Workbench [97]. Sharing data improves reproducibility, confidence in the data itself, and allows for reuse and re-analysis in the future by other teams. The reporting of the aforementioned data should also be standardized. Goodacre et al. attempted to address this issue by proposing minimum reporting standards for data analysis in metabolomics [98], but compliance with said standards is not absolute yet [99].

Another issue that hinders wide-scale adoption of single-cell metabolomics is the relative lack of established end-to-end, open access data analysis platforms that are especially suited for metabolomics. This forces each laboratory to develop their own tools which hampers collaborative efforts as well as attempts to reproduce the results by other groups. This is complicated further by the diversity of the analytical techniques utilized in single cell metabolomics and, by extension, the file formats as well as the type of generated data. Hence, the development of a universal software tool that comprehensively process, analyze, and translate the data into biologically meaningful context is yet to be achieved in MS-based metabolomic studies [100,101]. This proposed tool should be platform independent, possess an open license, and must be capable of importing raw files, processing them, performing data analysis and producing an output. To this end, several software tools that attempt to perform that role have been proposed (Table 1), all of the included methods are freely available and can import open-source MS file formats such as mzXML, mzML, NetCDF, etc., while some of them are capable of importing commercial file formats as well [102].

4. Conclusion

In conclusion, despite the multitude of isolation, analysis, and data analysis techniques developed, no single-cell metabolomic method is flawless in all regards. Instead, the appropriate isolation and analytical technique should be selected based on the individual research goal in mind. Balancing the degree of invasiveness, throughput, and success rate of the isolation method while selecting the analytical technique with the desired sensitivity, exhaustiveness, and accuracy is not an easy task but it is essential for the success of single-cell metabolomic studies. Despite the pitfalls of single-cell metabolomics, the information gleaned from single cell studies cannot be produced in population-based assays. Therefore, in combination with other single cell omics approaches, new insights can be gained about the behavior of individual cells, and subsequently be applied in the fields of biology, medicine, and pharmaceutics.

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