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Single-Cell Metabolomics by Mass Spectrometry for Drug Discovery: Moving Forward

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Abstract

Metabolomics is one omics approach that can be used to acquire comprehensive information on the composition of a metabolite pool to provide a functional screen of the cellular state. Single-cell metabolomics is proving useful at the molecular level for major biological research in drug development. One of the main goals of single-cell metabolomics research is to gain knowledge of cellular interaction with and in response to environmental influences on a molecular level, through a comprehensive understanding of cellular functions. In this short review, we discuss the importance of single-cell metabolomics, the concentrations of metabolites in cells, single-cell isolation, analytical methodologies for single-cell metabolomics, future prospects and a possible strategy for studying single-cell metabolomics by combining Fluorescence-activated cell sorting (FACS), Cytometry by time-of-flight (CyTOF) and Liquid Chromatography with tandem mass spectrometry (LC-MS/MS).

Keywords: Metabolomics; Single-cell; Cytometry by time-of-flight (CyTOF); Liquid chromatography with tandem mass spectrometry (LC-MS/MS)

Highlights

- All populations of living organisms show heterogeneity among their individual cells.
- Strategies for characterization of the metabolome of single cells are continually evolving.
- Current technologies allow detection of hundreds of metabolites from a single cell
- Sample preparation remains a significant challenge in single cell metabolomics
- Studies of metabolomics promise to provide a greater understanding of cellular fate, function, and homeostatic balance.

The Importance of Single-Cell Metabolomics and Its Impact on Drug Discovery

Metabolomics is an emerging field with tremendous potential to improve our understanding of human health and disease, and may led to the development of personalized approaches for disease diagnosis, patient monitoring and treatment response evaluation [1]. Metabolomics has the potential to uncover phenotypic differences in a very sensitive manner and may become a powerful tool for general drug screening studies. Single-cell analysis is necessary to understand the biological and physiological properties of single cells and multicellular organisms.

As an example of one major issue for metabolomics research, it is becoming apparent that studies of population-level characteristics may overlook crucial differences among individual cells. Because cell populations are not homogeneous, metabolomics will be even more critical to research at the single cell level [2-5]. In the same way that the people of one town, state or country display significant variations, whether among themselves or when compared to other cities, states or countries, the same applies to any given population of cells. Thus, the phenotype of any one cell may be significantly different from any other cell, as well as the average of the population. This variation presents a major challenge for the metabolomics researcher, as the biological "noise" represented by these variations can be every bit as valuable to such studies. Variations in timing, pathway, and response to specific stimuli provide important information about the history, present state, and imminent future of any particular cell at any given time. Such differences may be due to variations in factors such as genetics, microenvironment, cellular history, varied developmental or cell-cycle stages, or cell age.

This manuscript emphasizes the need for new approaches to single cell analysis which will expand our knowledge of biological functions and our ability to detect, monitor and treat diseases. Currently, the lack of techniques for study of single cells hinders our knowledge of metabolic phenotypes of individual cells in heterogeneous populations. New approaches will need to provide better information on the specific "state" of any given cell, such as the effects of environmental changes, relation of cellular responses to the larger tissue matrices and improved capability to measure these characteristics. As an example, population-level research on cell-cycle-synchronized yeast cells revealed significant changes in gene regulation and metabolite levels over cell life [2] and that the phenotypes of clonal cells were influenced by the age of parent cells. This indicates that synchronization and sorting will directly affect the metabolome under study. Currently, sorting methods do not account for these time dependent changes, to the degree that final results may well not represent the original metabolome of the sample. Thus, it should be evident that single-cell metabolomics technologies

can offer significant advances, even where information exists for metabolic phenotypes of cell subpopulations [1,2].

Single-cell metabolomics technologies have developed rapidly, reaching a critical point where they can be a valuable tool for systematic characterization of cellular heterogeneity, which in turn has important implications in a wide range of biomedical fields such as disease characterization. This development has been enabled to some extent by engineering sciences, among which microsystem technologies are a major driving force. One of the best examples of the need for single-cell metabolomics is cancer. It only takes one abnormal cell in the whole body that contains 30 trillion cells to cause cancer. Single-cell metabolomics analysis has the potential to help in early detection of medical conditions involving modifications in the cellular functions such as cancer genesis and progression. There is also a need to differentiate between cells with different metabolomes within the same cancer to evaluate phenotype heterogeneity, and in effect, prevent drug resistance or discover new, more effective therapeutics [1,2,6].

While adjacent cells in an organism originate from the same genomic information, varied circumstances around individual cells or epigenetic differences have different influences on each cell, leading to differing expression of genes, and thus differing levels and dynamics of metabolites, in single cells. Despite the promise of recent technologies, single-cell analysis remains a tough challenge, because of the small size of single cells. Unlike genes, metabolites cannot be amplified, and therefore metabolite analysis is another issue. To analyze such a tiny quantity of metabolites in a single cell, various techniques have been tried and developed. The marked improvements in both detection sensitivity and ionization techniques, especially in mass spectrometry, are providing answers to this challenge for the analysis of metabolites in single cells.

Metabolite Concentration in Cells

Metabolites are the by-products of metabolism. Their formation critically depends on enzymes that act on a parent structure. As such, they represent defined chemical intermediates in a pathway that is designed to modify the parent compound. Because they are intermediates, metabolites tend to be present in small amounts. The importance of metabolites in drug development cannot be understated. When an organism's biological systems are disturbed by disease, genetic mutations or environmental factors, the profile of metabolites produced often changes. This makes metabolites excellent candidates for biomarkers and particularly useful for understanding disease states, toxicities, drug interactions, mechanisms of action and other areas of biology. Nearly every internal and external factor impacting a living organism exerts its influence by subtly altering metabolite levels. Because the metabolome is at the heart of these factors, it serves as a surrogate to the phenotype itself.

In Figure 1 the pie graph presents the molar abundance of various metabolites in glucose-fed cells [7]. The metabolite content of cells varies, due to internal and external influences on the complex biochemical reactions which sustain the cell and produce both intermediary and end product metabolites. Variations in conditions or environment will produce different metabolic pathways for individual cells. Precise, sensitive measurement of metabolites allows the researcher to infer these pathways, and by extension, provides important information about cell state. For example, in the immune system, $CD4^+$ cells can differentiate into Th17 or TReg cells. These two cell types produce different responses from the immune system: Th17 cells lead to a powerful inflammatory action, while TReg cells produce the opposite effect. The increased energy demands of the Th17 response require glycolysis to fuel the reaction.

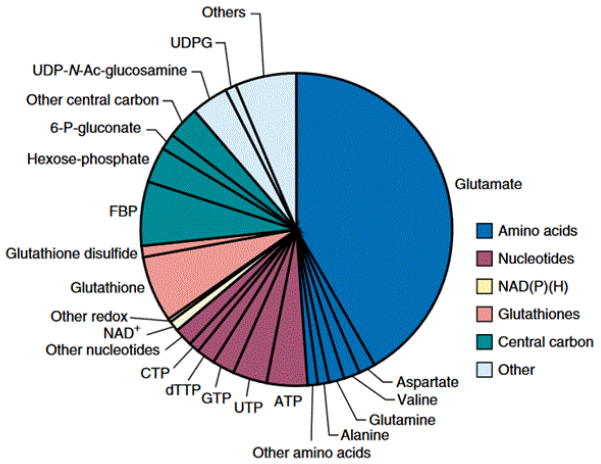

Figure 1: The composition of free metabolites for an *E. coli* cell growing on glucose, Metabolites are colored based on their functional group. In each category "other" refers to other metabolites in that category whose names are not shown due to small size. "misc." refers to other metabolites not part of any of the other categories such as UDP-N-Ac-glucosamine. FBP stands for fructose-1,6-bisphosphate. Abundant metabolites are labeled, among them FBP, fructose-1,6-bisphosphate; 6-P-gluconate, 6-phosphogluconate; hexose-phosphate, the combined pools of glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate; UDP-N-Ac-glucosamine, uridine 5-diphosphate N-acetylglucosamine; UDPG, uridine 5-diphosphate glucose (Adapted from Bennett et al. [7]).

Single-Cell Isolation

Before studying a single cell, one needs the ability to isolate it reliably. Several vendors offer technology for this, including the microfluidics-based C1 Single-Cell Analysis System from Fluidigm and DEPArray System from Silicon Biosystems, laser-capture micro dissection and fluorescence-activated cell sorting (FACS). Single cell sample isolation is an especially demanding procedure because the cellular metabolome is variable and sensitive to sampling-related perturbations such as temperature changes, enzyme or chemical treatments, and mechanical manipulations. Thus, it is vital to reduce intracellular biochemical activity and damage-induced analyte loss during this process. There are numerous approaches for reducing isolation-related perturbation in the cellular metabolome. For example, lower temperatures decrease metabolic activity. Additionally, a low-calcium medium may reduce undesirable release of analytes from neurons. Current methods for single cell isolation include serial dilution, micromanipulation, laser capture micro-dissection, fluorescence-activated cell sorting (FACS), microfluidics, manual picking and Raman tweezers (Figure 2) [8].

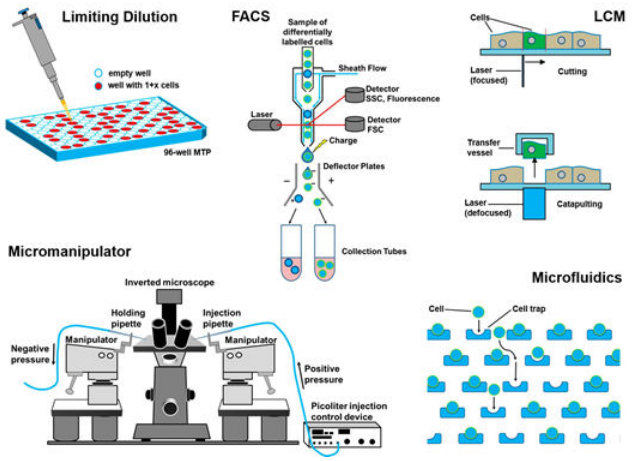

Figure 2: Schematic overview of single-cell separation technologies. The five technologies were identified through market studies as the most commonly used technologies for the handling of single cells (Adapted from Gross et al. [8]).

Analyte Extraction

Analyte extraction from the target cells becomes a crucial experimental step. This is because the measurement accuracy depends not only on the detection method but also on the efficiency of the analyte extraction. Likewise, the speed and efficiency of quenching the cell isolation-related changes impacting the intracellular metabolic activity is important. Recovery of intracellular analytes from single cells is often achieved either by lysis or by using chemical agents to make cellular membranes permeable to analytes. Physical lysis methods include high-strength electrical fields applied to cells in microfluidic devices, laser-induced lysis, lysis by freezing and/or thawing, and cell disruption by rapid decompression, osmotic shock, or mechanical means. Alternatively, enzymatic lysis or treatment with detergents is also used. Different cell types require different lysis protocols. Importantly, not all lysis methods are compatible with all of the analyte separation and detection modalities and thus must be carefully chosen. High detergent or inorganic salt concentrations may interfere with analyte desorption/ionization for mass spectrometric detection, and higher viscosity and salt content can detrimentally affect electrophoretic separations in capillaries or microfluidic devices. When working with hundreds of dynamically changing compounds within the cell, enzymatic activity must be quenched to avoid changes in the metabolome that occur during the cell sampling and analyte extraction processes. Methanol/water based mixtures are more frequently used for metabolite extraction, especially as methanol works well to quench metabolic activity. Often, multiple extraction protocols are used.

Analytical Methodologies for Single-Cell Metabolomics

Cell-to-cell heterogenicity prevails in virtually any given cell population and has fostered the development of advanced sampling and analytical approaches to enable studies at the single-cell level. To fully understand cell-to-cell variability, a complete analysis of an individual cell, from its live state to cell lysates, is essential. Highly sensitive detection of multiple components and high throughput analysis of a large number of individual cells remain the key challenges to realize this aim. In this context, microfluidics and lab-on-a-chip technology have emerged as the most promising avenue to address these challenges. The potential of the rapid expansion of high throughput single cell analysis is evident in its burgeoning impact on numerous applications such as drug discovery, diagnostics, cancer research, etc.

The detection and identification of defined analytes or their classes in single cells has been performed for many years using a range of histochemical and cytochemical methods (e.g. immunostaining, 4'6-diamidino-2-phenylindol staining of DNA), some of them developed more than a century ago and still in use today. There are four major methods used to quantify the metabolome of single cells: Fluorescence-Based Detection, Fluorescence Biosensors, Fluorescence resonance energy transfer (FRET) based-biosensors and Mass Spectroscopy. The first three methods listed use fluorescence microscopy to detect molecules in a cell [9]. Usually these assays use small fluorescent tags attached to molecules of interest. One issue with such techniques is that they have been shown to be too invasive for single cell metabolomics, as they alter the activity of the metabolites. The current solution to this problem is to use fluorescent proteins which will act as metabolite detectors, fluorescing whenever they bind to a metabolite of interest. Mass Spectroscopy is becoming the most frequently used method for single cell metabolomics. Its advantages are that there is no need to develop fluorescent proteins for individual molecules of interest, and it can detect metabolites in the femtomole range. Similarly, there has also been success in combining mass spectroscopy with separation techniques such as capillary electrophoresis to quantify metabolites. This method is also capable of detecting metabolites present in femtomole concentrations [9].

The accurate, precise and reliable measurement of metabolite levels in single cells is an excellent tool for discovery of metabolic differences in individual cells, and for system biology studies that aim at generating an understanding about the emergence of such phenotypic or genetic heterogeneity. Mass spectrometry (MS) is a label-free analytical technique which permits the identification of virtually any analyte along with additional structural information. MS is rapidly becoming one of the most widely used methods for ultrasensitive and simultaneous detection of many metabolites at the single-cell level. However single-cell analysis of metabolites by MS is challenging due to the very limited volume and inherent molecular complexity of the sample. Depending on the ionization method, MS can offer quantitative analysis for a broad class of metabolites exhibiting both high sensitivity and selectivity.

Figure 3 illustrates four different MS-based technologies that have the potential to generate data relevant for systems biology. Table 1 shows tools used in single-cell metabolomics studies, Figure 3a shows sampling the cell contents with a micropipette, followed by injection into a mass spectrometer using a nano-electrospray ionization (nano-ESI) source and 3b illustrates sample preparation on a microfluidic chip, followed by deposition on a sample plate for matrix-assisted laser desorption/ionization (MALDI or LDI) mass spectrometry. Figure 3c shows sample arraying MALDI-MS and Figure 3d shows imaging mass spectrometry. Metabolomic data are powerful because organisms have many metabolites including related precursors, derivatives, and degradation products with concentrations that vary dramatically and change rapidly. This complexity demands sophisticated separation and detection methods. MS and separations are clearly the most successful

single-cell metabolomics methods, both in terms of coverage of the metabolome and speed. Hundreds of metabolite signals have been discerned, although usually from fairly large cells such as snail neurons or plant cells [9,10].

Key point	Micropipette	Microfluidic chip	Sample Arraying	Imaging
Ionization	nano-ESI	MALDI or LDI	MALDI or LDI	MALDI, LDI or SIMS
Metabolite ID	MS/MS off-line	TOF	TOF	TOF
Live or pre-treated cells	Live, very large cells	Treated	Treated, Small cell	Treated
Sample preparation	Extensive	Extensive and off-line	Extensive	Extensive
High-throughput	No, few cells can be measured per hour	Has potential	Yes	Has potential
Metabolite	Histamine, serotonin and leukotriene	UDP, ADP, GDP, UTP, ATP, GTP, acetyl-CoA and butyryl/ isobutyryl-CoA	ADP, GDP, UTP, ATP, GTP, and GDP-Glc	Katempferol, quercetin isorhamnetin and their glycosides in individual cells. Distribution of sions such as Na+, K+, Ca+ and cationized cholesterol, lipids or their fragments.
References	10	11-13	14	15-19

nano-ESI: nano-Electrospray Ionization; MALDI-MS: MALDI-Mass Spectrometry; LDI: Laser Desorption Ionization; SIMS: Secondary Ion Mass Spectrometry; MS/MS: Tandem Mass Spectrometry TOF: Time-of-Flight; UDP: Uridine Diphosphate; ADP: Adenosine Diphosphate; GTP: Guanosine-5', Triphosphate; UTP: Uridine-5'-Triphosphate; ATP: Adenosine Triphosphate; GTP: Guanosine-5' Triphosphate

Table 1: Tools used in single-cell metabolomics studies.

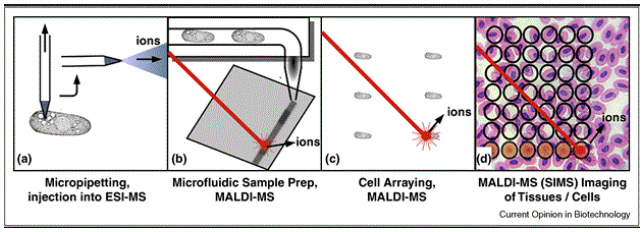

Figure 3: Schematics of the four MS-based approaches for single cell metabolomics. Heinemann et al. [2]).

As an example of the challenges ahead, identification of products from smaller cells such as yeast or bacteria is much more difficult. There are further limitations to either set of methods: both MS and Capillary electrophoresis (CE) are destructive of the sample; fluorescence and spectroscopic methods that detect metabolites non-destructively only cover single or very few metabolites. Moreover, with the exception of some high-resolution imaging methods, current single-cell analyses cannot distinguish molecular locations of compounds in subcellular compartments such as the membrane, cytoplasm, or nucleus. This leads to averaging over a single cell, which represents a limitation of current methodology. Despite these issues, the emerging field of metabolomics, aiming to characterize small molecule metabolites present in biological systems, promises immense potential for different areas such as medicine, environmental sciences, agronomy, etc. A promising new technique known as mass cytometry, or CyTOF, has been developed in Garry Nolan's lab at Stanford University. This method expands the capacity of the analyses to a range of tens, or even more than 100, specific mRNAs or proteins expressed by individual cells.

Figure 4 shows a strategy to study single-cell metabolomics by combining FACS, CyTOF and LC-MS/MS. Single-cell metabolic studies, optimization of each step-in Figure 4 workflows is essential for reaching sufficient sensitivity. Although the most abundant metabolites are in mM range, only fmol concentration can be observed due the small volume of the cells.

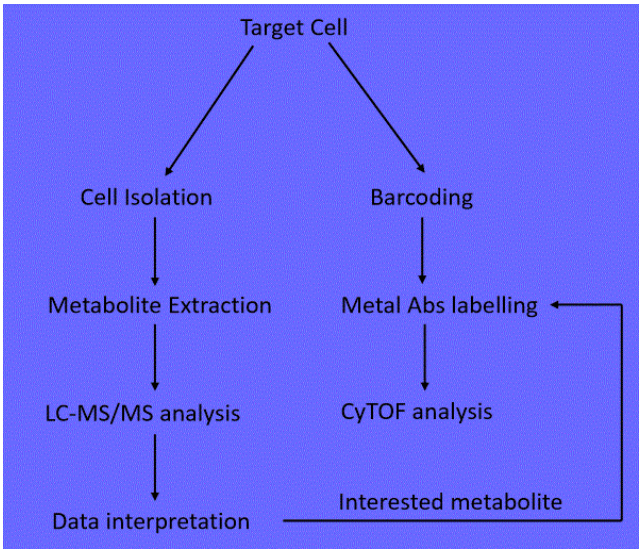

Figure 4: Workflow for single-cell metabolomic analysis outlining the experimental steps by combined fluorescence-activated cell sorting (FACS), cytometry by time-of-flight (CyTOF) and liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Conclusion

Metabolomics, a comprehensive metabolite analysis, is becoming an important method for understanding biological and physiological features of organisms. Single-cell metabolomics holds the potential to deliver unprecedented insights into the functioning of cellular metabolism, as cells are the fundamental units of all biological systems. Deep biological insight based on single-cell metabolomics has not yet been obtained, though important steps have been taken toward this goal. The analytical field has started to develop technologies for MS-based single cell metabolomics. Advances in mass spectrometry, MS imaging, capillary electrophoresis, optical spectroscopy, and in the development of fluorescence biosensors now allow the simultaneous determination of hundreds of metabolites in a single cell, with sensitivities in the attomole range. Modern array formats, in particular microfluidic platforms, contribute to our ability to perform such measurements rapidly and with high throughput. The data obtained from single-cell metabolomic studies will aid in the establishment of cellular metabolic models that will not be biased by averaging the metabolite concentrations over multiple cells.

Future Prospects

Single-cell analysis is a rapidly growing field of biology with much room for improvement, but many challenges remain to be addressed. There are several difficulties regarding sample considerations, isolation of single cells, and their subsequent analysis. Analytical chemists and biologists can work together to develop proper sample handling procedures for targeted measurement of certain metabolites and to specify metabolite classes to be measured. There are several further requirements for single-cell metabolomics to become truly useful for systems biology and medical diagnosis.

- More wide-ranging coverage of the metabolome.
- Faster identification of metabolites from single-cell data and in general measurements with high throughput.
- Continuous development of new protocols for discovery of new or unknown metabolites.
- Development of non-destructive methods of metabolite measurement.

Conflicts of Interest

The authors declare no conflict of interest.

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