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Real-time digitization of metabolomics patterns from a living system using mass spectrometry

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Abstract

The real-time quantification of changes in intracellular metabolic activities has the potential to vastly improve upon traditional transcriptomics and metabolomics assays for the prediction of current and future cellular phenotypes. This is in part because intracellular processes reveal themselves as specific temporal patterns of variation in metabolite abundance that can be detected with existing signal processing algorithms. While metabolite abundance levels can be quantified by mass spectrometry (MS), large-scale real-time monitoring of metabolite abundance has yet to be realized due to technological limitations for fast extraction of metabolites from cells and biological fluids. To address this issue, we have designed a microfluidic-based inline small molecule extraction system, which allows for continuous metabolomic analysis of living systems using MS. The system requires minimal supervision, and has been successful at real-time monitoring of bacteria and blood. Feature-based pattern analysis of *E. coli* growth and stress revealed cyclic patterns and forecastable metabolic trajectories. Using these trajectories, future phenotypes could be inferred as they exhibit predictable transitions in both growth and stress related changes. Herein we describe an interface for tracking metabolic changes directly from blood or cell suspension in real-time.

Introduction

Evidence suggests metabolite abundance exhibits phase like behavior when studied over time¹. This is best described in yeast, where glycolytic and other general metabolite oscillations are well documented². Metabolic oscillations have also been observed in bacteria³, plants^{4, 5}, insects⁶, mice⁷ and humans⁸⁻¹⁰. Despite the potential value to systems biology and a deeper understanding of metabolic networks in general, little effort has been made to observe these oscillations using traditional metabolomics. Current analyses generally focus on fold change values at distinct time points, similar to transcriptomics (mRNA) and proteomics (protein) studies^{11,12}. Metabolite abundance can change rapidly in response to even small perturbations, suggesting that important metabolic regulatory changes occur that current experimental and equipment set ups cannot capture due to low time resolution. Generally speaking, time course metabolic experiments are several orders of magnitude slower than the capability of a modern mass spectrometer (MS) to collect data. This is because the rate limiting step for long term metabolite monitoring is the extraction of metabolites and their subsequent computational interpretation.

The integration of lab on a chip technology (LOC) with mass spectrometry (MS-LOC) has the potential to revolutionize biology by directly integrating living systems with real-time computational analysis. LOC functions have the ability to extract and selectively separate metabolite and protein biomarkers from biological fluids for MS abundance measurement^{13, 14}. This makes MS-LOC an ideal candidate for real-time computational analysis of living systems, and while MS does not share the computational speed of microprocessors (GHz), it has mHz detection rates making it compatible with the current timescale of parallelized LOC valving¹⁵. Liquid chromatography mass spectrometry (LCMS) based untargeted metabolomic profiles provide an excellent source of phenotypic information, as phenotype specific patterns emerge from the quantification of thousands of molecular features which change in response to time and stress¹⁶. The majority of LCMS features are not easily identified; however, they still carry reproducible biological information which can be identified with pattern recognition algorithms. These algorithms allow us to predictively interpret the relationship between abundance patterns and phenotypes. Using a standardized system, metabolomic patterns could be used to monitor phenotypic transitions in real-time. However, one technological breakthrough which has inhibited metabolomics for real-time physiological analysis is an automated system to continuously separate the metabolites from a physiological fluid.

Here, we have engineered a system for extracting small molecules from biologically relevant fluids using a microfluidic based metabolite extraction chip (MEC) which is directly coupled to a mass spectrometer. This technology facilitates continuous automated biomolecule extraction, dramatically increasing the temporal resolution compared to standard metabolomics studies¹⁷. Analysis of cell growth and perturbation studies demonstrates the potential for metabolic forecasting using the MEC. This will vastly increase the information recovery from metabolomics experiments through the detection of oscillating metabolic transient states¹⁸. The overall setup is semi-supervised and can be

controlled wirelessly providing a framework for cloud based computational analysis and real-time predictive metabolomics.

Methods

Metabolite extraction chip

Rapid prototyping of the MEC was achieved using the method described by Duffy et al. 2008¹⁹. Polycarbonate molds for polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) chips were fabricated in a mini milling machine (Grizzly Industrial Inc. Model G8689). The PDMS layer for the MEC was mixed at a ratio of 10:1, and was cured in a vacuum oven for 4 hours at 65°C. MEC were then sealed with oxygen plasma (MARCH etcher, Nordson) to a glass slide. PEEK Tubing with 1/32" outer diameter 0.005 mm ID (Upchurch) was inserted and sealed into a modified 18 Ga needle (Supplemental Figure 1) using PDMS as glue, to interface with the MEC. The design of the chips was inspired by Bhagat et al. 2008, but optimized for diffusional separation of small molecules from cellular lysate^{20, 21}. The fabricated MEC consisted of a five loop logarithmic spiral microchannel with two inlets and two outlets (Figure 1). The spiral had an outer diameter of 17.78 mm, and an inner diameter of 7.62 mm. Each port was 2.54 mm in diameter, the inlet and outlet channels were 250 μ m wide, with the central microchannel 500 μ m in width. All port and channels heights were 10-12 μ m.

Metabolite Samples

E. coli strain BL21(DE3) was grown in Luria Bertani (LB) media (10 g tryptone, 5 g yeast extract, 10 g NaCl, pH=7 per Liter) in batch mode at 25 C with a beginning volume of 49 ml. Cultures were started by addition of 500 μ l of *E. coli* that were precultured to a cell density 2.96×10^8 cells/ml²². To evaluate whether the metabolites originated from the media or cells themselves 50 ml of the growing culture media was centrifuged in a 50 ml falcon tube at $4,300 \times g$ to pellet cells. Ions present in the cell free media were compared to the signal from the MEC. Data from the resuspended and diluted cell pellet was also checked to confirm the presence of metabolite signals. The cell free media was compared to the cell pellet resuspended in 1 ml acetonitrile (0.1% formic acid). The results of this were not so surprising; the metabolites in the media were also the most prevalent in the cells.

Ovine whole blood was collected in a sterile vial spiked with (2.6 g/ml) EDTA to prevent coagulation and injected directly onto the chip without any additional processing.

Phase contrast microscopy

Evaluation of *E. coli* lysis was done by phase contrast microscopy. Briefly, 100 μ l of *E. coli* (OD 600: 0.5) were mixed with 100 μ l of Acetonitrile (0.1% formic acid) and let sit at room temperature for 3 min in a 1.5 ml Eppendorf tube. Then 10 μ l of mixture was spread on coverslips and evaluated using an optical microscope (Nikon Eclipse E400) with both a 40 $\times$ and 100 $\times$ objective. Repeated evaluation indicated that no intact cells were present.

Mass Spectrometry

The oxidized glutathione (GSSG) diffusion experiment was performed on an ion trap (6340, Agilent Technologies). Analyses were performed using an isocratic flow (1100 HPLC, Agilent Technologies) of 50 μ l/min (50% A = 0.1% formic acid in water, 50% B = 0.1% formic acid in acetonitrile). The system was operated in positive electrospray ionization mode. In MS mode, the maximum accumulation time was 500 ms, scan range of 50-2200 m/z, and averages = 3. For multiple reaction monitoring (MRM), masses were selected and isolated for MS(n) with a width of 4.0. For *E. coli*, a direct injection of 30-592 cells (OD) generated a satisfactory signal in an injection volume of 2 μ l. ESI-TOF analyses were performed using an isocratic flow (1290 UPLC, Agilent Technologies) of 50 μ l/min (50% A = 0.1% formic acid in water, 50% B = 0.1% formic acid in acetonitrile) coupled to a Q-TOF (6538, Agilent Technologies). The system was operated in positive electrospray ionization mode, using extended dynamic range (50-1700 m/z), 1 scan per second. For *E. coli*, direct injection of 148-2960 cells in an injection volume of 10 μ l generated satisfactory signal, without saturating the chip or MS.

Interfacing the Metabolite extraction chip and mass spectrometer

MS for metabolomics is typically setup in tandem with liquid chromatography (LC) for separation of the complex mixtures of small molecules. However, our system utilizes an isocratic LC flow through the MEC at all times with periodic direct injections of sample into the continuously flowing stream. The speed, volume and periodicity of each injection can be adjusted using the syringe pump (NE-4000, New Era Pump Systems Inc.) whose control is synced with the valve (MXP-9900, Rheodyne) using an in house program designed with labview (National Instruments). Small molecule diffusion was controlled by adjusting the passive flow rate through the chip where slower flow rates allowed greater diffusion across the channel. The syringe pump valve combination allowed for metabolomics data to be generated in a continuous fashion from culture and directly from blood. The data manifested as ionization pulses in the MS, where pulse intensity could be controlled by the size of injections. Pulses had a periodicity of five minutes which was precisely controlled by the injection cycles of syringe pump valve combination. Testing showed that with the current tubing and valve set up the period could be decreased to 3 minutes without significant degradation in performance. By modifying injection volume and speed we could use a wide range of cell numbers to generate satisfactory results. This was necessary as pulse characteristics were not modified during the experiment except to avoid catastrophic failure of the system. The number of cells analyzed changed depending on how long the culture had been growing. During early phases of growth when the cell count is low (iontrap: 30, QTOF: 148, cells/injection) each pulse has less cell material than later on in growth (iontrap: 592, QTOF: 2960, cells/injection). Optical density growth curves for three replicates have been included as supplementary table 1, allowing the cell count to be deduced from the volume at the appropriate OD²².

Data analysis

Data was extracted using pymzML, a Python library for high-throughput bioinformatics on mass spectrometry data²³. MS pulses were approximately two minutes in length and the

intensity of each m/z was summed for the duration of the pulse and placed into a list. For *E. coli*, m/z were manually validated with Masshunter Qual (Agilent). The m/z intensities were imported into R where data visualization was done using principal component analysis (PCA) or plot, R function `prcomp` and `plot`. Data was mean centered (using function `prcomp` or `scale`) to negate the difference in pulse ionization abundance.

Results and Discussion

An engineered system for automated fast extraction of metabolites from cellular lysate that can operate in a continuous mode has great potential to revolutionize metabolic study by allowing direct analysis of a living system with high time resolution. Biofouling is a major issue with blood and cellular lysate even when injected after substantial dilution. Tests conducted on our system in which the waste outlet was directed to the instrument resulted in nearly immediate clogging of the ESI needle. Similar result occurred when diluted cellular lysate is injected directly from a syringe. To overcome this obstacle, a MEC that consisted of a five loop logarithmic spiral microchannel with two inlets and two outlets was fabricated (Figure 1). During operation, an isocratic flow of 50 $\mu\text{l}/\text{min}$ (50% acetonitrile, 0.1% formic acid) was continuously run through the MEC and into the MS, with periodic injections of sample into channel 2. The MEC took advantage of low Reynolds numbers in the microfluidic environment, which enabled the system to diffusively extract small molecules from cell lysate without turbulent mixing²⁴. As the injected sample traversed the MEC, diffusion of solute molecules from channel 2 to channel 1 occurred (Figure 1B). By design, only small molecules displayed rates of diffusion that allowed a significant accumulation in the channel directed for analysis. The outlet of the MEC sent the majority of the sample (cellular debris) to waste and the diffused small molecules directly to the MS for detection (Figure 1C). Channel diameter and flow was optimized to capture sufficient material for mass analysis, while minimizing buildup during long periods of continuous instrument operation (>8 hrs). Injection of sample into the MEC was facilitated by a dual syringe pump which was placed in line with a 2-way 6 port valve (Figure 2). In valve position 1, syringe 1 was set to draw MS solvent (50% acetonitrile, 0.1% formic acid) and syringe 2 the sample of interest (GSSG/*E. coli*/ovine blood) mixing with acetonitrile (Figure 2A). In position 2, the sample was injected into the respective channel of the MEC (Figure 2B). The periodicity of injections resulted in pulses of metabolites which were easily detected by ESIMS.

GSSG is a good molecular target for assessing cellular status as it is present in blood, during stress, disease and aging²⁵. It is also a mid-sized metabolite with a molecular weight of 612 daltons (m/z 613.159 $M+H^+$) see Figure 3. Therefore, GSSG was used for testing performance of the MEC. Quantification of diffusion was measured by the abundance of GSSG detected in each of the outlet channels. These experiments used a set period of 5 min., and testing showed that the period could be decreased to 3 minutes without significant carryover. The MEC had a diffusion efficiency of 23.23 $\pm$ 2.33% for GSSG from channel 2 to channel 1 at 50 $\mu\text{l}/\text{min}$ in 50% acetonitrile with 0.1% formic acid. Testing showed this efficiency of small molecule diffusion to be the optimal, as it ensured that the vast majority of cellular polymers and lysis debris had insufficient time to diffuse and remained in the waste channel preventing buildup in the mass spectrometer²⁴.

To test the capabilities of our system for monitoring a living system, we tracked a growing culture of *E. coli*. Automated sampling directly from the cell culture on a five minute cycle was used. The rate of diffusion was reproducible with total ion counts maintaining peak intensity at $5.58 \times 10^5 \pm 0.146 \times 10^5$ ions. A special lysis chamber that used a voltage gap to lyse cells was designed and tested. After use it was noticed that the process of mixing the cell culture broth with acetonitrile resulted in complete cell lysis (Supplementary Figure 2) so the additional module was removed. The cell lysate was then injected into the MEC for metabolite extraction and subsequent MS detection. Seventy-seven small molecules from media and cells could be detected consistently during the *E. coli* growth (Supplementary Figure 3). These 77 small molecules were observed in both cell free media and media free cells, so the contribution from each source was to known. However, only in the presence of growing cells was the abundance of small molecules observed to show smooth increases and decreases in abundance (Figure 4A) and oscillatory behavior during growth (Figure 4B). Because each of these small molecules displayed unique abundance changes over time, the oscillations were thought to be related to metabolic changes and not an instrument artifact. Interestingly the oscillations were not smooth, but bore a striking resemblance to the chaotic glycolytic oscillations observed in yeast². Using the abundance of the seventy-seven small molecules in each pulse, the changes over time were visualized using PCA (Figure 5). Time points were separated in chronological order, where each was most similar to those closest to it in time (Figure 5A, B). During one time course the system was paused and restarted after twenty minutes. This left a gap in the data which appears as a void in the PCA spacetime (Figure 5C). To provide evidence that the changes in metabolomic patterns were a product of growth, *E. coli* was allowed to progress to stationary phase and pulses were plotted by PCA (Figure 5D). The data now showed that sequential samples were no longer clearly distinguished by temporal linkage. Combined the PCA analysis revealed time dependent patterns could be clearly distinguished in the growing *E. coli* cultures.

Replicate cultures of the same cells under similar conditions would be expected to have similar metabolic fingerprints and somewhat reproducible changes over time. To test that this was in fact the case, data from the independent batch cultures of *E. coli* were analyzed together. A PCA plot of the data showed that there was distinct conservation in the trajectory of individual plots, however, the beginning and ending points differed (Figure 5E). This variation is probably due to the fact that cells used to inoculate the batch culture were taken from a continuous culture on different days. The goal of the experiments was not to minimize the variation between growths, but to instead observe the natural variation of the organism devoid of strict experimental control. In this way the degree of conservation could be attributed to natural metabolic constraints not human experimental control. In order to test if small changes in the metabolic state of the cells could be detected, 100 μ M hydrogen peroxide was added to one replicate in the middle of the run. A change in the trajectory of the growth can be clearly seen (Figure 5E). The time points after the addition deviated from the smoothly curved overlaid trajectories before stress.

Plots in the abundance of the 77 small molecules over time were compared to find consistencies between the three replicates and difference which could be indicative of stress. Temporal changes in small molecule abundance were conserved across three different growths (Figure 6). This explains the similar trajectories presented in the PCA above.

However, markers of stress are also present. The feature at 132.1001 m/z was present in all growths, however, in contrast to the other features, it had a very distinct abundance change after the addition of hydrogen peroxide. MS/MS of this feature was consistent with the amino acids leucine/isoleucine. Given the experimental conditions, the detection of leucine/isoleucine consumption is highly relevant. Leucine supplementation, can be used to protect cells from damage caused by hydrogen peroxide in *E. coli* cultures²⁷. Supplementation of branched chain amino acids (leucine, isoleucine and valine) is used for the treatment of burns, trauma and sepsis, making them all attractive candidates for physiological monitoring²⁸.

Combined, this evidence demonstrates this system is suitable for automated monitoring of metabolic processes. It is capable of quickly identifying growth patterns and phenotypic changes such as those caused by oxidative stress. Further development of this technology could be especially useful for large scale monitoring of cell culture during mass production of pharmaceutical therapeutics and protein nanomaterials²⁷.

The ability to monitor metabolic regulatory patterns could have implications far beyond the scope of tracking microbial growth phenotypes. This is because metabolomics abundance data can be a highly sensitive to a wide variety of environmental influences including both disease and stress^{16, 28-29}. Therefore a miniaturized system such as the MEC used here could one day be used for extracting detailed information about the human body. Real-time monitoring of metabolic patterns from a population could allow preemptive identification of disease and/or monitoring of pharmaceutical intervention. To test the applicability of the current MEC setup and data analysis for mammalian metabolic monitoring, samples of whole blood were analyzed. Two μ l samples of blood mixed with acetonitrile (0.1% formic acid) were injected into the system every five minutes for 1.5 hours without a significant change in sensitivity or fouling. Total ion counts over the 1.5 hours were $1.46 \mu 10^6 \pm 0.214 \times 10^6$, indicating that diffusion rate is constant and reproducible (Supplementary Figure 4). Over one hundred small molecules could be detected. The system was capable of monitoring for extended periods of time, but because we were analyzing a blood sample that was previously collected, compared to an interavenous draw, no changes in metabolic activity were observed.

Conclusion

Metabolomics technology is often used for identification of biomarkers that can be indicative of disease or stress. However, because the low time resolution of typical metabolomics data it is easy to see how transient changes in metabolism could cause erroneous biomarker identification. Here we have designed a system to vastly increase the time resolution of metabolomic study²³ by automating metabolite extraction from living cells. The results indicate we can identify oscillatory regulatory patterns in real-time (while the organism is growing) from as few as thirty cells. However, the system does have pitfalls. The direct injection of small molecules decreases the number of molecular features we can see. Typically hundreds to thousands of small molecules can be viewed with MS when chromatography is used, but our system is limited as we only detected between ~77-100 small molecules per injection. This shortcoming could be overcome by integrating fast

chromatography between injections, as injections only take place approximately every 5 minutes. In addition, the system is very large would need to be miniaturized for portable use. Portable capability could be close however, as most functions already exist in miniaturized forms including microfluidic pumps, valves and mixers, and MEMS mass spectrometers³⁰. Here we have provided the first system for observing real-time metabolomic patterns from a living system and raise the bar for future metabolomics time course study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Metabolite extraction chip (MEC). (a) photo of the PDMS chip, (b) schematic of inlet: *port 1*: 25% ACN, 0.1% FA; *port 2*: cell lysate and (c) schematic of outlet: extracted metabolites are directed to the mass spectrometer (MS) while cellular debris goes to waste (W).

Figure 2. Binary setup of integrated syringe pump, valve, solvents, MEC, and mass spectrometer, (a) valve is in position 1, syringe pump is set to draw from cells and solvents; (b) valve is in position 2, syringe pump is set to pump.

Figure 3.

Diffusion of GSSG (613.1 m/z) in MEC on ESI-Trap, injections are made every 5 minutes in a 30 minute run. (a) Extracted ion chromatogram (EIC) of GSSG. EIC of GSSG standard (higher) versus EIC of GSSG diffusion in MEC (lower). (b) Resolved isotopic peaks of GSSG.

Figure 4. Change in *E. coli* metabolite abundance over 6 hrs (x-axis: time). Extracted intensities for eight representative small molecules. (a) Abundance changes of four of small molecules which exhibit smooth transitions over time. (b) Abundance changes of four small molecules which exhibit oscillatory changes over time.

Figure 5.

Real-time analysis of *E. coli* growth by MS, colors of PCA indicate sequential subsets of time points (b, c, d). (a) Periodic injections of cellular lysate onto MEC yields total ion count (TIC) as the small molecules are detected by the MS (left). Difference in TIC depending on injection time point (right). (b) PCA of fifteen sequential time points during growth. (c) PCA of fifty time points during growth, red circle indicates missing time points when syringe pump ceased injections. (d) PCA of *E. coli* in stationary. (e) PCA of three growth replicates, each represented with a different color. (black) 8.5 hour growth, (red) 8.5 hour growth, (green) 6 hour growth, stressed with H_2O_2 after 4 hours, resulting in a different trajectory on the plot indicated with red circle.

Figure 6.

Change in small molecule abundance during *E. coli* growth for five small molecules in three growth replicates (Y-axis: abundance, X-axis: time). Abundance changes for four small molecules which exhibit similar changes over time. Leucine and/or Isoleucine displays a much different pattern. A ~10-fold decrease in abundance occurs in response to H_2O_2 . It should be noted that plots have been scaled to capture changes. The change in abundance observed for Leucine/Isoleucine appears less dramatic because of the scaling used.