ABSTRACT

Title of Thesis:	ENHANCED THROUGHPUT SINGLE-CELL CAPILLARY ELECTROPHORESIS MASS SPECTROMETRY
	John Udara Mendis, Master of Science, 2023
Thesis Directed By:	Associate Professor, Peter Nemes, Chemistry & Biochemistry

Mass spectrometry (MS) has allowed for the analysis of small molecules and metabolites with high specificity and sensitivity. Capillary Electrophoresis mass spectrometry (CE-MS) is an ultrasensitive analytical technique to process amount-limited samples. Robust high-throughput ultrasensitive CE-MS methods and technologies are needed to be developed to comprehensively study the metabolome or proteome of a sample with a limited amount of material. In this study, we developed an enhanced-throughput multi field amplified sample stacking (M-FASS) method. The resulting approach has a sample processing throughput of 5–10 time that of conventional CE methods. FASS voltage duration and strength were optimized for peak area and peak resolution. The M-FASS CE-MS method was then applied for single cell analysis (SCA) of metabolic differences and gradients in the developing embryo of Xenopus Laevis. The statistical analysis: PCA and Fuzzy-c means clustering analysis revealed cell-to-cell differences among D11, V11, D12, and V12 cells and uncovered 6 distinct metabolite gradients between the four cells in X. laevis 16-cell embryos. The findings showcase inherent metabolic gradients in the developing embryo.

ENHANCED THROUGHPUT SINGLE-CELL CAPILLARY ELECTROPHORESIS MASS SPECTROMETRY

by

John Udara Mendis

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2023

Advisory Committee:
Associate Professor Dr. Peter Nemes, Chair
Professor Ian White
Professor Yang Tao
Professor David Goodlett

Acknowledgments

I would like to express my deep appreciation to all those who have supported and encouraged me throughout my studies at the University of Maryland. It was definitely not an easy or linear journey, but I am grateful to have met many great people, learned interesting topics and life lessons, and had many great experiences over the past few years in Maryland and the DMV.

First and foremost, I would like to thank my supervisor Dr. Nemes for your invaluable guidance, encouragement, and support throughout the research process. Your expertise and insights were critical in shaping this work and helping me navigate the challenges along the way.

I also want to thank Dr. David Goodlett, my first graduate school advisor, for introducing me to the wonders of mass spectrometry and for providing me with a home to do research, albeit for a year, at the UMB Dental School.

I want to thank everyone from the Nemes Lab for helping me learn different lab techniques, bounce off research ideas, collaborate on different projects, and review my written work, especially Dr. Jie Li, Ryan O'Neal, Dr. Erika Portero, Dr. Aparna Baxi, Leena Pade, Steven Woolford, Dr. Kellen Delany, Bowen Shen, Dash Jia, Ailing Li, Yayra Turani, and Fei Zhou. Without your help and support, this thesis work would not have been possible.

I would also like to extend my gratitude to the faculty and staff of the Bioengineering Department especially Bill Churma, Dr. Ian White, Dr. Helim Aranda-Espinoza, and Dr. Yang Tao, some who also were my committee members, who provided me with professional guidance and emotional support during periods of my time at Maryland.

Also, I want to thank my classmates and friends in Maryland and the DMV area (and outside) who were helpful outside of the lab and classroom and helped me to develop personally and professionally, including Dr. Brandon Gaitan and Dr. "Niv"edita Hegdekar.

Leaving the best for last, I am deeply indebted to my family for their unwavering support and encouragement throughout my academic career and studies. A big thanks to my Thatha (Rohan), Amma (Maureen), Akka (Anne Thanya), and Nangi/Chuchu (Natasha). Your love and encouragement kept me going through difficult times, and I cannot thank you enough for your unconditional support.
As with science and in life, expect the unexpected and enjoy the process no matter how difficult it may seem (especially with people closest to you – professionally and personally). Once again, I thank everyone for being part of my journey, and I look forward to a new chapter in my life.

Table of Contents

Acknowledgments.....**Error! Bookmark not defined.**
Table of Contents.....iii
List of Figures.....**Error! Bookmark not defined.**v
Chapter 1: Background & Motivation 1
Section 1.1 Research Motivation, Significance & Overview 1
Section 1.2 Mass Spectrometry for Devleopment and Metabolomics 2
Section 1.3 Sample Preparation for Metabolomics..... 7
Section 1.4 Capillary Electrophoresis..... 8
Section 1.5 Single Cell MS..... 13
Section 1.6 Research Model: Xenopus Laevis for MS-Based Metabolomics 14
Chapter 2: Development of High-Throughput Single Cell CE-ESI-MS (M-FASS) Workflow 16
Section 2.1 Introduction..... 16
Section 2.2 Methods & Materials 17
Section 2.3 BGE Spacer Gap Apporach 19
Section 2.4 M-FASS Approach 20
Section 2.5 Analytical Validation and Optimization of M-FASS Method..... 21
Chapter 3: : Uncovering Spatiotemporal Metabolic Heterogeneity and Gradients in a 16-cell Embryo..... 26
Section 3.1 Introduction..... 26
Section 3.2 Methods & Materials 26
Section 3.3 Application to Assess Metabolic Heterogeneity in the Developing Embryo..... 29
Chapter 4: Summary & Future Directions..... 34
Section 4.1 Summary..... 34
Section 4.2 Future Directions 35
References..... 36

Table of Figures

Figure 1.1 General Mass Spectrometry Workflow for Metabolomics 3

Figure 1.2 Diagram of Capillary Electrophoresis 10

Figure 1.3 Diagram of Field Amplified Sample Stacking 12

Figure 2.1 Ultrasensitive Multi-Plug CE-MS workflow 17

Figure 2.2 FASS vs M-FASS Injection 20

Figure 2.3 5kV vs 15kV M-FASS injection 21

Figure 2.4 Effect of Increasing FASS Voltage to Peak S/N Ratio 22

Figure 2.5 Effect of FASS Voltage on Separation Resolution 23

Figure 2.6 Calibration Curve of Selected Metabolites in a 5-plug M-FASS CE Run 24

Figure 2.7 Histogram of Metabolite Peak Area RSD for 16-cell embryo 27

Figure 3.1 HCA Heat Map 30

Figure 3.2 PCA plot and PCA loadings plot 31

Figure 3.3 Fuzzy-C means clustering analysis 32

Chapter 1: Background & Motivation

Section 1: Research Motivation, Significance & Overview

CE is an ultrasensitive technology that can be utilized to study cellular biology, especially from the metabolomics perspective of the cell. For applications such as single-cell analysis which require the analysis of many samples with high statistical confidence for compound identification and quantification, it is important to develop high-throughput methods. **In this thesis work, I developed an enhanced-throughput Multi-FASS (M-FASS) CE method, which has been applied to explore metabolite gradients and cellular heterogeneity in *X. laevis* during early embryonic development.** In Chapter 2, I will discuss the analytical development and optimization of the M-FASS method, and Chapter 3 will explore how it was applied to study a developmental biology problem and uncovered inherent metabolite gradients between adjacent cells at a particular stage of development. This novel and scalable approach increased the throughput, sample processing, and analysis speed of an ultra-sensitive custom-built CE system by 5-10x for volume-limited samples. This can be adapted to many different cell types or biological samples. Advances in throughput can allow for the analysis of many samples, which is an important development for biological mass spectrometry single-cell analysis and allowed for the discovery of spatial metabolite gradients in the developing embryo.

Section 2: Mass Spectrometry for Development and Metabolomics

Understanding and making sense of the molecular complexity of biological cells, at the multi-omics level, is important to know about the state of the cell and its associated dynamic processes. Having this knowledge will aid in a better understanding of different biological processes, especially with regard to normal embryonic development and diseases such as cancer and diabetes. Traditionally, there is a plethora of knowledge concerning different kinds of genes, proteins, and transcripts and their role in multiple processes biological in cell biology, developmental biology, and pathological processes. However, understanding the metabolome is important for studying molecular phenotypes. The metabolome refers to the complete suite of metabolites that exist within an organism and can provide a snapshot of its metabolic state, which is closely related to the molecular phenotype. A main reason to study metabolomics is that metabolites are the best descriptor of the molecular phenotype. Furthermore, the molecular architecture refers to a dynamic and intricate network of molecular pathways and interactions that effect biological functions. The metabolome represents many intermediates and final products in this molecular architecture. Understanding the metabolome, at the single cell level, will be key to gaining a more complete and robust understanding of human health, diseases, and development.[1].

Currently, the Human Metabolome database[2] states that there are over 114,000 small molecules that occupy a dynamic range between 7 to 10 log-order concentration range[3], which only represent a sliver of total molecules in the cells**.** These metabolites are biologically relevant and endogenous small molecules, usually

with a molecular weight less than 1500 Da, that are involved in driving, regulating, and propagating biological processes and pathways with many associated intermediates[1]. Detecting metabolites has many challenges. They have a large dynamic concentration range, diverse physiochemical properties, a large turnover rate, and overall limited coverage from many analytical techniques[4-6]. These aspects make the general detection, quantification, and identification complex. Therefore, it is imperative to have highly sensitive, reproducible, and high throughput technologies and methods to analyze many cells for metabolite detection with high statistical confidence.

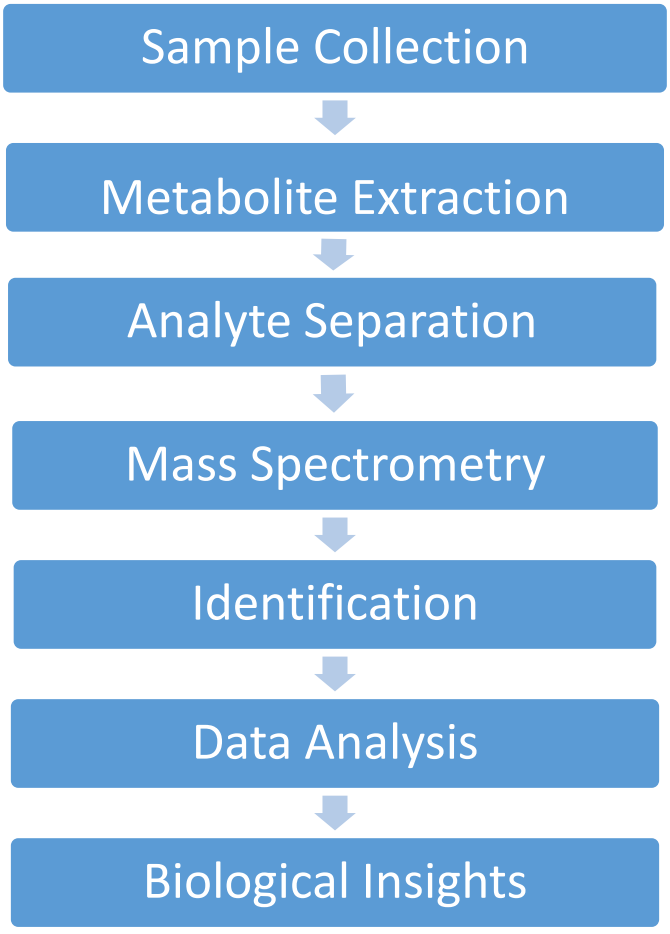


Figure 1.1: General Mass Spectrometry Workflow for Metabolomics

Recent advances in Mass spectrometry (MS) have allowed for the detection and identification of hundreds of different kinds of metabolites, lipids, and biologically relevant small molecules in numerous animal model and sample types[7,8]. Fast metabolite analysis has been seen with frog[9,10], mouse[11], human[12], and bovine[13] embryos and oocytes, with different ionization techniques, such as desorption electrospray ionization (DESI)[13] laser ablation electrospray ionization (LAESI)[10]**,** and Electrospray Ionization (ESI)[14]. Furthermore, MS techniques have been used to detect and identify metabolites in tissues and single cells[15-18].

Efficient sample preparation and processing is very important for metabolite analysis from cells and tissues. The main way of extracting metabolites from cells and tissues is the addition of cold organic solvents. The sample preparation should involve limited transfer steps, since small molecules can stick to surfaces thereby reducing number of metabolites in samples, especially volume limited samples[19].

To have the most accurate detection of metabolites, it is advised to couple the mass spectrometer to a suitable separation technology. Integrating a separation technology can lead to better IDs and deeper omics by limiting interferences of isobaric species. Separation technologies can facilitate separating compounds prior to detection by the MS and can provide additional information for identification, such as migration or retention time[8]. Separation technologies can facilitate the seperation of sample components by chemical and physical properties, which reduces the sample complexity. This aids in the detection of compounds that are in low in abundance by

reducing the masking effect that would occur by the presence of more abundant compounds. Additionally, the use of seperation technologies can improve peak capacity or the number of resolved peaks, while reducing ion suppression and co-elution. Ion suppression occurs when signal from one compound is quenched or reduce by the presence of different ions. Seperation technologies can reduce this effect, which in turn increases sensitivity and more accurate quantification. The reduction in co-elution of analytes can lead to better MS-MS fragmentation, which can improve identification of compounds[8]. Notable separation technologies include liquid chromatography (LC), capillary electrophoresis (CE), and gas chromatography (GC), and ion mobility (IM). Each of these separation technologies have multiple advantages and disadvantages and must be selected based on the overall application[8]. GC-MS usually has very reproducible retention times and relatively high separation efficiency, but require chemical derivatization for different kinds of molecules, which could be difficult for some compounds such as polar metabolites[20]. LC-MS methods have been used extensively in medicinal and biopharmaceutical chemistry applications and have been tailored to analyze lipids[21], metabolites[22],[23], and peptides[19]. However, they require large sample volumes. CE-MS is a newer technology that has high separation efficiency, especially for metabolites, and requires only a small volume of sample[24]. Advantages of CE are that it has a high separation efficiency and relatively quick analysis time compared to other methods, such as LC. Comparing CE and LC, CE has better separation efficiency since it operates at higher flow rates with plug flow, therefore the contribution of longitudinal diffusion to the plate height is smaller resulting in better separation efficiency.

Seperation is CE is done in a narrow capillary, which has limited interactions with the capillary wall. This leads to sharp, symmetrical peaks with excellent peak resolution and peak symmetry, which is in direct contrast to LC which has band broadening due to the packed stationary phase.[24] Therefore, CE is ideal for rare and volume-limited samples, such as individual single cells. A deeper review of CE-MS will be covered in the next section.

MS is a powerful analytical tool that can be used for metabolite identification and quantification. Fast MS/MS with high *m/z* resolution can lead to better IDs. A major strength of using this technology is that it has relatively high sensitivity, great specificity, and detection can be used in a label-free manner[14]. Many MS-centered workflows for metabolomics analysis have very high reproducibility and can allow for both very accurate relative and absolute quantification. The two main approaches of MS workflows for metabolomics are untargeted or discovery based analysis and targeted metabolomic analysis. In the former, a certain type of metabolite or a group of metabolites in a pathway are preselected prior for analysis. This kind of analysis is common in medicinal and pharmaceutical applications[25]. Conversely, an untargeted approach is ideal for maximizing the number of metabolites detected in unknown or hypothesis generating studies. In single-cell metabolomics studies, the standard workflow is cell isolation and sample preparation followed by MS-based detection and identification of metabolites and data analysis[9].

Another important consideration and bottleneck when running MS-metabolomics is metabolite identification. Many metabolites and their intermediaries have very similar mass-to-charge (*m/z*) ratio, which is the main parameter of

detection in MS[26]. Other information that can be used to identify is MS/MS fragmentation data, migration time (in CE-MS) and retention time (in GC and LC), and ion mobility values (in IM-MS)[25]. There is a plethora of metabolite identification databases and software from MS and MS/MS data, such as the Human Metabolome Database[2], METLIN[27] and mzCloud[28]. Additionally, a generally accepted minimum reporting standards for metabolomics data has suggested and outlined in the Metabolomics Standards Initiative (MSI)[26]. For these reasons, MS is a robust choice for analytical cellular biology and metabolomics.

Section 1.3: Sample Preparation for Metabolomics

Efficient sample preparation is need for ultrasensitive MS metabolomic analysis. The most important first step in sample preparation is quenching or rapid stopping of metabolic activity. This is usually done with the addition of cold organic solvents. The extraction process should create an unvaried extract that is representative of endogenous metabolite levels of the cell of tissues[19]. Therefore, minimally invasive metabolic perturbation during extraction process is preferred. For biofluids, such as serum and urine, and cells and media, quenching happens with introduction cold organic solvents.

For tissues, it is recommended to have quick excision coupled with snap freezing via liquid nitrogen followed by addition of extraction solvent. Freeze clamping may be needed for bulky tissues to ensure proper tissue freezing[19]. Capillary microsampling, where a glass capillary is attached to a vacuum, can be used to aspirate contents of a living cell. Two advantages of this approach are that it is a minimally invasive sampling approach with minimal perturbation to the cell and the

cells are sampled in the natural spatiotemporal environment[29]. The aforementioned conditions must be considered when deciding on sample preparation techniques for metabolomics MS analysis.

Section 1.4: Capillary Electrophoresis

Capillary Electrophoresis (CE) is a separation method where under the application of an electric field charged analytes move and migrate within a capillary. It has been used as a powerful separation method between different types of compounds such as biological macromolecules, peptides, vitamins, dyes, proteins, etc[30]. The primary mechanism of CE separation lies in electric field (E) induced ion velocities (v) differences, as seen in **Eq. 1**. The Factors that influence the ion electrophoretic mobility (μ_e), as described in **Eq. 2**., are the buffer viscosity (η) and charge-to-radius ratio[31].

$$v = \mu_e E \quad \textbf{(Eq. 1)}$$

$$\mu_e = \frac{q}{6\pi\eta r} \quad \textbf{(Eq. 2)}$$

This means that charged and smaller molecules will have higher ion mobilities as compared to lesser charged, neutral, and larger molecules. Another factor that CE separation is electroosmotic flow (EOF). The EOF is liquid bulk flow inside the separation capillary, which is determined by the surface charge inner capillary wall of the fused silica capillary (FSC). The surface charge can change with the pH. Therefore, the amount of EOF can be adjusted by changing the coating of the inner capillary wall, the buffer's ionic strength, and or the pH buffer within the capillary. Depending on the charge of the inner wall, positively and negatively charged ions

will migrate differently to the anode or cathode. However, because of the EOF, all charged and neutral molecules will move in EOF direction towards the detector, if the electrophoretic velocity is less than EOF. Conversely, the ions will move in the opposite direction from the EOF, if the electrophoretic velocity is greater than the EOF. In our study, analyte migration would be the cathode due to the use of a low pH background electrolyte and resulting negative inner capillary charge. As opposed to LC, which has a laminar flow profile, EOF in CE has a relatively flat flow velocity profile, which allows for separations that are very efficient[32]. The efficiency of separations is based on number of theoretical plate numbers and plate height values. Separations that are very efficient have a high number of theoretical plates and low plate height values. CE, which has limited peak broadening, has an estimated 1 million theorical plate number, as opposed to ~10x less plates in LC[33]. Chromatographic efficiency can be defined by the theoretical plate height (H) through the Van Demeter equation[34] (**Eq. 3)**. With no solute-wall interactions or column packing, both the eddy diffusion (A) and mass-transfer term (C), the only influence on the plate height in CE would be longitudinal diffusion (B) term.

$$H = A + \frac{B}{u} + Cu \text{ (Eq. 3)}$$

There are various methods to concentrate the sample on-line through velocity gradient techniques. This is used to increase sensitivity and lower detection limits. Four main kinds of on-line sample concentration techniques are stacking, sweeping, dynamic pH junction, and transient isotachophoresis (ITP) and electrokinetic supercharging[35].

For stacking, there are three major approaches. These are field amplified sample stacking (FASS), Large Volume Sample Stacking (LVSS), and pH-mediated stacking[35].

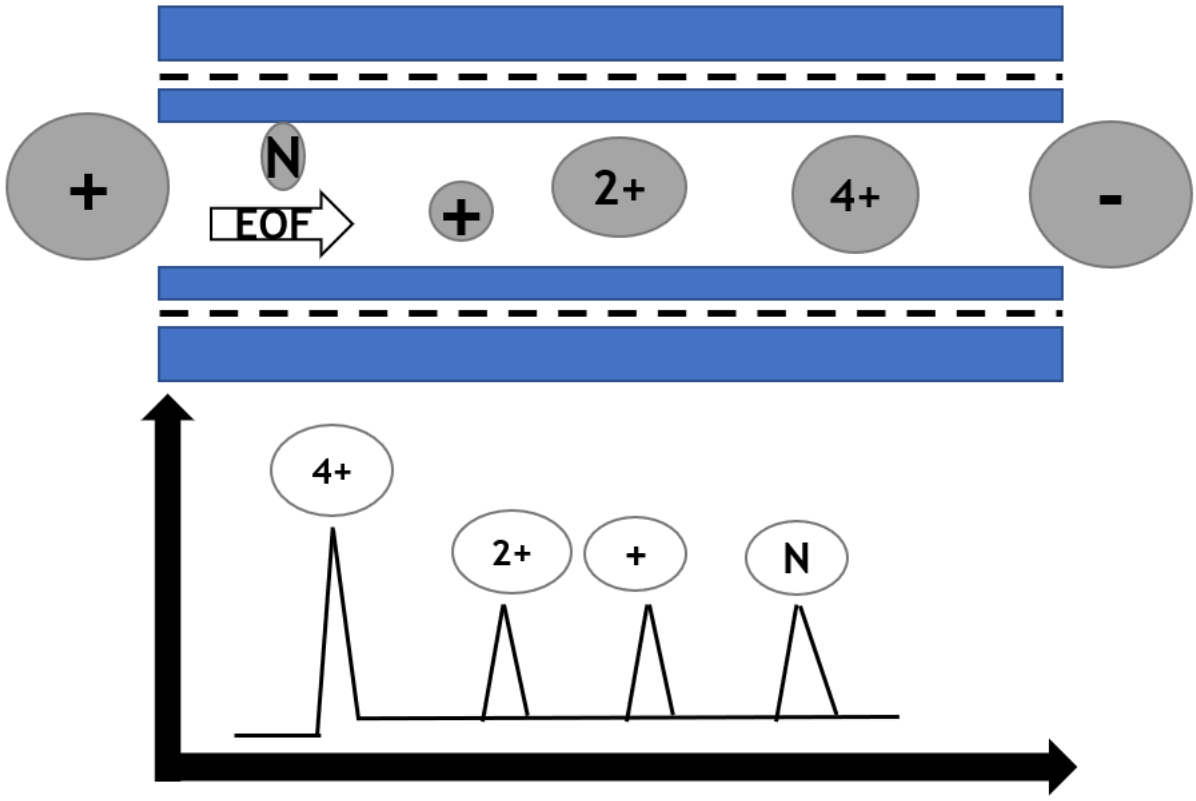


Figure 1.2: Diagram of Capillary Electrophoresis with a representative CE-MS chromatogram

FASS method is considered one of the easiest techniques for online sample concentration, where the sample is made in a matrix with low-conductivity, and the background electrolyte (BGE) has high-conductivity. The sample is injected in a capillary that is already filled with BGE. Then, a high positive voltage is applied, there is a larger electric field that allows the ionic analytes to move more quickly towards the sample zone and BGE boundary. Then, the electric field decreases greatly

making migration slower. This makes the sample ions concreated on the boundaries. As the EOF mobility is greater than of the ionic analytes and the EOF is greater than the electrophoretic velocity, all the analytes will go towards the detection window, with cations migrating at a quicker rate than anions. The analytes will be separated in capillary zone electrophoresis (CZE) mode. The volume of injected sample is between 1-3% of capillary length, since a larger sample volume would make focusing difficult and have limited length for CE separation[35]. FASS has been used for a wide range of detection applications from alkaloids[37] and metabolites[38], with 10-10,000x sensitivity. LVSS is like FASS, but the polarity of the electrode is reversed to create a reversed EOF. When a negative polarity is applied to a capillary filled with high-conductivity BGE and injected with sample, EOF is moving towards the inlet. Anionic analytes will migrate towards the outlet and detection end and stack towards the sample zone and BGE boundary. Conversely, the neutral species and cations will migrate towards the inlet injection end. Two downsides with this method are that both anions and cations cannot be separated simultaneously and can be only used with low mobility analytes. Both in the LVSS and FASS approaches, the sample is prepared in a low conductivity buffer, but certain samples, such as ones containing urine or blood, have salts and high conductivity, which would limit the effectiveness of stacking. To combat this, pH-mediated stackings methods can be employed. In this approach, samples are made within a medium which has high-ionic strength and then injected electrokinetically to the separation capillary. This is followed by an electrophoretic injection of a strong acid, which is then applied with positive voltage. This creates a greater electric field along the neutral zones thereby increasing ion migration.

Consequently, the analytes were concentrated and stacked on the boundary between the BGE and the titrated zone. This is then followed by separation in CZE mode[35]. LVSS method has been used for anions[39] and peptides[40].

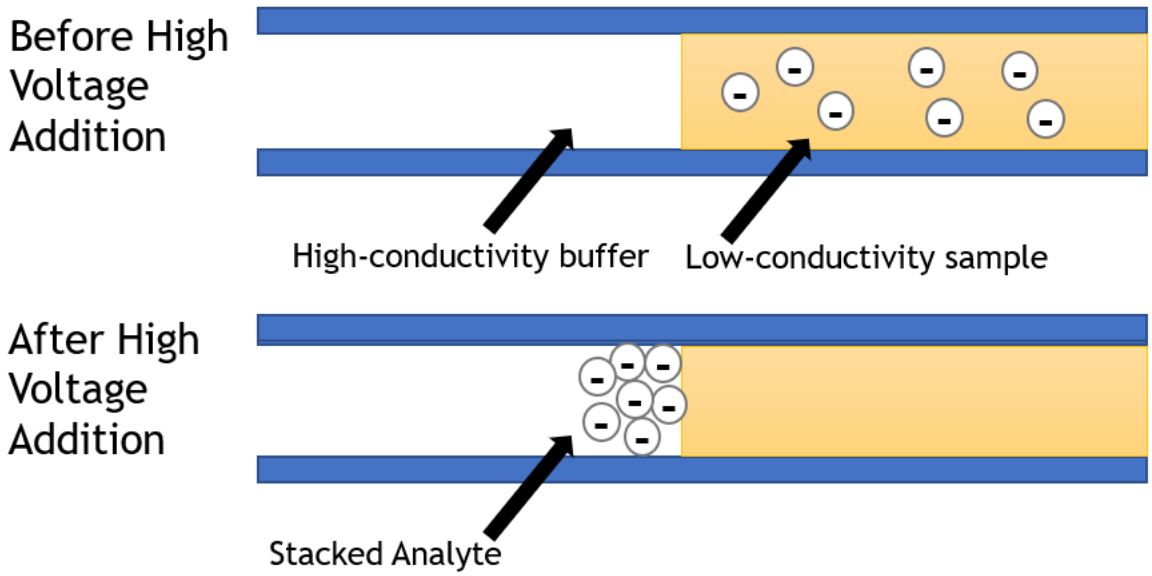


Figure 1.3: Diagram of Field Amplified Sample Stacking (FASS)

Another sample concentration approach in CE is dynamic pH junction. A pH junction is between sample zone and BGE. The separation capillary is filled with BGE solution with a higher pH than the sample solution. The interface between these sections creates a pH junction. In alkaline solution, the analytes gain a negative charge, whereas in an acidic solution the analytes are neutral. When a large positive voltage is applied, there is a creation of a discontinuous electrolyte zone. Negative ions will migrate to the sample zone, while the EOF will move in the opposite direction towards the BGE solution zone. Consequently, the sample analytes are focused on the pH junction area and are then separated by CZE model. Factors that affect the focusing and analyte local velocity in the different segments are the pH,

concentrations, and pKa values of the BGE and sample[35]. This method has been used recently to improve sensitivity for analysis of single mouse neurons[44]. Two downsides of this approach are the that only one sample can be theoretically injected, thereby limiting the overall throughput capability, and that it cannot be used for cationic analytes[35]. These preconcentration methods coupled with the sensitivity robustness of CE for volume-limited samples make it especially attractive for biomolecule analysis, especially for single cells.

Section 1.5: Single Cell MS

In recent years, analysis of single cell has been pivotal in understanding the intricacies of cellular molecular programming and how that relates to functional differences between cells. An advantage of looking at the single-cell level is that analysis at this level can provide novel insights that would be not seen by traditional approaches due to signal averaging of pool samples of many cells. Characterizing the metabolome at the single-cell resolution presents many analytical challenges. With more than 100,000 known molecules, metabolites have a broad range of physicochemical properties, are very dynamic, and have abundance ranges that span many orders of magnitude[45]. Furthermore, single cells come in many shapes, sizes, and morphologies, and vary immensely even intraorganismically and offer very limited amounts of sample to analyze. Therefore, analysis of volume-limited samples requires specialized techniques that have very high molecular specificity, are ultrasensitive, and are fast to examine quick metabolic changes.

Single-cell mass spectrometry (SC-MS) is a primer technology that is used in metabolite characterization at the single-cell resolution. SC-MS technologies have

been refined to have their measurements scalable, have large molecular coverage, and/or preserve biologically relevant, spatial-temporal resolution parameters. Several review papers outline an overview of SC-MS[45-50]. Usually, SC-MS is paired with various separation methods to broaden the single-cell metabolome coverage. Liquid phase separation (CE & LC) provides additional compound-dependent identification information, as retention time in LC and migration time in CE. It also facilitates interference removal during the generation of ions and subsequent spectral detection. A gas-phase-based separation within an ion mobility analyzer facilitated the reduction of spectral inferences. This approach resulted in the detection of roughly 400 ion signals and identification of 23 lipids and metabolites from *A. thaliana* cells[16].

Trace-sensitive CE-MS has several advantages for single cell analysis. CE requires only a small amount of sample (~nL), which is compatible with the volume-limited samples that are acquired from single cells. It has excellent separation peak capacity, which can, also differentiate between isobaric species[47]. Several groups[18,52-54] including the Nemes Lab[14,17,55] have devised workflows that coupled CE to electrospray ionization (ESI)-MS, which has allowed for the detection of hundreds of molecular features from single cell samples.

Section 1.6: Research Model: Xenopus Laevis for MS-Based Metabolomics

There are many different models for studying cellular biology, especially with respect to developmental biology. The South African clawed frog (*Xenopus laevis*) is a popular model to study developmental biology due to numerous experimental advantages. Since evolutionary speaking, frog and humans are very similar, findings from *X.laevis* studies can be translated into a clinical context[56]. Furthermore, *Xenopus*

laevis have standard and well-known and described cell-fate maps[57,58]. From an experimental standpoint, the stereotypical embryos have large cell size (90 uM), which aids in direct cellular manipulation and extraction without the aid of microscope. The cells also have noticeable natural pigmentation difference between different kinds of cells[58]. The timespan for cellular differentiation is quick, and the frog mating produces many eggs, hundreds to few thousands, externally, which facilitates with sample collection[59]. Since the cell-fate maps are well established, MS-based metabolomics with *X. laevis* give a unique opportunity to see how the cellular metabolome changes in the developing embryo, which is important in understanding normal and disease development. The Nemes Lab has previously shown there is inherent metabolite heterogeneity between different cell types and that enrichment of metabolites in the developing embryo can alter a cell's fate change[14]. For example, methionine and acetylcholine were enriched in V11 (midline ventral animal) cell, which is epidermically fated, while threonine and histidine were enhanced in D11 (midline dorsal animal) cell, which is neuronally fated. Interestingly, however, neuronally fated D11 cell became epidermally-fated tissue after injecting acetylcholine and methionine[14]. Understanding inherent metabolite-gradients can be very helpful in understanding in the dynamic changes to the metabolome that give rise to cellular differentiation.

Chapter 2: Development of High-Throughput Single Cell CE-ESI-MS (M-FASS)

Section 2.1: Introduction

CE-MS has many advantages for the analysis of single cells. Single cells produce small workable sample volumes. Suitable analytical technologies for single cell need to be very sensitive and work well with limited volumes. CE-MS is both ultrasensitive and only requires very small amount of sample, such as nanoliters. Analyzing many different single cells are important in order to make statistically relevant conclusions regarding their composition. Having more samples from more cells will make the analyses more robust and account for variation between biological and technical replicates. Our custom-built microanalytical CE-ESI-MS, is highly sensitive with a ~25 amol lower limit of detection for metabolites[49]. One limitation with the platform is that it is relatively low throughput with roughly 1 sample/hr injected and analyzed. One of the bottle necks for single cell CE-MS is the ability to measure samples in a high-throughput manner. Here we explored various methods of injecting multiple sample plugs in one CE-MS runs to increase throughput of the workflow, as described in **Figure 2.1**, which was validated and optimized with an amino acid mixture.

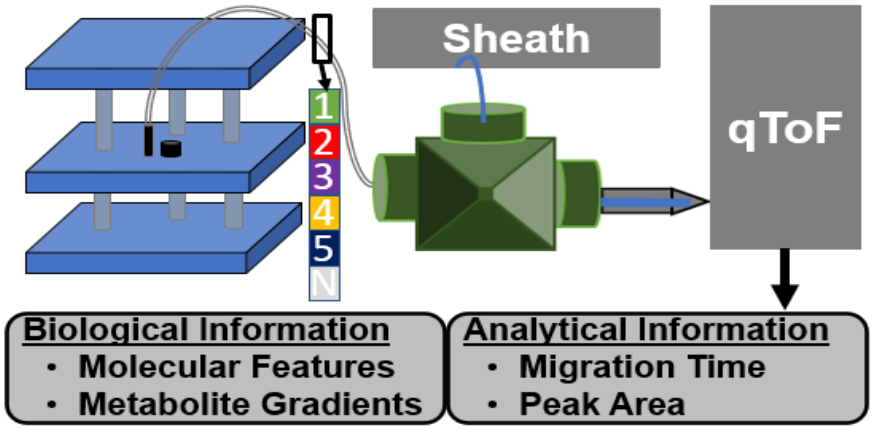


Figure 2.1: Figure Ultrasensitive Multi-Plug CE-MS workflow

Section 2.2: Methods & Materials

Chemicals and Materials

Water, formic acid (FA), acetic acid, isopropanol, and methanol were LC-MS grade from Fisher Scientific (Fair Lawn, NJ). All other chemicals were reagent grade or higher and from Sigma Aldrich (St. Louis, MO). All Samples were prepared with EPPS and HEPES (99% purity) as an internal standard. The amino acid standards include acetylcholine, alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, tyrosine, and valine were from Sigma Aldrich (St. Louis, MO). 100 cm fused silica capillary (130/260 µm inner/outer diameter) was used as CE separation capillary and from Polymicro Technologies (Phoenix, AZ). The ESI Ion source components, such as ferrules, T-unions, and sleeves were sourced from IDEX Corporation (Lake Forest, IL). The stainless metal emitter (130/260 µm inner/outer diameter) were from Hamilton Company (Reno, NV).

Solutions

The CE background electrolyte (BGE) contained 1% v/v formic acid (FA) in LC-MS grade water (measured pH 2.1). The CE-ESI sheath solution was made of 0.1% v/v FA in 50% MeOH (measured pH 3.2).

CE-ESI-HRMS

The single-cell metabolite extracts were measured on a custom-built microanalytical CE-ESI platform that was identical to the platform that was previously reported[49]. An ~10 nL of an amino acid standard, was electrophoresed in a BGE-filled 1-meter-long fused silica capillary (40/105 μm inner/outer diameter) at +21 kV (applied at the capillary inlet), yielding ~7.5 μA CE current. Multiple sample plugs were injected with varying spacers at varying potentials during method development. The CE separation capillary was connected to a CE-ESI interface. The ion source was operated in the cone-jet regime for maximal ion production under the following conditions: co-axial *sheath liquid*, 600 nL/min; ESI potential –1,700 V (applied to front plate of the mass spectrometer); ESI-to-mass spectrometer distance was ~ 2 mm. The generated ions were detected and identified using a quadrupole time-of-flight high-resolution mass spectrometer equipped with a collision-induced dissociation cells (HRMS; Impact HD, Bruker, Billerica, MA). MS measurements employed the following operational conditions: dry gas, 2 L/min nitrogen at 100 °C; survey scan rate, 2 Hz; mass range (MS^1 and MS^2), *m/z* 50–550; collision-induced dissociation, 18–20 eV in nitrogen. The mass spectrometer was mass-calibrated to <5 ppm accuracy using sodium formate clusters, which were formed by injecting 100 mM NaCl and electrospray ionization. Hydrodynamic injection of by raising sample

and BGE into the capillary by physically lifting up a sample loading stage 15 cm, for approximately 60 seconds for a 10 nL injection and 30 seconds for a 5 nL injection.

Data Analysis

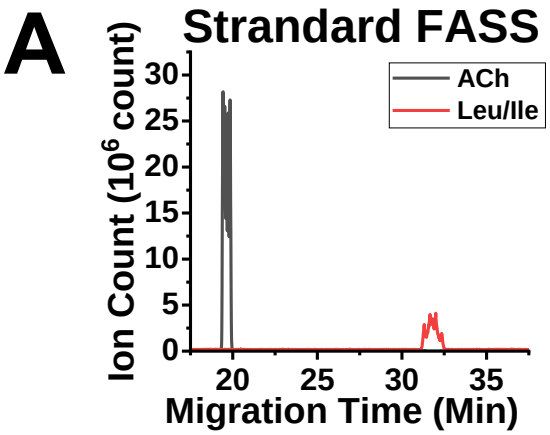
The primary MS data was processed using Compass DataAnalysis 4.3 (Bruker). To identify standard metabolites, molecular features were searched manually, and relative migration time-warping analysis and accurate mass was used. Under-the-peak-area was manually integrated via Compass Data Analysis 4.3 (Bruker).

Section 2.3:BGE Spacer Gap Approach

The original way of using the CE-ESI-MS platform is to hydrodynamically inject 10nL of sample (~30 seconds) followed by 5nL plug BGE for FASS, which would 60 seconds and 30 seconds respectively. Then CE high voltage is applied to the capillary and analytes are separated then fed into the MS for detection. We first tried BGE spacer gap approach by loading 5 samples of an amino acid mixture consecutively (10 nL each) in one CE-MS run using the original method resulted in peaks of the same species that did not fully resolve. The BGE spacer gap was expanded to 1.5 min (15 nL) and 3 min (30 nL) between injections. With the 3 min BGE spacer, the peaks between different injections in the same run were resolved. However, having 3 min BGE spacer separation yielded a long total injection period, (i.e., for 5-plug sample would have total 20-25 min injection time, which is time prohibitive.)

Section 2.4: M-FASS Approach

To cut down on the injection period to improve throughput, we sought instead to use a multi-FASS (M-FASS) approach, where a FASS voltage was applied in between sample injection in a multi-plug run. 30s FASS voltage coupled with a 30 s BGE spacer provided good resolution between peaks of multiple CE sample intrarun injections with a >50% reduction in injection time as compared to the large BGE space gap approach. **Figure 2.2** shows a 5-multi plug injection with and without M-FASS application. With the M-FASS, peaks were able to be resolved with the same sample (10nL) and BGE spacer length (5nL), thereby reducing the total injection volume and length of the sample loading zone, making the process much more time efficient as compared to the Long BGE Spacer Gap Approach.



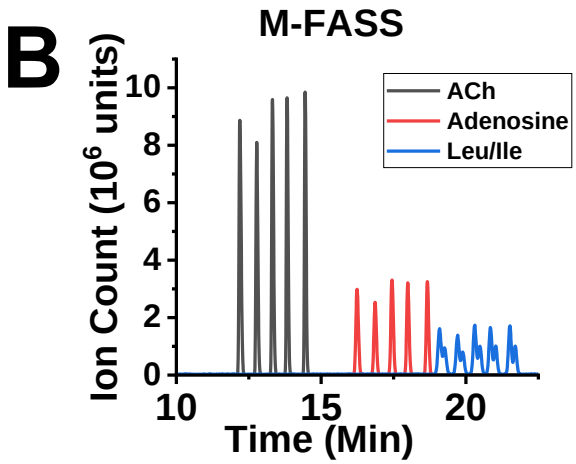


Figure 2.2: A) 5-multiplug standard FASS injection

B) 5-multiplug M-FASS injection

Section 2.5: Analytical Validation and Optimization of M-FASS Method

Analyzing different FASS KV application and duration settings were examined to optimize peak area and resolution. The M-FASS voltage application of 0, 5, 10, 15, and 20 KV was tested in between injections in a 5-plug multi-injection for a standard amino acid mixture. Two conditions of 5kV and 15 kV M-FASS voltage are shown as an example in **Figure. 2.3**.

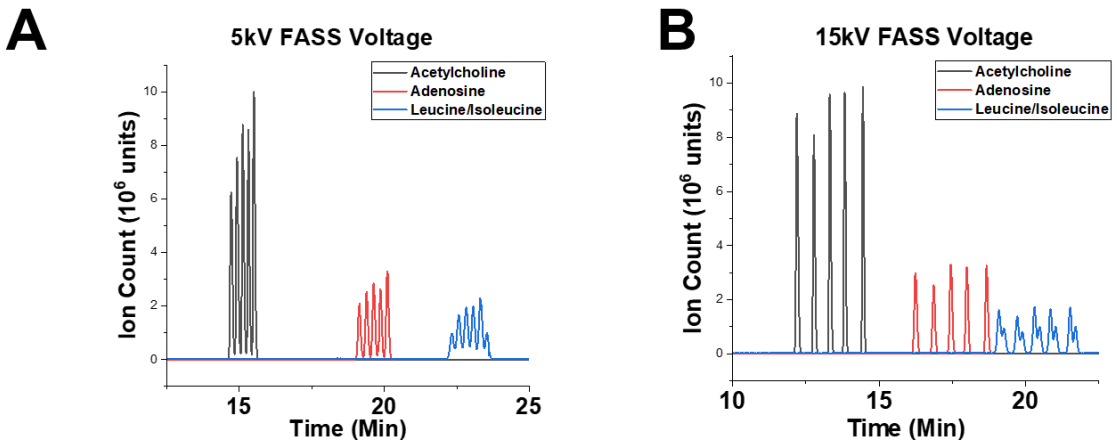


Figure 2.3 A) 5-plug 5kV M-FASS injection B) 5-plug 15kV M-FASS injection

The signal-to-noise (S/N) ratio for most compounds increased greatly from 0 to 10 KV then had steady increases thereafter (**Figure 2.4)**. The peak separation resolution increased with increasing FASS voltage up until the 10-15 KV range then subsequently decreases (**Figure 2.5)**. We further tested the effect of FASS duration on S/N and separation resolution, we found 10 s, 20 s, and 30s had no major difference. Therefore, we chose a 30s M-FASS duration. With these considerations along with better intra-run peak-to-peak resolution, a M-FASS voltage of 15 kV was chosen. After optimizing M-FASS parameters, up to 7-10 samples could be comfortably loaded in one run, thereby increasing throughput by 7-10x as compared to the original method.

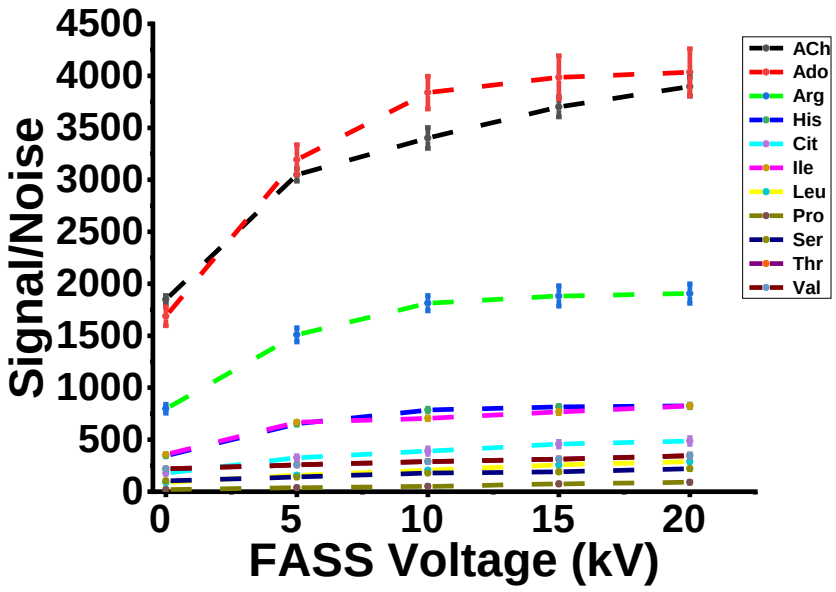


Figure 2.4. Effect of Increasing FASS Voltage to Peak S/N Ratio

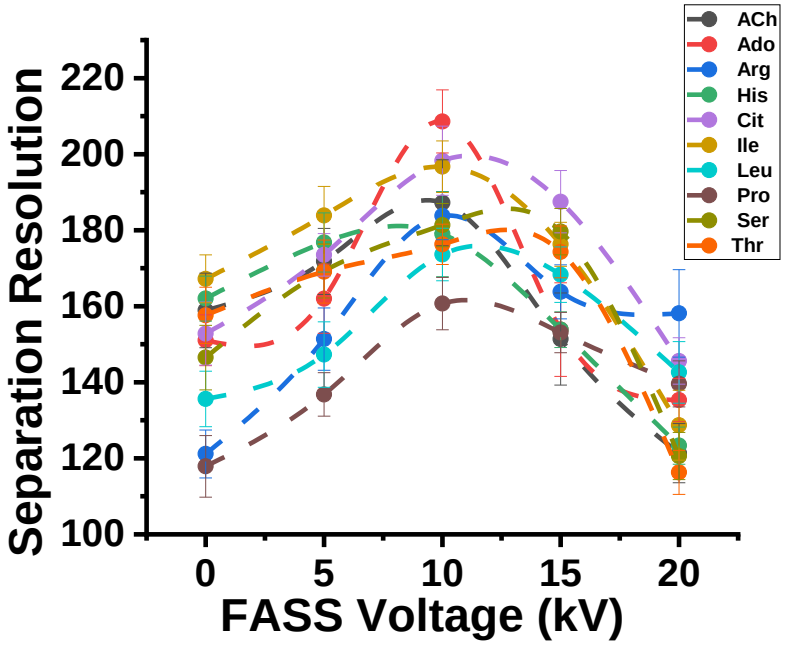


Figure 2.5. Effect of FASS Voltage on Separation Resolution

The analytical performance of this M-FASS method for the CE-μESI-MS was evaluated by metabolite standards and biological embryonic extracts. Specifically, we evaluated the lower limit of detection, linear dynamic range of peak area, and quantification reproducibility for amino acid standards.

A calibration curve (**Figure. 2.6**) was generated using a concentration series of a mixture of standard amino acids (0.5 nM, 1 nM, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM, and 500 nM).

The lower limit of detection was determined to be the concentration of tested metabolite when signal to noise ratio (S/N) equals to 3. The signal to noise ratio was the root mean square ratio of the peak height to signal noise (**Fig. 2.6**). The S/N of acetylcholine was 3 at 10 nM (~70 amol), so the lower limit of detection (LLOD) was 10 nM. Small molecules (as indicated in **Fig. 2.6**) such as histidine and arginine had

similar LODs, while other metabolites like proline had a higher LLOD, around 100 nM. This validated that this method could be used for high-throughput measurements of low-abundant metabolites in volume limited samples single cells (where the endogenous concentration ranges from 100 μM to 2 mM).

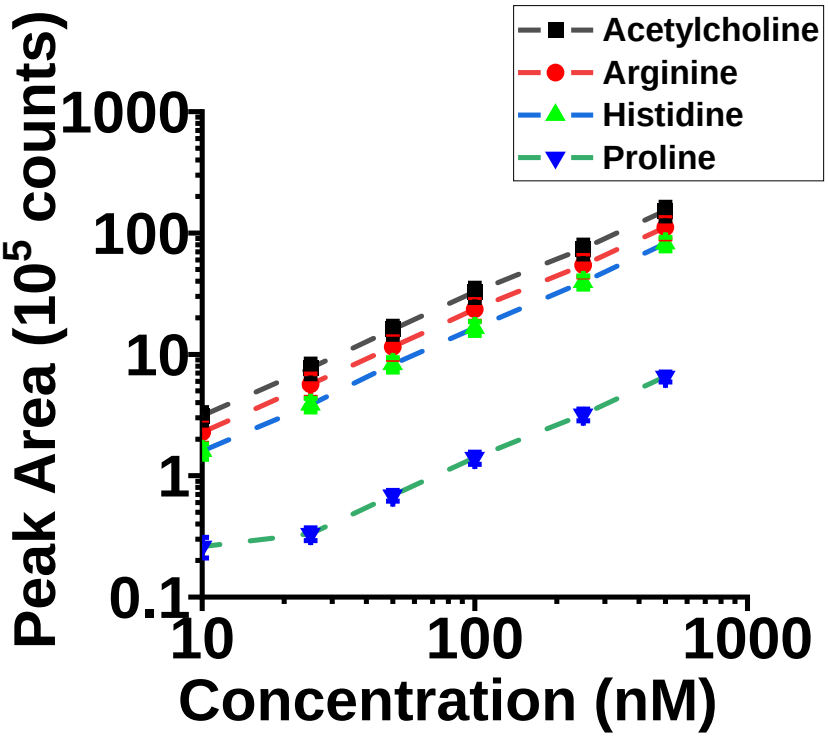


Figure 2.6. Calibration curve of selected metabolites in a 5-plug M-FASS CE Run

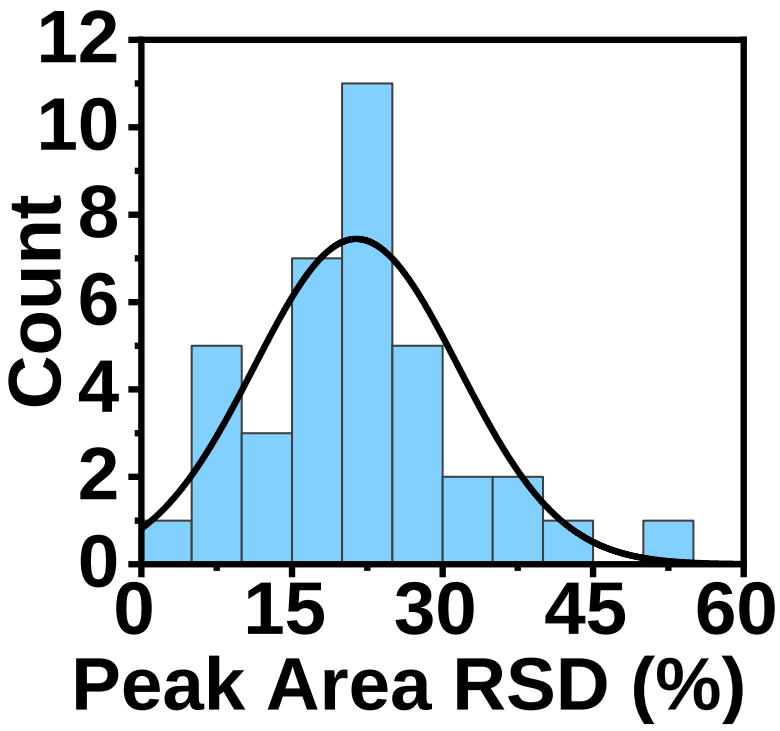


Figure 2.7. Histogram of metabolite Peak Area RSD for 16-cell embryo

The reproducibility of peak areas between multiple injections for multiple metabolites in the same run, ranged from 6 – 27% RSD for a run of chemical standards. The peak area RSD of intrarun metabolite peak areas in a 5-plug M-FASS CE of whole 16-cell *X.laevis* embryo extract was 21.4% and followed a normal distribution (**Figure 2.7)**. This demonstrated the capability of the developed M-FAAS method for quantitative analysis of complex biological sample. In summary, these results demonstrate the analytical robustness and sensitivity of this method and platform, which is suitable for the single-cell metabolomics applications and analysis.

Chapter 3: Uncovering Spatiotemporal Metabolic Heterogeneity and Gradients in a 16-cell Embryo

Section 3.1:Introduction

The purpose of increasing throughput through the M-FASS approach was to develop an ultrasensitive CE-ESI-MS method to study multiple volume-limited biological samples in a shorter period of time. To demonstrate the power and scalability of the M-FASS approach, a SCA study with applications to developmental biology was chosen, where many single cells were studied in a relatively short period of time. Previous studies were limited to studying 2 or 3 different cell types due to long run time to analyze many technical and biological replicates one-by-one [60,61,62] which hindered the ability of the original single injection method to study spatial metabolic differences and trends among many kinds of adjacent cells in the developing embryo.

The specific biological application chosen to demonstrate the capability of the M-FASS method was to explore spatial metabolic differences between adjacent left-sided dorsal and ventral cells (V11, V12, D11, and D12) in a developing embryo.

Section 3.2: Methods & Materials

Chemicals, Materials, Solutions

All solvents were LC-MS grade from Fisher Scientific (Fair Lawn, NJ). All other chemicals were reagent grade or higher and from Sigma Aldrich (St. Louis, MO). All Samples were prepared with EPPS and HEPES (99% purity) as an internal standard. Same materials for CE-MS as described in 2.2 were used.

The metabolite extraction solution included 40% v/v acetonitrile (ACN) and 40% v/v methanol (MeOH) in and LC-MS grade water with 2.5 μM EPPS and HEPES. The CE background electrolyte (BGE) contained 1% v/v formic acid (FA) in LC-MS grade water (measured pH 2.1). The CE-ESI sheath solution was made of 0.1% v/v FA in 50% MeOH (measured pH 3.2). For embryology, 2% cysteine solutions for dejellying and Steinberg's solution (SS) embryos were prepared following established protocols.

Animal Care and Embryology.

Adult *Xenopus laevis* frogs from Nasco (Fort Atkinson, WI) were maintained in a breeding colony. Protocols related to humane handling, use, and care of the frogs were conducted under approval by the University of Maryland Institutional Animal Care and Use Committee (approval no. R-FEB-21-07). Embryos were obtained through gonadotropin-induced natural mating following established protocols[63]. The embryos were dejellied in 2% cysteine (pH 8) and cultured in 100% SS. Two-cell stage embryos exhibiting stereotypical cleavage and pigmentation were cultured to the 16-cell stage in 50% SS solution. Cells on the left side of the 16-cell embryo were identified based on pigmentation and reproducible cell-fate maps[58].

Single-Cell Metabolomics.

The content of each identified cell, ~40 nL per cell, was collected from a different embryo using capillary microsampling. For each cell, a clean (unused) microfabricated borosilicate capillary was used to avoid chemical cross-contamination between subsequent sampling events. Each single-cell aspirate was individually processed in a microvial (Eppendorf). Metabolites from the collected

samples were extracted in 10 µL of metabolite extraction solution (4 °C) and vortex-mixed for 30s. The extracts were stored at −80 °C until analysis. To account for biological variability and enhance statistical power, n = 5 biological replicates were collected for each cell type, each from a different embryo. Each single-cell extract was analyzed in 5 technical replicates within one separation experiment using M-FASS.

M-FASS CE-ESI-MS

Using the M-FASS method and same CE-ESI-MS parameters described in Chapter 2, 5 technical replicates (10nL) and 5 BGE spacers (5nL) of a particular cell sample were injected in the same run. A 15kV M-FASS voltage was selected to optimize for S/N and peak resolution.

Data Analysis

The primary MS data was processed using Compass DataAnalysis 4.3 (Bruker). Molecular features were searched using semi-automatic method following a previously established protocol[5]. To identify molecular features, relative migration time time-warping analysis and accurate mass was used. Under-the-peak-area was manually integrated via Compass Data Analysis 4.3 (Bruker).

Statistics

Using DataAnalysis, selected-ion electropherograms were generated and the under-the-curve area was manually integrated for each peak. The peak areas were log-transformed and median-normalized in MetaboAnalyst 5.0[64]. Statistical data analysis employed ANOVA with an adjusted p-value (FDR) < 0.05 from a Fisher's LSD post-hoc analysis marking statistical significance of the selected metabolites for

analysis. Hierarchical cluster analysis (HCA) was performed to create heatmaps (**Figure 3.1**) using Euclidean distance measure and Ward method as the cluster method. The measured patterns of cell-by-cell metabolite abundances were grouped using Fuzzy-C clustering in VSClust[65]. Mfuzz software, which is based on-source statistical language R from Bioconductor repository, was used for fuzzy-c means clustering analysis to group statistically significant samples together in clusters based on peak area information.

Section 3.3: Application to Assess Metabolic Heterogeneity in the Developing Embryo

Using the peak area information of 36 molecular features from all the M-FASS runs, principal component analysis (PCA) showed differential clustering of the four different cell types. These 36 molecular features were selected because of limited interference from isobaric and interfering species when inspecting the EIC and their identities were identified via dynamic time warping analysis. Furthermore, unsupervised hierarchical cluster analysis (HCA) from statistically significant molecular features was performed and the corresponding dendrogram (**Figure 3.1**) showed differential clustering of the 4 different cell types as well. One challenge of this strategy was the overlapping of isobaric species interference for peaks of similar *m/zs,* which rendered some EICs unusable for quantitative analysis.

Analyzing 36 different metabolites in the loadings plot generated by MetaboAnalyst, 4 major predominated gradient patterns emerged (**Figure 3.2**). In Q1 (16 metabolites), a midline apex pattern shows, where the metabolite concentration is high near midline and decreases in cells further away from midline. Conversely, Q4

(4 metabolites) shows a midline nadar pattern, where the metabolite concentration decreases as it gets closer to the midline. Q2 (11 metabolites) shows a pattern where there is a metabolic sink at the V12 cell, while Q3 (5 metabolites) shows a metabolic source (enrichment) from the V11 cell. The findings suggest there are inherent metabolic gradients in the developing embryo.

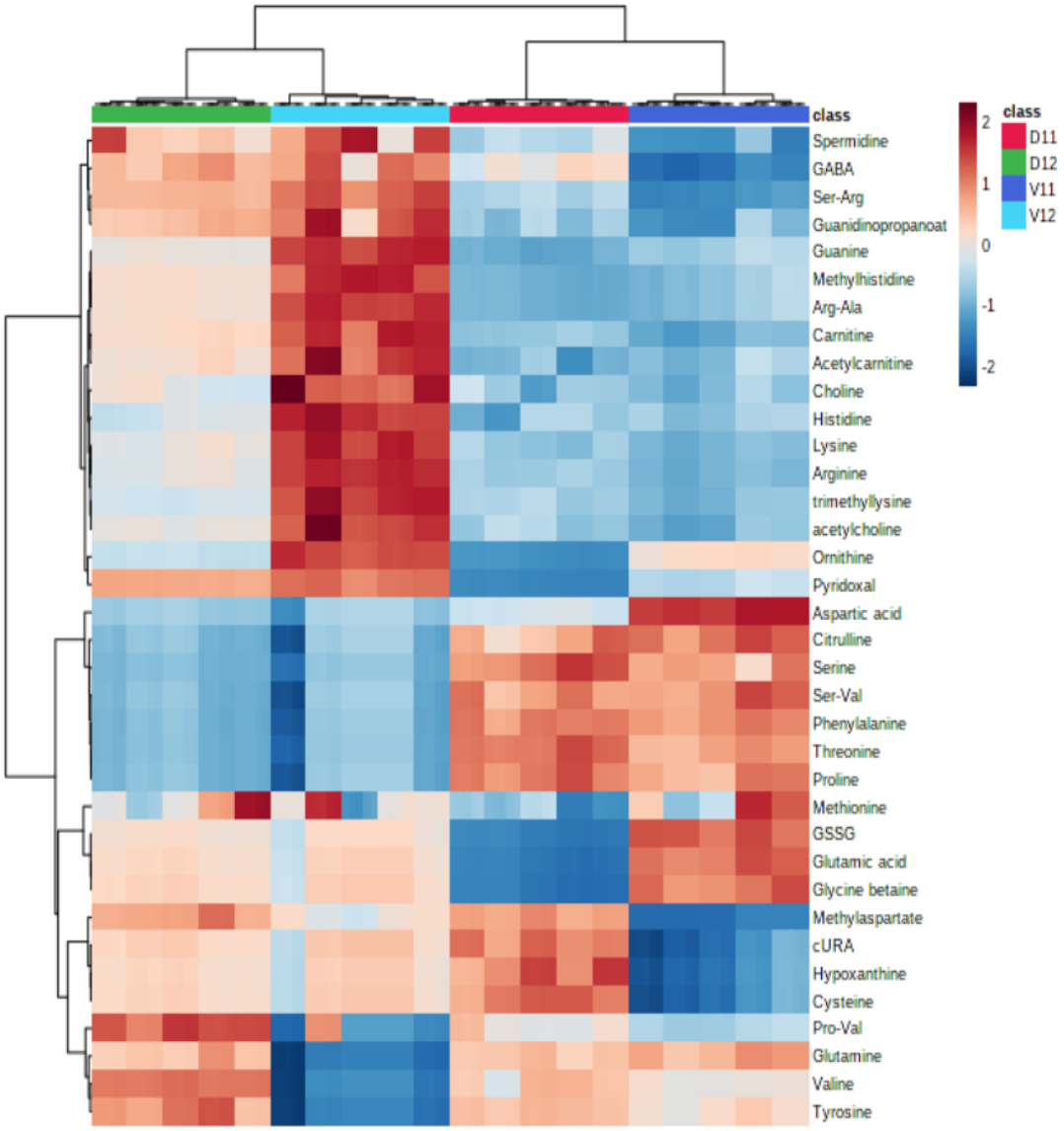


Figure 3.1 HCA heatmap shows the clustering V11, V12, D11, and D12 cell types and suggests that there is cellular heterogeneity between the 4 cell types.

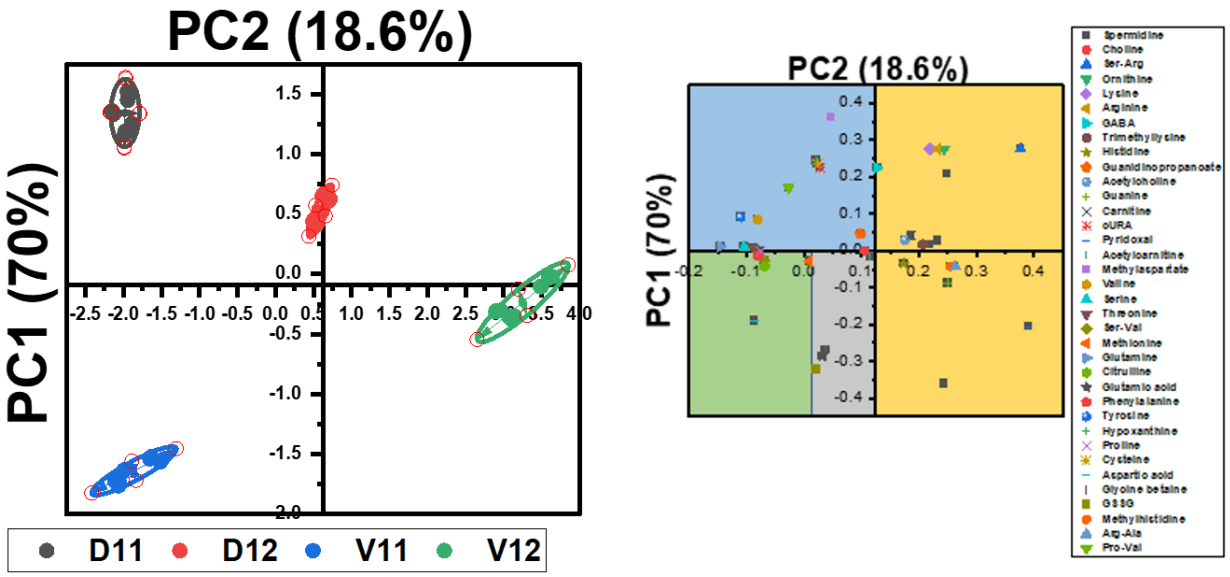


Figure 3.2 A) PCA's loading plot shows that four potential major patterns for metabolite gradients (Q1 Yellow - Midline Apex), (Q2 Teal - V12 Sink), (Q3 Green - midline nadar), (Q4 gray – V11 source) **B)** PCA analysis shows that there is unsupervised differential clustering of the same cell type based on molecular feature peak area

To better analyze trends of higher resolution and statistical certainty between the different cell types and their biological replicates, fuzzy-c clustering analysis, via Mfuzz software, was utilized for the 36 identified metabolites in order to cluster metabolites into statistically significant clusters based on metabolite peak area information. With this analysis, as seen in **Figure 3.3**, it was determined 6 distinct metabolite trends emerge. 12 metabolites had a source from one cell (8 for V12, 4 for V11). 8 metabolites had cell sink trends (5 with V12 sink and 3 with D12 sink). Lastly, there was 15 metabolites with a sink at the midline, where the V12 and D12

cell meet. Identified metabolites allowed for pathway enrichment analysis. 36 metabolites were mapped via the KEGG metabolic database. Several different pathways were deemed to be enriched to levels of statistical significance ($p<0.05$). For example, all cell types exhibited aminoacyl-tRNA biosynthesis and arginine biosynthesis with high statistically significance. However, V11 had high match rate for glutathione metabolism, while V12 and D12 had high match for arginine and proline metabolism. Furthermore, D11 had strong match for phenylalanine, tyrosine, and tryptophan biosynthesis.

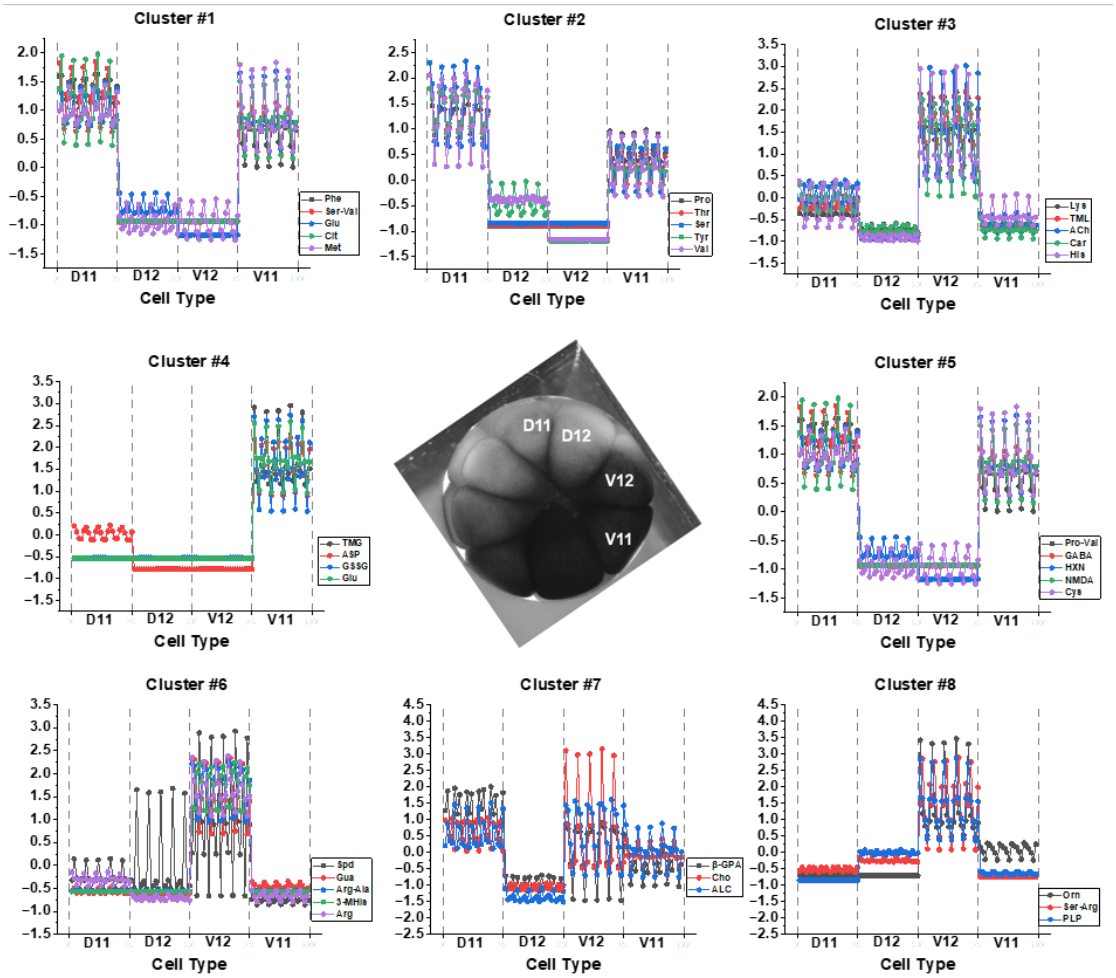


Figure 3.3 Cell-by-cell analysis of metabolite distribution. Fuzzy-C means cluster analysis uncovered different spatial metabolite gradient trends.

In conclusion, the M-FASS method was successfully used to measure multiple samples, at a 5x throughput rate as compared to more traditional methods, in a short time frame (20 runs instead of 100 for this study). We were able to collect data and found cell heterogeneity among 4 different cells with different cell fate type in a living embryo and discover spatial metabolite gradients.

Chapter 4: Summary & Future Directions

Section 4.1: Summary

Through this investigation, we further advanced CE-ESI-MS technology to enable enhanced throughput analysis of single cells by developing a multiplug injection method called M-FASS. This was applied to metabolite analysis in single embryos. Microprobe sampling allowed for direct whole cell sampling from developing embryos. Multiple samples were loaded and separated via a M-FASS method in the CE capillary. Optimization of FASS Voltage (15kV) and duration (30s) allowed for good peak area and peak resolution. The resulting workflow was able to identify metabolites reproducibly with trace sensitivity in the sub nM range, which would be necessary for volume-limited samples and single-cell analyses.

The resulting CE-ESI-MS workflow allowed for analysis of the measurement of 100 samples from 20 single cells in 20 M-FASS CE runs, reducing MS run time by 80% from ~150 hours to ~30 hours. This resulted in the discovery of 6 metabolite gradient patterns between 4 adjacent cells. Developing enhanced-throughput methods is important for single-cell analysis because it would allow for more measurements in a short period of time, which would allow for more different kinds of biological and technical replicates to be ran and the discerning results could be held with higher statistical confidence.

Section 4.2: Future Directions

The M-FASS approach integrated with the in-situ microprobe sampling improved the analytical ability of CE-MS for single-cell biological studies, as it

allows for an increased number of processed samples to be analyzed. The resulting metadata from this approach can improve both the quantification and identification of metabolites and small molecules from volume limited biological samples. Future studies using the M-FASS enhanced-throughput approaches could include analyzing the metabolome of all the cells in the embryo as it develops from 2-cell to later cell stages or perturbing the developing cell chemically and seeing how the metabolite gradients differ in abnormal vs normal development. This kind of microprobe M-FASS CE-ESI-MS can be scalable to different cell types of thereby furthering cell biology and single cell analysis for many different kinds of applications. The method can also be used for other purposes such as environmental monitoring and pharmaceutical research applications in addition to cell biology. Small metabolites can be found using this higher throughput approach in challenging, complex samples like soil or water. This can be used to study drug metabolism and pharmacokinetics studies relating to drug discovery and development. This robust method can be used to monitor many samples in an ultrasensitive with a decent throughput and has implications in many different areas of research.

Chapter 4: Summary & Future Directions

1. Dunn WB, Bailey NJC, Johnson HE. Measuring the metabolome: current analytical technologies. *Analyst*. 2005;130(5):606-625. doi:10.1039/b418288j
2. Wishart DS, Feunang YD, Marcu A, et al. HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Research*. Jan 2018;46(D1):D608-D617. doi:10.1093/nar/gkx1089
3. Kim MS, Pinto SM, Getnet D, et al. A draft map of the human proteome. *Nature*. May 2014;509(7502):575-+. doi:10.1038/nature13302
4. Sokol SY. Maintaining embryonic stem cell pluripotency with Wnt signaling. *Development*. Oct 2011;138(20):4341-4350. doi:10.1242/dev.066209
5. Budnik B, Levy E, Harmange G, Slavov N. SCoPE-MS: mass spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation. *Genome Biology*. Oct 2018;19161. doi:10.1186/s13059-018-1547-5
6. Thompson A, Schafer J, Kuhn K, et al. Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Analytical Chemistry*. Apr 2003;75(8):1895-1904. doi:10.1021/ac0262560
7. Jang, C.; Chen, L.; Rabinowitz, J. D., Metabolomics and Isotope Tracing. Cell 2018, 173 (4), 822-837
8. Wang SY, Blair IA, Mesaros C. Analytical Methods for Mass Spectrometry-Based Metabolomics Studies. In: Woods AG, Darie CC, eds. *Advancements of Mass Spectrometry in Biomedical Research, 2nd Edition*. 2019:635-647. *Advances in Experimental Medicine and Biology*.
9. Wang M, Dubiak K, Zhang ZB, Huber PW, Chen DDY, Dovichi NJ. MALDI-imaging of early stage Xenopus laevis embryos. *Talanta*. Nov 2019;204:138-144. doi:10.1016/j.talanta.2019.05.060
10. Shrestha B, Sripadi P, Reschke BR, et al. Subcellular Metabolite and Lipid Analysis of Xenopus laevis Eggs by LAESI Mass Spectrometry. *Plos One*. Dec 2014;9(12)e115173. doi:10.1371/journal.pone.0115173
11. Dettmer K, Aronov PA, Hammock BD. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews*. Jan-Feb 2007;26(1):51-78. doi:10.1002/mas.20108
12. Rauh M. LC-MS/MS for protein and peptide quantification in clinical chemistry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. Feb 2012;883:59-67. doi:10.1016/j.jchromb.2011.09.030
13. Gonzalez-Serrano AF, Pirro V, Ferreira CR, et al. Desorption Electrospray Ionization Mass Spectrometry Reveals Lipid Metabolism

of Individual Oocytes and Embryos. *Plos One*. Sep 2013;8(9)e74981. doi:10.1371/journal.pone.0074981
14. Onjiko RM, Moody SA, Nemes P. Single-cell mass spectrometry reveals small molecules that affect cell fates in the 16-cell embryo. *Proceedings of the National Academy of Sciences of the United States of America*. May 2015;112(21):6545-6550. doi:10.1073/pnas.1423682112
15. Rauh M. LC-MS/MS for protein and peptide quantification in clinical chemistry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. Feb 2012;883:59-67. doi:10.1016/j.jchromb.2011.09.030
16. Onjiko RM, Portero EP, Nemes P. Single-cell Metabolomics with Capillary Electrophoresis–Mass Spectrometry. The Royal Society of Chemistry; 2018
17. Wang M, Dubiak K, Zhang ZB, Huber PW, Chen DDY, Dovichi NJ. MALDI-imaging of early stage Xenopus laevis embryos. *Talanta*. Nov 2019;204:138-144. doi:10.1016/j.talanta.2019.05.060
18. Shrestha B, Sripadi P, Reschke BR, et al. Subcellular Metabolite and Lipid Analysis of Xenopus laevis Eggs by LAESI Mass Spectrometry. *Plos One*. Dec 2014;9(12)e115173. doi:10.1371/journal.pone.0115173
19. Alseekh, S., Aharoni, A., Brotman, Y. *et al.* Mass spectrometry-based metabolomics: a guide for annotation, quantification and best reporting practices. *Nat Methods* **18**, 747–756 (2021). https://doi.org/10.1038/s41592-021-01197-1
20. Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer L. Metabolite profiling for plant functional genomics. *Nature Biotechnology*. Nov 2000;18(11):1157-1161. doi:10.1038/81137
21. Hu CX, van Dommelen J, van der Heijden R, et al. RPLC-Ion-Trap-FTMS Method for Lipid Profiling of Plasma: Method Validation and Application to p53 Mutant Mouse Model. *Journal of Proteome Research*. Nov 2008;7(11):4982-4991. doi:10.1021/pr800373m
22. Tolstikov VV, Lommen A, Nakanishi K, Tanaka N, Fiehn O. Monolithic silica-based capillary reversed-phase liquid chromatography/electrospray mass spectrometry for plant metabolomics. *Analytical Chemistry*. Dec 2003;75(23):6737-6740. doi:10.1021/ac034716z
23. Plumb RS, Rainville P, Smith BW, et al. Generation of Ultrahigh peak capacity LC separations via elevated temperatures and high linear mobile-phase velocities. *Analytical Chemistry*. Oct 2006;78(20):7278-7283. doi:10.1021/ac060935j
24. Onjiko RM, Portero EP, Nemes P. Single-cell Metabolomics with Capillary Electrophoresis–Mass Spectrometry. The Royal Society of Chemistry; 2018
25. Sun LL, Zhu GJ, Zhang ZB, Mou S, Dovichi NJ. Third-Generation Electrokinetically Pumped Sheath-Flow Nanospray Interface with Improved Stability and Sensitivity for Automated Capillary Zone

Electrophoresis-Mass Spectrometry Analysis of Complex Proteome Digests. *Journal of Proteome Research*. May 2015;14(5):2312-2321. doi:10.1021/acs.jproteome.5b00100
26. Sumner LW, Amberg A, Barrett D, et al. Proposed minimum reporting standards for chemical analysis. *Metabolomics*. Sep 2007;3(3):211-221. doi:10.1007/s11306-007-0082-2
27. Guijas C, Montenegro-Burke JR, Domingo-Almenara X, et al. METLIN: A Technology Platform for Identifying Knowns and Unknowns. *Analytical Chemistry*. Mar 2018;90(5):3156-3164. doi:10.1021/acs.analchem.7b04424
28. Marina ML, Ríos A, Valcárcel M. *Analysis and Detection by Capillary Electrophoresis*. 1st ed. vol 45. Elsevier Science; 2005.
29. Onjiko RM, Plotnick DO, Moody SA, Nemes P. Metabolic comparison of dorsal versus ventral cells directly in the live 8-cell frog embryo by microprobe single-cell CE-ESI-MS. *Analytical Methods*. Sep 2017;9(34):4964-4970. doi:10.1039/c7ay00834a
30. Harris DC. *Quantitative Chemical Analysis*. vol 8th. W. H. Freeman; 2010.
31. vanDeemter JJ. Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography - Commentary (Reprinted from Current Contents, no 3, January 19, 1981). *Chemical Engineering Science*. Dec 1995;50(24):3867-3867. doi:10.1016/0009-2509(96)81812-4
32. Lin CH, Kaneta T. On-line sample concentration techniques in capillary electrophoresis: Velocity gradient techniques and sample concentration techniques for biomolecules. *Electrophoresis*. Dec 2004;25(23-24):4058-4073. doi:10.1002/elps.200406172
33. Liu SH, Li QF, Chen XG, Hu ZD. Field-amplified sample stacking in capillary electrophoresis for on-column concentration of alkaloids in Sophora flavescens Ait. *Electrophoresis*. Sep 2002;23(19):3392-3397. doi:10.1002/1522-2683(200210)23:19<3392::aid-elps3392>3.0.co;2-x
34. Alnajjar A, McCord B. Determination of heroin metabolites in human urine using capillary zone electrophoresis with beta-cyclodextrin and UV detection. *Journal of Pharmaceutical and Biomedical Analysis*. Oct 2003;33(3):463-473. doi:10.1016/s0731-7085(03)00298-x
35. Quirino JP, Terabe S. Sample stacking of fast-moving anions in capillary zone electrophoresis with pH-suppressed electroosmotic flow. *Journal of Chromatography A*. Jul 1999;850(1-2):339-344. doi:10.1016/s0021-9673(99)00204-6
36. Almeda S., Arce L. and Valcarcel M., The More and Less Common Approaches to Enhancing Sensitivity in Capillary Electrophoresis, Current Analytical Chemistry 2010; 6(2) . https://dx.doi.org/10.2174/157341110790945508
37. Siri N, Riolet P, Bayle C, Couderc F. Automated large-volume sample stacking procedure to detect labeled peptides at picomolar concentration using capillary electrophoresis and laser-induced

fluorescence detection. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. Aug 2003;793(1):151-157. doi:10.1016/s1570-0232(03)00372-6
38. Quirino JP, Terabe S. Exceeding 5000-fold concentration of dilute analytes in micellar electrokinetic chromatography. *Science*. Oct 1998;282(5388):465-468. doi:10.1126/science.282.5388.465
39. Markuszewski MJ, Britz-McKibbin P, Terabe S, Matsuda K, Nishioka T. Determination of pyridine and adenine nucleotide metabolites in Bacillus subtilis cell extract by sweeping borate complexation capillary electrophoresis. *Journal of Chromatography A*. Mar 2003;989(2):293-301. doi:10.1016/s0021-9673(03)00031-1
40. Monton MRN, Otsuka K, Terabe S. On-line sample preconcentration in micellar electrokinetic chromatography by sweeping with anionic-zwitterionic mixed micelles. *Journal of Chromatography A*. Jan 2003;985(1-2):435-445. Pii s0021-9673(02)01654-0. doi:10.1016/s0021-9673(02)01654-0
41. DeLaney K, Jia DS, Iyer L, et al. Microanalysis of Brain Angiotensin Peptides Using Ultrasensitive Capillary Electrophoresis Trapped Ion Mobility Mass Spectrometry. *Analytical Chemistry*. Jun 2022;94(25):9018-9025. doi:10.1021/acs.analchem.2c01062
42. Zenobi R. Single-Cell Metabolomics: Analytical and Biological Perspectives. *Science*. Dec 2013;342(6163):1201-+. 1243259. doi:10.1126/science.1243259
43. Svatos A. Single-cell metabolomics comes of age: new developments in mass spectrometry profiling and imaging. *Analytical Chemistry*. Jul 2011;83(13):5037-5044. doi:10.1021/ac2003592
44. Heinemann M, Zenobi R. Single cell metabolomics. *Current Opinion in Biotechnology*. Feb 2011;22(1):26-31. doi:10.1016/j.copbio.2010.09.008
45. Rubakhin SS, Lanni EJ, Sweedler JV. Progress toward single cell metabolomics. *Current Opinion in Biotechnology*. Feb 2013;24(1):95-104. doi:10.1016/j.copbio.2012.10.021
46. Ong TH, Tillmaand EG, Makurath M, Rubakhin SS, Sweedler JV. Mass spectrometry-based characterization of endogenous peptides and metabolites in small volume samples. *Biochimica Et Biophysica Acta-Proteins and Proteomics*. Jul 2015;1854(7):732-740. doi:10.1016/j.bbapap.2015.01.008
47. Spiller DG, Wood CD, Rand DA, White MRH. Measurement of single-cell dynamics. *Nature*. Jun 2010;465(7299):736-745. doi:10.1038/nature09232
48. Comi TJ, Do TD, Rubakhin SS, Sweedler JV. Categorizing Cells on the Basis of their Chemical Profiles: Progress in Single-Cell Mass Spectrometry. *Journal of the American Chemical Society*. Mar 2017;139(11):3920-3929. doi:10.1021/jacs.6b12822
49. Aerts JT, Louis KR, Crandall SR, Govindaiah G, Cox CL, Sweedler JV. Patch Clamp Electrophysiology and Capillary Electrophoresis-Mass

Spectrometry Metabolomics for Single Cell Characterization. *Analytical Chemistry*. Mar 2014;86(6):3203-3208. doi:10.1021/ac500168d
50. Lapainis T, Rubakhin SS, Sweedler JV. Capillary Electrophoresis with Electrospray Ionization Mass Spectrometric Detection for Single-Cell Metabolomics. *Analytical Chemistry*. Jul 2009;81(14):5858-5864. doi:10.1021/ac900936g
51. Zhang W, Gulersonmez MC, Hankemeier T, Ramautar R. Sheathless Capillary Electrophoresis-Mass Spectrometry for Metabolic Profiling of Biological Samples. *Jove-Journal of Visualized Experiments*. Oct 2016;(116)e54535. doi:10.3791/54535
52. Onjiko RM, Portero EP, Moody SA, Nemes P. Microprobe Capillary Electrophoresis Mass Spectrometry for Single-cell Metabolomics in Live Frog (Xenopus laevis) Embryos. *Jove-Journal of Visualized Experiments*. Dec 2017;(130)e56956. doi:10.3791/56956
53. Onjiko RM, Morris SE, Moody SA, Nemes P. Single-cell mass spectrometry with multi-solvent extraction identifies metabolic differences between left and right blastomeres in the 8-cell frog (Xenopus) embryo. *Analyst*. 2016;141(12):3648-3656. doi:10.1039/c6an00200e
54. Onjiko RM, Plotnick DO, Moody SA, Nemes P. Metabolic comparison of dorsal versus ventral cells directly in the live 8-cell frog embryo by microprobe single-cell CE-ESI-MS. *Analytical Methods*. Sep 2017;9(34):4964-4970. doi:10.1039/c7ay00834a
55. Wheeler GN, Brandli AW. Simple Vertebrate Models for Chemical Genetics and Drug Discovery Screens: Lessons From Zebrafish and Xenopus. *Developmental Dynamics*. Jun 2009;238(6):1287-1308. doi:10.1002/dvdy.21967
56. Wheeler GN, Brandli AW. Simple Vertebrate Models for Chemical Genetics and Drug Discovery Screens: Lessons From Zebrafish and Xenopus. *Developmental Dynamics*. Jun 2009;238(6):1287-1308. doi:10.1002/dvdy.21967
57. Moody SA. FATES OF THE BLASTOMERES OF THE 32-CELL-STAGE XENOPUS EMBRYO. *Developmental Biology*. Aug 1987;122(2):300-319. doi:10.1016/0012-1606(87)90296-x
58. Moody SA. FATES OF THE BLASTOMERES OF THE 16-CELL STAGE XENOPUS EMBRYO. *Developmental Biology*. Feb 1987;119(2):560-578. doi:10.1016/0012-1606(87)90059-5
59. Peuchen EH, Sun LL, Dovichi NJ. Optimization and comparison of bottom-up proteomic sample preparation for early-stage Xenopus laevis embryos. *Analytical and Bioanalytical Chemistry*. Jul 2016;408(17):4743-4749. doi:10.1007/s00216-016-9564-2
60. Budnik B, Levy E, Harmange G, Slavov N. SCoPE-MS: mass spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation. *Genome Biology*. Oct 2018;19161. doi:10.1186/s13059-018-1547-5

61. Thompson A, Schafer J, Kuhn K, et al. Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Analytical Chemistry*. Apr 2003;75(8):1895-1904. doi:10.1021/ac0262560
62. Hu CX, van Dommelen J, van der Heijden R, et al. RPLC-Ion-Trap-FTMS Method for Lipid Profiling of Plasma: Method Validation and Application to p53 Mutant Mouse Model. *Journal of Proteome Research*. Nov 2008;7(11):4982-4991. doi:10.1021/pr800373m
63. Moody, S. A. *Methods Mol Biol*. 2000;135:331−347.
64. https://www.metaboanalyst.ca/.
65. Schwammle V, Jensen O. VSClust: feature-based variance-sensitive clustering of omics data. Article. *Bioinformatics*. SEP 1 2018 2018;34(17):2965-2972. doi:10.1093/bioinformatics/bty224