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In Situ Microprobe Single-Cell Capillary Electrophoresis Mass Spectrometry: Metabolic Reorganization in Single Differentiating Cells in the Live Vertebrate (*Xenopus laevis*) Embryo

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Abstract

Knowledge of single-cell metabolism would provide a powerful look into cell activity changes as cells differentiate to all the tissues of the vertebrate embryo. However, single-cell mass spectrometry technologies have not yet been made compatible with complex three-dimensional changes and rapidly decreasing cell sizes during early development of the embryo. Here, we bridge this technological gap by integrating capillary microsampling, microscale metabolite extraction, and capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS) to enable direct metabolic analysis of identified cells in the live frog embryo (*Xenopus laevis*). Microprobe CE-ESI-MS of <0.02% of the single-cell content allowed us to detect ~230 different molecular features (positive ion mode), including 70 known metabolites, in single dorsal and ventral cells in 8-to-32-cell embryos. Relative quantification followed by multivariate and statistical analysis of the data found that microsampling enhanced detection sensitivity compared to whole-cell dissection by minimizing chemical interferences and ion suppression effects from the culture media. In addition, higher glutathione/oxidized glutathione ratios suggested that microprobed cells exhibited significantly lower oxidative stress than those dissected from the embryo. Fast (5 s/cell) and scalable microsampling with minimal damage to cells in the 8-cell embryo enabled duplicate and triplicate metabolic analysis of the same cell, which surprisingly continued to divide to the 16-cell stage. Last, we used microprobe single-cell CE-ESI-MS to uncover previously unknown reorganization of the single-cell metabolome as the dorsal progenitor

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.7b00880. Molecular features detected and identified in single embryonic cells; significant metabolite differences between microprobe CE-ESI-MS and whole-cell dissection; Fisher's least significant discriminant analysis; results from GProX Cluster Analysis; representative molecular features detected from the embryo culture media (PDF)

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Author Contributions

P.N. and R.M.O. designed the study. S.A.M. provided the *Xenopus* embryos and commented on the manuscript. R.M.O. and E.P.P. measured the cells. R.M.O., E.P.P., and P.N. analyzed the data. P.N. and R.M.O. interpreted the results and wrote the manuscript.

Notes

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cell from the 8-cell embryo formed the neural tissue fated clone through divisions to the 32-cell embryo, peering, for the first time, into the formation of metabolic single-cell heterogeneity during early development of a vertebrate embryo.

Graphical abstract

In situ characterization of the metabolome as individual cells differentiate is a major gap in mass spectrometry (MS) technology that promises to empower a systems cell biology understanding of normal and impaired development. With dynamic response to both intrinsic and extrinsic effects, the metabolome provides a unique look into the activity state of the cell. Recent studies found metabolism to actively contribute to key developmental processes, ranging from the viability of the preimplantation embryo¹ and establishment of cell fates,^{2,3} body patterning,⁴ and organs^{5,6} to transitioning between stem cell and cancer phenotypes.^{7–9} Contemporary MS enabled the detection of gross metabolic changes in whole developing embryos of powerful vertebrate models in cell and developmental biology and health studies, such as the zebrafish (*Danio rerio*)¹⁰ and the South African clawed frog (*Xenopus laevis*).¹¹ However, there is limited information available on how the metabolome is reorganized as embryonic cells undergo differentiation to establish cell heterogeneity and give rise to all the different types of tissues in the body.

The challenge has been mainly a lack of single-cell MS tools capable of unbiased measurement of the single-cell metabolome *directly in the live, morphologically complex* vertebrate embryo. Current single-cell MS tools are confined to denaturing conditions or cells dissected free from other cells or cultured or patterned into flat arrays (see reviews in refs 12–17). For example, metabolites have been measured in single cells in plant or animal tissues or cell cultures under vacuum using MALDI,^{13,18,19} SIMS,^{19,20} NIMS,²¹ and NAPA²² and at ambient conditions using DESI,²³ LAESI,²⁴ live single-cell video MS,²⁵ and combinations of in situ microsampling with direct electrospray ionization (ESI)^{26–30} or MALDI.³¹ To enhance detection sensitivity and molecular identification, we and others have developed high-sensitivity capillary electrophoresis (CE) ESI-MS platforms capable of separating molecules from dissected single neurons^{32–34} and embryonic cells.^{2,16,35–38} Using whole-cell dissection, we recently uncovered previously unknown metabolic cell heterogeneity in the 8- and 16-cell frog (*Xenopus laevis*) embryo along all three primary developmental axes^{2,35} and discovered metabolites capable of altering dorsal–ventral specification.² However, cell dissection sacrifices live embryonic development, is too slow to track fast metabolic changes, and becomes increasingly difficult for smaller and more tightly

adherent cells in the morphologically complex developing embryo. To better understand the molecular basis of cell differentiation, there is a high and yet unmet need for single-cell MS tools capable of scalable, fast, in situ, and preferably minimally invasive characterization of the metabolome in the live vertebrate embryo.

Here, we address this technological gap by developing “*microprobe single-cell CE-ESI-MS*” to enable, for the first time, the in situ mass spectrometric analysis of metabolites in single cells in the freely developing vertebrate embryo. After establishing technical performance metrics for single-cell analysis of the *X. laevis* embryo, we validate microprobe CE-ESI-MS against whole-cell dissection, which is the closest neighboring single-cell MS technology for the vertebrate embryo. Additionally, we employ microprobe CE-ESI-MS to determine how the metabolome is altered as a single dorsal embryonic cell forms a neural-fated clone in the 8- to 32-cell *X. laevis* embryo. The presented work demonstrates that in situ single-cell CE-ESI-MS is sensitive, is scalable to broad spatial and temporal dimensions, is compatible with the complex three-dimensional body of the vertebrate embryo, and enables discovery or targeted analysis of the single-cell metabolome. We expect this technology to be also adaptable to other types of cells and biological models, opening new potentials to advance our systems cell biology understanding of normal and impaired development.

METHODS

Materials and Reagents

LC-MS-grade methanol, formic acid, water, acetonitrile, sodium chloride, potassium chloride, and magnesium sulfate were from Fisher Scientific (Fair Lawn, NJ). Calcium nitrite, cysteine, Trizma hydrochloride, and Trizma base were from Sigma-Aldrich (Saint Louis, MO). Acetylcholine and amino acid standards were purchased at reagent grade or higher purity from Acros Organics (Fair Lawn, NJ).

Solutions

Steinberg’s solution (100%) and fresh 2% cysteine solution were prepared following established protocols.³⁹ The “*embryo culture solution*” contained 50% Steinberg’s solution. The metabolite “*extraction solvent*” contained 40% acetonitrile and 40% methanol in LC-MS-grade water, which we previously found to be able to efficiently extract small polar metabolites from *X. laevis* cells.³⁵ The CE “*background electrolyte*” consisted of 1% formic acid in LC-MS-grade water. The CE-ESI “*sheath solution*” contained 50% methanol and 0.1% formic acid in LC-MS-grade water.

Animals and Cell Identification

Adult male and female *X. laevis* frogs were purchased from Nasco (Fort Atkinson, WI) and housed in a breeding colony at the George Washington University (GWU). All protocols related to the handling and manipulation of animals were approved by the GWU Institutional Animal Care and Use Committee (IACUC #A311). Fertilized eggs were obtained by gonadotropin-induced natural mating of male and female adult frogs as described elsewhere.^{39,40} The jelly coats surrounding the embryos were removed using 2% cysteine solution as described elsewhere.⁴¹ Dejellied embryos were transferred to 100%

Steinberg's solution in a Petri dish and monitored until they reached the two-cell stage. Two-cell stage embryos in which asymmetric pigmentation marked the stereotypical dorsal–ventral axis with high accuracy (in reference to established cell fate maps^{42–47}) were isolated into a separate Petri dish and monitored; only these embryos were used in this study. On the basis of pigmentation and location in the *X. laevis* embryo with regards to established cell fate maps,^{42–47} we identified the right V1 (V1R) and right D1 (D1R) cell in the 8-cell embryo, the right D11 (D11R) and right D12 (D12R) cell in the 16-cell embryo, and the right D111 (D111R) and right D121 (D121R) cell in the 32-cell embryo. For microdissection studies, embryos were collected at the 8-cell stage into a separate Petri dish coated with 2% agarose gel and containing 50% Steinberg's solution at room temperature.

Dissection of Single Identified Cells and Metabolite Extraction

For technology validation, the identified cells were dissected free of other cells using protocols reported elsewhere.⁴¹ To quench enzymatic reactions, each dissected cell was immediately transferred into a separate microvial containing 20 μL of methanol chilled on ice. Contents of the vials were lyophilized at 4 °C, and metabolites were extracted in 5 μL of *extraction solvent* at 4 °C for 3 min, facilitated by periodic sonication and incubation on ice, following our recent protocol.^{2,35} The single-cell extracts were then centrifuged at 8000*g* for 5 min at 4 °C and stored together with the cell debris in the same vial at –80 °C until measurement by CE-ESI-MS.

Microprobe Sampling of Single Identified Cells and Metabolite Extraction

We designed an optically guided microsampling platform to enable the collection of a portion of the cell content from select identified cells in the live embryo. Embryos were immobilized in individual wells made of 2% agarose gel in a 50 mm Petri dish containing 50% Steinberg's solution at room temperature. Borosilicate capillaries (part number B100-50-10, 0.5/1 mm inner/outer diameter, Sutter Instrument Co., Novato, CA) were pulled to a taper with a barrel length of ~850 μm in a capillary puller (model P-1000, Sutter Instrument Co.) using the following settings: heat = 355; pull = 65, velocity = 80; time = 150. The pulled capillaries were manually cleaved using sharp forceps and a high-resolution stereomicroscope (model SMZ18, Nikon, Melville, NY) to produce “*micropipettes*” with an ~20 μm -diameter tapered-tip. To enable accurate positioning of the micropipette tip, the micropipette was mounted on a motorized three-axis micro-manipulator (model TransferMan 4r, Eppendorf, Hauppauge, NY) capable of <20 nm resolution and remote control with a joystick. With guidance from the high-resolution stereo-microscope, the tip of the micropipette was fine-positioned into individual identified single-cells in the live embryo that were selected for analysis. To aspirate a portion of the cell cytoplasm, –30 psi pressure was applied to the microcapillary using a microinjector (model PLI-100A, Warner Instrument, Hamden, CT). Thereafter, the tip of the microcapillary was retrieved from the cell, and the aspirated cell content was expelled using one second pressure surge (at 80 psi) into a microvial (Fisher Scientific, Pittsburgh PA) containing 4 μL of *extraction solvent* chilled to 4 °C on ice. Each sampling event utilized a separate micropipette to eliminate potential sample carryover between cells. These microvials were vortex-mixed for ~1 min at room temperature to facilitate metabolite extraction and centrifuged at 8000*g* for 5 min at 4 °C to pellet cell debris and precipitates (Sorvall Legend X1R; Thermo Scientific, Waltham,

MA). The resulting cell extracts were stored together with the cell debris and precipitates in the same vial at -80°C until measurement by CE-ESI-MS.

CE-ESI-MS Instrument

The cell extracts were analyzed using our custom-built CE-ESI-MS platform following protocols that we have reported elsewhere.^{2,32,35} Briefly, 10 nL of each cell extract was hydrodynamically injected into a fused silica capillary (40/105 μm inner/outer diameter, 1 m length) filled with the *background electrolyte*. Compounds were electrophoretically separated by applying 21 kV across the capillary until glutathione was detected, which was used to mark the end of separation experiments (~ 45 min). The compounds were ionized in a custom-built CE microflow ESI interface, which coaxially supplied the sheath solution at 1 $\mu\text{L}/\text{min}$ around the CE capillary in an earth-grounded metal capillary to complete the CE electrical circuit. The ESI needle was positioned ~ 2 mm from the mass spectrometer's orifice to maintain the electrospray in the stable cone-jet regime for efficient ionization.⁴⁸ Generated ions were analyzed in the positive ion mode between m/z 50 and 500 at 40 000 (fwhm) resolution by a quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Impact HD, Bruker Daltonics, Billerica, MA). The mass spectrometer was tuned and calibrated following vendor instructions and then further refined for acetylcholine and *S*-adenosylmethionine to maximize detection sensitivity. External mass calibration provided 5 ppm mass accuracy. The CE-ESI-MS instrument provided a 60 amol lower limit of detection and 4–5 log-order quantitative range. To ensure robust instrumental performance, we required a signal-to-noise (S/N) > 15 for sensitivity and $< 10\%$ relative standard deviation (RSD) in migration time and $< 25\%$ RSD in peak area for 300 amol of acetylcholine (6 nL of 50 nM analyzed) each day.

Experimental Design

All embryos were collected from natural mating of 2 pairs of parents to minimize genomic variability in this study. Each cell type was identified and then sampled in a random order. To account for biological variance between embryos and to enhance statistical analysis, at least $n = 4$ –7 biological replicates were collected for each cell type. Additionally, single-cell extracts were measured in a randomized order to minimize the effect of potential instrumental biases on results interpretation. Each cell extract was measured in 2–4 interday technical replicates.

Data Analysis

Raw mass spectrometric data files were processed in Compass Data Analysis version 4.3 (Bruker Daltonics) using custom-written scripts. Each file was externally mass-calibrated using sodium formate clusters that were formed in situ in the CE-ESI interface as sodium ions were separated by CE. Molecular features (signals with unique m/z vs migration time values) were semiautomatically searched following a protocol described elsewhere.^{2,32} *To relatively quantify metabolite abundances*, selected-ion electropherograms were generated for the detected molecular features with ± 5 mDa window, and the under-the-curve peak areas were integrated, which serve as a quantitative proxy for metabolite concentration.² *Statistical and multivariate data analysis* was performed using ANOVA and principal component analysis (PCA) in MetaboAnalyst ver. 3.0⁴⁹ with the following settings:

normalization by sum; log transformation; data scaling, with mean centering. Data normalization allowed us to compare metabolism in different portions of the total cell volume that were collected by whole-cell dissection and microprobe CE-ESI-MS. A p value 0.05 was chosen to mark statistical significance (Student's t -test). *Cluster analysis* was performed in GProX ver. 1.1.16⁵⁰ with the following settings: cluster number, 4; upper limit, 1.5; lower limit, 0.67; fuzzification value, 2; minimum membership for plot, 0.5; standardizations and 100 iterations.

Safety Considerations

Standard safety procedures were followed when handling chemicals and biological samples. Capillary micropipettes, which pose a potential needle-stick hazard, were handled with gloves and safety goggles. High voltage presents electrical shock hazard; therefore, to prevent users from exposure, all connective parts were earth-grounded or isolated in a Plexiglas enclosure equipped with a safety interlock-enabled door.

RESULTS AND DISCUSSION

Our single-cell analysis strategy integrates live imaging, in situ microsampling, and high-sensitivity CE-ESI-MS. As shown in Figure 1, we began with raising the embryo to the desired developmental stage, followed by the identification of individual cells using bright-field microscopy. Alternatively, epifluorescence may be used to identify cells that express fluorescent markers. Figure 1 demonstrates how we used morphological cues and fate maps^{42–47} to identify the right ventral cell (called V1R) in the 8-cell *X. laevis* embryo. To sample a desired cell in situ, the tip of a sharpened capillary was precision-positioned into the cell under translational control by a multiaxis micromanipulator and guidance by the stereomicroscope, and a calibrated portion of the cell content was aspirated by an online connected microinjector delivering negative pressure pulses to the capillary. Using a borosilicate capillary pulled to an $\sim 20\ \mu\text{m}$ tip, we were able to collect $\sim 10\text{--}15\ \text{nL}$, viz., 10% of the V1R cell in $<5\ \text{s}$. After retracting the capillary tip, the collected cell material was pressure-ejected into a microvial, where proteins were denatured and small molecules were extracted in $4\text{--}5\ \mu\text{L}$ of aqueous organic solvent mixture following our established protocols.^{2,35} The next steps performed metabolic analysis of the extract to infer the metabolic state of the cell. An $\sim 10\ \text{nL}$ portion of the extract, corresponding to $<0.02\%$ of the cell metabolome, was injected into a fused silica capillary, where molecules were electrophoretically separated by a custom-built CE system, efficiently ionized by a custom-designed CE-ESI interface operated in the cone-jet spraying regime, and generated ions were mass-analyzed by a high-resolution mass spectrometer. Metabolites were identified and quantified using stringent protocols that we developed for CE-MS.^{2,35} While sharpened capillaries have been used with MS to analyze metabolites in single cells in culture^{26–30} or tissues, such as single neurons,^{32,51} or plants,^{25,30,51–53} *microprobe CE-ESI-MS* from this study enabled, for the first time, the in situ characterization of metabolites in single identified cells in the live, freely developing vertebrate embryo.

Microprobe Single-Cell CE-ESI-MS versus Whole-Cell Dissection

We compared microprobe CE-ESI-MS to whole-cell dissection (Figure 2), which is the closest neighboring single-cell MS technology for the *X. laevis* embryo.² As reference, $n = 5$ different V1R cells were dissected over ~ 5 min/cell, each from a different embryo (biological replicates). In parallel, a 10–15 nL portion of the V1R cell was aspirated in < 5 s in situ from $n = 7$ live embryos. Unlike whole-cell dissection, 50–100-times faster sampling and minimal damage to the cell allowed us to repeatedly sample the same cell, including duplicate and triplicate analysis of the same cell. For example, as shown in Figure 2A, locations where the V1R cell was probed twice are marked by spots that formed as the cell membrane healed. Intriguingly, the singly or even doubly probed V1R cell continued to divide in synchrony with other cells as the embryo progressed to the 16-cell stage over the next ~ 20 min, as expected for normal development. In theory, microprobe CE-ESI-MS also raises a technical capability to characterize multiple cells in the same embryo, thus empowering statistics of single-cell analysis by reducing known metabolic variability between embryos.¹¹ Additionally, microprobe single-cell CE-ESI-MS is scalable to smaller cells and compatible with the complex three-dimensional body of the embryo, which we also demonstrate using the 16- and 32-cell embryos in this study.

Microprobe CE-ESI-MS enhanced the qualitative and quantitative characterization of the single-cell metabolome in comparison to whole-cell dissection. Typically, we detected ~ 230 distinct molecular features in each single cell, ~ 170 of which were repeatedly detected in at least 50% of the biological replicates. We identified ~ 70 metabolites by comparing the accurate mass, tandem mass spectrum, and migration time of molecular features that were detected in the cells to data measured using authentic chemical standards, available in metabolite MS databases (Metlin⁵⁴ or HMDB⁵⁵), or previously identified in *X. laevis* using our CE-ESI-MS platform.^{2,35} Detected molecular features and metabolite identifications are listed in Table S1. Representative separation of identified metabolites is shown in Figure 2B. Surprisingly, despite sampling an ~ 10 – 20 -times smaller portion of the single-cell metabolome, microprobe sampling yielded comparable levels of signals to whole-cell dissection (e.g., compare spermidine and putrescine) and higher separation performance (e.g., respective theoretical plate numbers were $\sim 71\,800/\text{m}$ vs $\sim 3800/\text{m}$ for spermidine and $137\,600/\text{m}$ vs $110\,800/\text{m}$ for carnitine). We attribute this improvement by microprobe CE-ESI-MS to collecting substantially lowered amounts of salts, buffering agents, and other additives from the embryo culture media (Table S1 and Figure S1), which are known to interfere with separation (e.g., less efficient field amplified sample stacking in CE) and ion generation (e.g., ion suppression during ESI).

Next, we evaluated the repeatability of quantification (Figure 3). To relatively quantify metabolite abundances, we generated selected-ion electropherograms for the detected molecular features with ± 5 mDa window and integrated the under-the-curve peak areas, which serve as a quantitative proxy for concentration.² These metadata were used to calculate the mean quantitative error (μ), expressed as relative standard deviation (RSD), on the basis of technical (same extract analyzed multiple times) and biological (same or different V1R cells analyzed multiple times) replicates. The technical repeatability of the CE-ESI-MS platform was characterized with $\mu = 8.2\%$ RSD by triplicate analysis of the

same cell extract (see “CE-MS” in Figure 3A). Microprobe sample collection followed by metabolite extraction before CE-ESI-MS slightly increased the quantitative error to $\mu = 13.9\%$ RSD with this difference being significant ($P = 7.4 \times 10^{-5}$). In comparison, biological replicates yielded higher quantitative variability. Triplicate microsampling of the same V1R cell (considered biological replicate here) yielded $\mu = 21.5\%$ RSD, which was significantly higher than the technical repeatability of microprobe CE-ESI-MS ($P = 2.1 \times 10^{-3}$). Because microsampling was completed long before cell division to the 16-cell stage embryo (compare <5 s/cell vs 5 min/cell in Figure 2A), greater biological than technical repeatability may reflect highly active cell metabolism (even before cell division), rapid metabolic response to perturbation caused by the microcapillary (viz., within seconds), and/or subcellular metabolic heterogeneity. Indeed, metabolism is known to be highly active during early embryonic development in many vertebrates, such as *Xenopus*, in which 50% of transcripts⁵⁶ and 60% of proteins³⁶ have been associated with metabolic processes. In contrast, metabolites in V1R cells were quantified with $\mu = 42.5\%$ RSD using whole-cell dissection (see “Diss”), and importantly, this error was statistically significantly higher than repeated microsampling of the same cell ($P = 5.7 \times 10^{-5}$). Therefore, repeated analysis of the same cell by microprobe sampling eliminated metabolic variability that exists between dissected cells as a result of, e.g., different stages of the cell cycle or embryo-to-embryo differences. With higher detection sensitivity and quantitative repeatability than dissection, microprobe CE-ESI-MS raises the potential to detect differences between cells that would be masked by embryo-to-embryo differences, within even the same cell type.

The single-cell MS data revealed differences in metabolic activity between the dissected and in situ microprobed cells. For example, Figure 2B suggests a higher ratio of lysine/arginine, valine/serine, and glutathione (GSH)/oxidized glutathione (GSSG) in the microprobed cells. To systematically compare the metabolomes, we performed unsupervised principal component (PC) analysis of the metadata. The first three most significant principal components explained 51.9%, 11.1%, and 8.2% variance in the data; therefore, PC1 and PC2 were sufficient for the PCA model used in this study. Data corresponding to the dissected versus microprobed cells formed separate clusters in the scores plot (Figure 3B), revealing reproducible metabolic differences between the approaches. In the corresponding loadings plot, distant clustering of the molecular features from the origin revealed enrichment in the dissected (e.g., GSSG) or the microprobed cells (e.g., GSH), but not both. To further evaluate these apparent chemical differences, we normalized the signal areas in each sample and then calculated the average ratio of small molecule abundance between microsampled-to-dissected cells and the corresponding statistical significance. The results, presented in Figure 3C, exposed clear metabolic differences between these single-cell analysis strategies. Molecular features with significantly different abundances are tabulated in Table S2. Notably, the majority of small molecules were relatively enriched during microprobe sampling, including cysteine, methionine, and GSH, whereas GSSG along with compounds originating from the embryo culture media (see filled circles) were significantly more abundant in the dissected cells.

Therefore, we proposed that dissection and microprobe sampling differentially influenced the metabolic state of the V1R cells. To test this, we compared the oxidative status of the microprobed versus dissected V1R cells. GSH is an abundant antioxidant that is known to

provide defense against oxidative damage by conversion to GSSG; thus, the GSH/GSSG ratio is an effective metric of oxidative stress. As noted earlier, microprobe-generated extracts contained systematically (Figure 3B) and significantly (Figure 3C) higher amounts of GSH and lower levels of GSSG compared to whole-cell dissection. These data suggested a higher level of oxidative stress in the dissected cells. Indeed, the ratio between the average GSH/GSSG ion signal was 45 ± 27 by dissection and 1045 ± 76 by microprobe sampling with this difference being statistically significant ($P = 2.8 \times 10^{-5}$). We credit significantly less oxidative stress imposed on the V1R cell by microprobe CE-ESI-MS to a combination of factors, including rapid sampling (<5 s by microprobe vs ~ 5 – 10 min by dissection), minimal physical damage to the cell (membrane heals and the cell divides during microprobe sampling), and no/undetectable perturbation to neighboring cells (compare with dissection, which sacrifices the remaining cells in the embryo) (see Figure 2A). These results revealed that microprobe CE-ESI-MS provided a direct, in situ snapshot of innate single-cell metabolism in the live, freely developing vertebrate embryo.

Reorganization of the Metabolome within Neural Cell-Clones

Last, we used *microprobe CE-ESI-MS* to quantify, for the first time, metabolic changes as an embryonic cell forms a neural cell lineage in the live *X. laevis* embryo (Figure 4). Using whole-cell dissection, we previously uncovered spatial metabolic cell heterogeneity in the 8- and 16-cell embryo,² suggesting that single-cell CE-ESI-MS is also able to provide insights on cell differentiation in the broader spatial and temporal domain. To test this hypothesis, we used microprobe *CE-ESI-MS* to determine how the metabolome is reorganized as cells of the dorsal cell clone are formed between the 8-cell and 32-cell embryo (Figure 4A); this clone is the main precursor of neural tissues (e.g., brain, spinal cord, and retina) with moderate contributions to other organs, such as the notochord, gut, heart, and liver.^{42–47} We quantified metabolites in the D1R cell ($n = 6$ biological replicates) in the 8-cell embryo, its midline D11R ($n = 4$) and lateral D12R ($n = 5$) daughter cells in the 16-cell embryo, and their respective animal pole daughter cells in the 32-cell embryo, which are the D111R ($n = 4$) and D121R ($n = 4$) cells. Cells in the clone become progressively smaller as the volume of the cell is halved during each cell division; the volume of the average cell corresponds to a sphere with an ~ 550 μm diameter in the 16-cell embryo and ~ 450 μm diameter in the 32-cell embryo (Figure 4A). Each cell extract was measured in technical duplicate–quadruplet, yielding 50 single-cell measurements that quantified the production profiles for ~ 230 different molecular features, including the ~ 70 metabolites that were identified across the cell lineage in this study (Table S1).

The quantitative data revealed complex spatial and temporal metabolic changes as the progenitor D1R cell gave rise to its descendants. A brief survey of metabolite abundances using analysis of variance based on Fisher's least significant analysis found significant changes in the single-cell metabolome for close to 100 molecular features, including ~ 50 identified metabolites (Table S3). For example, the daughter cells of D1R contained increasing levels of γ -aminobutyric acid (GABA) and decreasing amounts of aspartate, whereas creatine was comparably abundant across all cells in the lineage (Figure 4B). In agreement, aspartate has been found to rapidly decay in the whole developing *Xenopus laevis* embryo.^{11,57} To systematically evaluate metabolic profiles across the cell lineage, we

examined the quantitative metadata using unsupervised clustering by fuzzy-c means (see Methods). A total of 122 small-molecule features (excluding compounds from the culture media) were grouped into 4 different clusters based on different spatial and temporal abundance profiles across cells of the dorsal clone (see Figure 4C). A complete list of these molecular features and their cluster assignments are listed in Table S4. As a general trend, metabolites in Cluster 0, including creatine, were present at steady abundance across the cells. Metabolites in Cluster 1 yielded stable signal abundance in cells in the 8- and 16-cell embryo and then became enriched in D121R with significant accumulation in D111R of the 32-cell embryo. In comparison to Cluster 1, metabolites in Cluster 2 followed a seemingly anticorrelated profile with enrichment in D11R but rapid decay in the daughter cells in the 32-cell embryo. Progressively decreasing concentration across all cells of the lineage was characteristic for metabolites in Cluster 3, including aspartate, asparagine, carnitine, and acetylcholine. This finding is in agreement with recent isotopic pulse-chase experiments for aspartate in whole *Xenopus* embryos.¹¹ Stable production until the 16-cell stage followed by a rapid decrease in abundance characterized metabolites in Cluster 4. While understanding the functional significance of these metabolic profiles goes beyond the scope of this study, these results demonstrate the first example of using single-cell MS to track metabolic cell heterogeneity in space and time in the live, developing vertebrate embryo. The data resulting from these measurements show how the metabolome is reorganized as embryonic cells commit to different tissue fates during early vertebrate embryonic development.

CONCLUSIONS

Microprobe single-cell CE-ESI-MS enables, for the first time, the in situ characterization of metabolic changes as a single embryonic cell forms a cell clone in the developing vertebrate embryo (*X. laevis*). Key advantages of the technology over classical whole-cell dissection include rapid and direct (in situ) operation with a capability for in vivo studies (see Figures 1 and 2A), scalability over broad spatial and temporal dimensions (see Figure 4A), and compatibility with the morphologically complex structure of the vertebrate embryo. Compared to whole-cell dissection, microprobe CE-ESI-MS enhanced detection sensitivity and quantitative repeatability (see Figure 2) and imposed significantly lower oxidative stress on cells. Repeated analysis of the same cell in the same embryo raises a technical capability to profile metabolite production between multiple cells in the same embryo. By minimizing or eliminating biological and cell-cycle variability between cells dissected from different embryos, microsampling CE-ESI-MS provides higher quantitative fidelity than whole-cell dissection, which in turn affords a potential to find metabolic cell heterogeneity that would otherwise be masked by biological variability using whole-cell dissection. Single-cell metabolomic MS complements single-cell transcriptomics and recent advances in single-cell proteomics,^{36–38,58} helping to pave the way to multiomic analysis of single cells. With future developments in speed, throughput, automation, and streamlined data analysis, we anticipate microprobe single-cell CE-ESI-MS to enable one to measure metabolites in larger populations of single cells and other model organisms. In situ microprobe single-cell CE-ESI-MS extends the bioanalytical toolbox of systems cell biology¹⁶ and empowers exciting new research directions to help understand the metabolic underpinnings of cell differentiation during normal and impaired development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Microprobe single-cell capillary electrophoresis (CE) mass spectrometry (MS) enabling in situ metabolic characterization of live *Xenopus laevis* embryos (1). A 10–15 nL portion of single cells identified under a stereomicroscope (2) were aspirated into a pulled capillary (3) using a multi-axis translation stage (4) and a microinjector (5) delivering vacuum ($-P$). The collected cell content (6) was pressure-injected ($+P$) into a vial for metabolite extraction (7). The extract was measured by a microloading CE platform (8) connected to a CE electrospray ionization (ESI) source (9) operated in the cone-jet mode (see Taylor-cone, Tc). Metabolite ions were identified and quantified using a high-resolution tandem mass spectrometer (10). Scale bars = 500 μm (dark/gray); 10 mm (white). Key: SP, syringe pump.

Figure 2. Metabolite detection by microprobe CE-ESI-MS vs whole-cell dissection. (A) As reference, the whole right-ventral (V1R) cell was dissected from the 8-cell *X. laevis* embryo. In comparison, the V1R cell was analyzed once (1×) and twice (2×) in the live embryo using the microprobe (spots mark analysis locations; see white arrows). Intriguingly, microprobed cells divided to form the 16-cell embryo. (B) Electropherograms exemplifying metabolites identified in a V1R cell using whole-cell dissection (top panel) and microsampling (bottom panel). Scale bars = 250 μ m. Key: HPX, hypoxanthine; SAM, *S*-adenosylmethionine; AcCho, acetylcholine; AcCar, acetylcarnitine. Peaks in gray correspond to compounds from the embryo culture media. See detected molecular features and metabolite identifications in Table S1.

Figure 3.

Microprobe quantification of metabolites in single cells. (A) Technical repeats by CE-ESI-MS (CE-MS) and microprobe CE-ESI-MS (“ μ P” techn.) versus biological repeats by microprobe CE-ESI-MS (μ P on the same cell) and whole-cell dissection (Diss). Key: ** $P < 0.005$ (B) Unsupervised principal component (PC) analysis scores plot (top panel) and loadings plot (bottom panel) revealing small-molecule differences between microprobe sampling and cell dissection. Key to scores plot: each data point is a different cell; cells numbered by sampling order; 95% confidence ellipses in dashed lines. Key to loadings plot: each data point is a detected molecular feature (see Table S1); signals from culture media in filled circles. (C) Statistical analysis of fold change (FC) differences between microprobed/dissected cells. Signals from media in filled circles. Horizontal line marks $P = 0.05$. Metabolites are listed in Table S1.

Figure 4.

Single-cell metabolism in the dorsal cell lineage of the *X. laevis* embryo. (A) Optical images of the progenitor right dorsal (D1R) cell, its daughter cells, the D11R and D12R cells, which then respectively are precursors to animal pole D111R and D121R cells. (B) Fisher's least discriminant analysis finding significant metabolic changes between cells in the lineage. Key: * $P < 0.05$; ** $P < 0.001$; ns = no significant difference. (C) Unsupervised fuzzy-c means clustering of single-cell metabolite profiles between cells in the clone. See molecular features and their cluster assignments in Table S4. Scale bars = 250 μm .