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Analysis of endogenous nucleotides by single cell capillary electrophoresis-mass spectrometry

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Abstract

Analytical technologies that enable investigations at the single cell level facilitate a range of studies; here a lab-fabricated capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS) platform was used to analyze anionic metabolites from individual *Aplysia californica* neurons. The system employs a customized coaxial sheath-flow nanospray interface connected to a separation capillary, with the sheath liquid and separation buffer optimized to ensure a stable spray. The method provided good repeatability of separation and reliable detection sensitivity for 16 mono-, di- and triphosphate nucleosides. For a range of anionic analytes, including cyclic adenosine monophosphate (cAMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP), the detection limits were in the low nanomolar range (<22 nM). A large *Aplysia* R2 neuron was used to demonstrate the ability of CE-ESI-MS to quantitatively characterize anionic metabolites within individual cells, with 15 nucleotides and derivatives detected. Following the method validation process, we probed smaller, 60-μm diameter *Aplysia* sensory neurons where sample stacking was used as a simple on-line analyte preconcentration approach. The calculated energy balance ($[\text{ATP}] + 0.5 \times [\text{ADP}] / ([\text{AMP}] + [\text{ADP}] + [\text{ATP}])$) of these cells was comparable with the value obtained from bulk samples.

Keywords

single-cell CE-MS; *Aplysia californica*; nucleotides; nanospray

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Introduction

Metabolism refers to the set of chemical and physical processes in which small endogenous molecules, the metabolites, are generated, modified and/or destroyed. Both anabolites and catabolites are involved in the formation of cellular and organismal phenotypes, as well the development of physiological and pathological states of cells and their reactions to physical and chemical factors. Qualitative and quantitative investigations of the composition of the cellular and organismal metabolome are important for understanding a variety of phenomena, including single-cell biological variability and functional heterogeneity.¹ In particular, heterogeneity plays an important role in nervous system functioning,² stem cell-dependent regeneration,³ and the etiology of many diseases.⁴

Single cell metabolomics is a promising but challenging area of measurement science.⁵ Cells sizes typically range from 15–100 μm in diameter, and so represent volume-limited samples containing a wide variety of metabolites with different physico-chemical properties and concentrations that span orders of magnitude. Current bioanalytical approaches and instrumentation enable targeted metabolomics investigations of a variety of single cell types^{6–8} using spectroscopic (e.g., fluorescence and vibration-based), electrochemical, nuclear magnetic resonance and mass spectrometric methods.^{6,9} Chemical separations hyphenated to mass spectrometry (MS) offer powerful options for metabolite analysis that often increase analyte coverage compared with direct MS, while providing high resolution separation, sensitive detection and ample structural information at the molecular level.^{5,7–13} Various separation techniques hyphenated to MS, such as gas chromatography,¹⁴ liquid chromatography (LC),¹⁵ and capillary electrophoresis (CE),¹⁶ have been shown to increase analyte coverage in metabolomics investigations.^{17,18}

Among the separation methods, CE, with its nano- and picoliter sample-volume requirements and high separation efficiency, enables microanalyses that include profiling and quantitation of metabolites in single cells.^{9,16,19,20} We developed a CE-MS platform that has a small volume inlet vial and a nanospray interface and used it in several single cell and small-volume metabolomics studies.^{1,16,21} Cationic metabolites have been the main targets in our prior studies involving identified neurons and cytoplasmic sampling from *Aplysia californica*^{1,16,22} and *Rattus norvegicus*.^{21,23}

The investigation of anionic metabolites, such as nucleotides in individual cells, is not as well developed as the cationic measurements. Nucleotides play a plethora of roles inside and outside of cells. Ribonucleotides and deoxyribonucleotides are building blocks of RNA and DNA, and adenosine triphosphate (ATP) is a key energy molecule. Purine nucleotides have intercellular (e.g., ATP) or intracellular (e.g., guanosine-5'-triphosphate (GTP) and cyclic adenosine monophosphate (cAMP)) signaling functions. Moreover, some coenzymes (such as nicotinamide adenine dinucleotide (NAD⁺), nicotinamide adenine dinucleotide phosphate (NADP⁺) and flavin adenine dinucleotide (FAD)) are important endogenous electron carriers in redox cycles. Nucleotides can be detected and quantified in complex biological matrices using LC-MS,²⁴ CE-UV,²⁵ and CE-MS.^{26–30} However, significant method optimizations are required in order to apply these approaches to single cell nucleotide analysis.

Here we describe a method for qualitative and quantitative anionic metabolite analysis using CE-MS. We optimized the CE analyte stacking and injection process, as well as the separation conditions, to ensure compatibility with nanospray ionization. We validated the approach using a variety of individual *Aplysia* neuron types in which we detected and identified a number of nucleotides.

Experimental

Chemicals

Stock solutions (10 mg/L) of NAD⁺, cAMP, FAD, adenosine monophosphate (AMP), cytidine monophosphate (CMP), NADP⁺, guanosine monophosphate (GMP), uridine monophosphate (UMP), adenosine diphosphate (ADP), guanosine diphosphate (GDP), cresyl diphenyl rphosphate (CDP), adenosine triphosphate (ATP), guanosine-5'-triphosphate (GTP), uridine diphosphate (UDP), cytidine triphosphate (CTP) and uridine-5'-triphosphate (UTP) were prepared using LC-MS grade water and stored at –80°C. The stock solutions were diluted with LC-MS grade water before injection. These 16 anionic standards as well as isolated individual neurons were used in the method validation process, including quantitative analysis. Both the standard solutions and neurons were placed in 0.5 mL Protein LoBind microcentrifuge tubes (No. 022431064, Eppendorf North America, Hauppauge, NY). All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). Solvents were analytical grade or higher purity.

Animals and single cell sample preparation

Aplysia californica (180–250 g body weight) were maintained in constantly aerated and circulating seawater at 14°C; animals were anesthetized by injection of 390 mM MgCl₂ solution into the vascular cavity. Injection was equal by mass to about one-third of each animal's body weight. Ganglia and adjacent nerves were surgically dissected and placed into artificial seawater (ASW) containing 460 mM NaCl, 10 mM KCl, 10 mM CaCl₂, 22 mM MgCl₂, 26 mM MgSO₄ and 10 mM HEPES (pH 7.7). To reduce mechanical connections between cells, ganglia were enzymatically treated for 30–50 min at 34°C in 1% protease type IX Bacterial dissolved in ASW containing penicillin G, gentamycin and streptomycin.

Single neurons were isolated manually from ganglia with sharp tungsten needles and quickly (2–5 s) rinsed with deionized water to remove ASW salts and extracellular metabolite contaminants. Analytes from individual neurons were extracted using 2–5 µL (dependent on cell sizes) of a 1:1 cold mixture of methanol and 0.85% ammonium bicarbonate (all v/v).¹⁶ Methanol-based media is widely used for enzymatic activity quenching and analyte extraction in metabolomics.³¹ Typically, less than 30 s are needed for single cell sample preparation. Samples were stored at –80°C until analysis, with the frozen samples assayed within 2 weeks. Our CE-MS analysis of control samples (e.g., cell rinsing solution) confirmed the absence of detectable losses of metabolites from cells due to leakage or contamination of the biological samples from plastic ware and reagents (data not shown). More details about sample preparation can be found in earlier publications.^{1,23}

CE-electrospray ionization (ESI)-MS set-up and sample analysis parameters

Experiments were performed on a custom-built CE-ESI system hyphenated to a micrOTOF mass spectrometer (Bruker Daltonics, Billerica, MA) using a previously described modified coaxial sheath-flow nanospray interface.^{1,16} Briefly, the interface for this work was constructed using a microtee assembly (part number P-875, IDEX Health & Science, Oak Harbor, WA) that housed a platinum alloy emitter (10% iridium, 0.0055" inner diameter (i.d.) $\times$ 0.003" wall $\times$ 1" length, part 27525A, Johnson Matthey Inc., Wayne, PA). Compared to the stainless-steel emitters used in our prior studies, platinum withstands corrosion and helps to achieve a higher sensitivity of detection.²⁶ A light microscope (model SZ40, Olympus, Japan) was used to visually observe the quality of the electrospray plume and assist in making appropriate adjustments. The electrophoretic analyte separations were performed in 60-cm long, 40- μ m i.d., and 105- μ m outer diameter (o.d.) fused silica capillary (part TSP040105, Polymicro Technologies, Phoenix, AZ) using 20 mM ammonium bicarbonate (NH_4HCO_3) as the background electrolyte (BGE) and 0.2 mM NH_4HCO_3 in 50% IPA (v/v) as the sheath liquid (SL). All buffers were prepared fresh each day, and were also replenished after each separation to improve reproducibility. A 600 nL/min SL was delivered by a syringe pump (model 70-2000, Harvard Apparatus, Holliston, MA) through another 75-cm long, 75- μ m i.d., and 365- μ m o.d. fused silica capillary (part TSP075375, Polymicro Technologies). The nanospray emitter was always grounded, and the spray voltage (+1600 V) was applied at the inlet of the mass spectrometer. Dry gas was kept at 180°C with a flow rate of 3 L/min.

The mass spectrometer was calibrated regularly in a mass range of m/z 300–800 by infusing sodium acetate (150 $\mu\text{g/mL}$) via a commercial CE-ESI-MS sprayer (G7100-60041, Agilent Technologies, Inc., Santa Clara, CA). Recalibration of the acquired data sets was also performed, if needed, using Data Analysis software (version 4.0, Bruker Daltonics). Calibration was accomplished via external calibration with sodium acetate clusters performed before the CE acquisition, and by using internal calibration via offline recalibration with sodium bicarbonate clusters (that were endogenous to the sample).

Large-volume sample stacking

To analyze smaller neurons (e.g., a 60- μ m diameter *Aplysia* sensory neuron), an online concentration method was used in which a large volume of sample was injected and excess buffer was pumped out by applying a negative high voltage across the separation capillary. In the sample stacking or regular separation experiments, the BGE vial was electrically connected to two high voltage power supplies (HVPSs) (Model PS/MJ30N0400-11, Glassman High Voltage Inc., Whitehouse Station, NJ, and Bertan model 30R, Spellman High Voltage Electronics Corp., Hauppauge, NY) through a high voltage relay (model E60-DT-80, Ross Engineering Corp., Campbell, CA) connected to an isolation transformer (model N-67A, Triad Magnetics, Perris, CA). The HVPSs were set for operation at opposite polarities. The system could change the BGE vial potential in seconds, even without the use of a dedicated fast polarity switching HVPS. Water-diluted sample was injected hydrodynamically by elevating the separation capillary inlet by 15 cm, creating a height difference between the capillary inlet and outlet, for 10 min. After sample loading, the capillary inlet was returned to its normal level in the buffer vial before the application of -3

kV to the separation capillary. The sample buffer was removed from the capillary inlet by reversed electroosmotic flow (EOF), which was maintained for 3 min. Analytes were separated during 40 min runs while 10 kV was applied across the separation capillary. These experimental conditions provided optimal resolution and signal response for CE analysis with sample stacking.

Results and discussion

CE-MS analysis of negatively charged compounds has proven to be more difficult than with positively charged analytes, in part, because of frequent corona discharge formation, which affects the quality and repeatability of MS detection. Different electron scavenging gases, such as SF₆ or CO₂, and chlorinated or nonaqueous solvents, are used to improve negative ion detection with ESI-MS.³²⁻³⁴ Nebulizer gas flow and relatively low electrospray voltages are maintained in commercial ESI interfaces operating in negative MS mode to facilitate solvent nebulization and suppress electrical discharge. Here we utilized a modified lab-fabricated nanosprayer²³ for the analysis of single cells in negative mode MS. The coaxial sheath-flow nanospray interface has a smaller diameter capillary outlet (40- μ m vs. a typical 75- μ m i.d., and 105- μ m vs. a typical 365- μ m o.d.), allowing slower sheath flow rates (e.g., <1 μ L/min vs. 4–10 μ L/min in commercial designs) and avoiding the use of a nebulizer gas. These modifications reduced sample dilution and improved reproducibility and detection limits for anions.¹⁶

Optimization of CE-ESI-MS for investigation of anionic species

CE-MS is an effective method for the analysis of anionic metabolites.^{26,29,35-37} Single cell investigation requires low detection limits as well as highly efficient separation. To support high throughput analysis, pressure-assisted electrophoresis can be applied to speed up the migration of analytes through the fused silica capillary.^{27,38} For many biological samples, proteins and peptides adsorbed onto the silica surface during separation reduced the repeatability of measurement. Different coatings have been applied to the inner surface of the capillary to improve the repeatability;^{27,39-42} however, they reduced the quality of the nanospray in our experiments. Generally, a number of parameters are associated with a stable spray and a successful CE-MS measurement, including the emitter geometry, interface design, buffer composition, SL flow rate, spray voltage and cleanliness of the interface. As previously reported,^{1,43} we measured the total ion current to monitor the stability of ion generation.

Ammonium salts are typically used in BGEs for CE-MS, in part because of their volatility, which is a prerequisite for efficient formation of gaseous ions in electrospray, as well as minimization of deposits forming on the sprayer and inlet of the mass spectrometer. In this work, BGEs containing ammonium formate (NH₄HCO₂), ammonium acetate (NH₄Ac) and ammonium bicarbonate (NH₄HCO₃) were evaluated for their ability to provide acceptable separation of anionic standards. Lower concentrations of basic electrolytes in BGEs are beneficial for increasing the EOF in bare fused silica capillaries and minimizing ion suppression in negative mode MS detection. Thus, a BGE containing a 20 mM ammonium salt, titrated to pH 10 with ammonium hydroxide, was utilized in this work. A 60-cm long fused silica capillary provided the compromise between separation speed and resolution.

The formation and quality of the nanospray was optimized by testing SLs containing commonly used organic solvents: methanol (MeOH), acetonitrile (ACN), or isopropanol (IPA) (Table 1). The stability of the electric current and proper geometry of the cone-jet spray⁴³ are two important parameters for ensuring nanospray stability and CE repeatability. Both parameters were monitored and optimized. For example, we determined that 50–100% methanol-based SLs demonstrated good electrical conductivity; however, the stability of the electrospray was inadequate, even at the highest methanol concentrations. With lower percentages of methanol (50% and 70%), cone-jet sprays were not formed, as visually monitored through a light microscope. A comparative analysis of ACN- and IPA-based SLs, in the same percentage, revealed that IPA performed better in generating a stable nanospray and forming a finely shaped spray cone. Some of these results could be predicted, taking into account the properties of the solvents, such as dielectric constant, surface tension and gas-phase basicity. As reported by Straub and coworkers,³⁴ solvent properties play key roles in the efficiency of negative ion generation in the electrospray ionization process.

In our coaxial interface, CE effluents and pumped SL were mixed to form a liquid meniscus on the tip of the emitter. The nanospray emitter was positioned axial to the MS inlet at a distance that allowed the gas phase ions to enter the mass spectrometer's inlet. Heated by both the dry gas (180 °C) coming from the MS inlet and Joule heat from the CE separation, the solution around the platinum emitter easily formed gas bubbles. Most of this solution is SL, therefore, a lower content of organic solvents in the SL is preferred so as to reduce bubble formation. The results of our experiments demonstrated that an SL containing 50% IPA (v/v) performed better than SLs composed of either the MeOH or ACN mixtures (Table 1).

Solution conductivity plays an important role in CE analyte separation and nanospray stability. Fig. 1 summarizes results of experiments where a 50% IPA (v/v)-based SL was supplemented with different electrolytes and four anionic standards. The mixtures were directly infused into the mass spectrometer through the nanospray interface, with most experimental parameters (such as temperature, emitter position, analyte concentrations) kept constant during these tests. As shown in Fig. 1, the signal intensities of four standards dissolved and electrosprayed in 50% IPA (v/v) were maximal. Because of a variety of factors, including ion suppression and interface design, other signals from SLs with added electrolytes were lower than in the control. The optimal solution conductivity was determined by balancing the quality of the spray and the stability of the electric circuit. An SL with higher conductivity, e.g., 2 mM NH_4HCO_3 and a low flow rate (0.6 $\mu\text{L}/\text{min}$), produced an efficient spray but resulted in ion suppression. This led to poor analyte signal (Fig. 1). In contrast, the lower conductivity of solutions having low concentrations of electrolytes (e.g., 0.2 mM NH_4HCO_3) allowed the formation of a cone-jet spray and the detection of analytes with reduced background.⁴⁴ In a nebulizer gas-free nanospray system, the SL should contain electrolytes to maintain acceptable current through the CE capillary and therefore, allow spray. Using an SL containing 0.2 mM NH_4HCO_3 resulted in an ~50% decrease in the relative signal intensities of four standards, but was still better than other tested solutions. In contrast to our observations, Kok et al.³⁵ reported significant signal improvements when salts were added to the SL with increased electric conductivity. However, in their study, a commercial SL electrospray interface and nebulizer gas were

used, which led to an increase in signal intensity but also background noise level. Based on our test results, we selected NH_4HCO_3 as the electrolyte for both the BGE (20 mM) and SL (0.2 mM).

An optimal SL flow rate is necessary for the formation of a stable electrospray to ensure low limits of detection (LODs) and high sensitivity. In prior studies we have demonstrated that a stable nanospray can be obtained at SL flow rates lower than 1 $\mu\text{L}/\text{min}$. After optimization of all of the CE-MS parameters, the SL flow rate in this work was set at 0.6 $\mu\text{L}/\text{min}$, without apparent influence on the CE analyte separation (EOF $\sim 60 \text{ nL}/\text{min}$).

CE-ESI-MS method validation

Our optimized CE-ESI-MS method was validated using 16 anionic analyte standards. Fig. 2 shows extracted ion electropherograms of the standards at 100 $\mu\text{g}/\text{L}$ concentrations. Calibration curves for peak area (A) versus concentration (c) were fitted using a linear equation $\log(A) = s \cdot \log(c) + B$ (see the calibration curves in the Supplementary Information). Correlation coefficients of the calibration curves covering a wide concentration range (20, 100, 250, 5000 and 10000 $\mu\text{g}/\text{L}$) were between 0.977 and 0.999. The relative standard deviations (RSDs) for peak areas at the 250 $\mu\text{g}/\text{L}$ concentration point were $<15\%$ (Table 2). The LODs and limits of quantitation (LOQ) of the CE-ESI-MS method for many tested analytes were in the low nanomolar range, similar to, or better than, prior reports.^{26,27} This performance has lower LODs compared with other nucleotide characterization approaches such as LC-MS (in $\mu\text{g}/\text{ml}$) and HILIC-MS (in ng/ml)^{45,46} and is well suited for small-volume samples such as cells.

Quantitative measurements of endogenous nucleotides in individual neurons

Widespread physiologically and pathologically important cellular heterogeneity and biological variability demonstrates the requirement for single cell analysis.⁸ In this study, CE-ESI-MS was used to investigate anionic metabolite content in large individual neurons of the sea slug *Aplysia californica*. Samples containing metabolites extracted from single neurons were diluted 20-fold, bringing intrasample analyte concentration into the linear range of the calibration curves. A single cell extract (100–500 nL) was placed into the sample vial, an aliquot was injected and the metabolites measured using the CE-ESI-MS system.

While many other peaks were present, we verified 16 anionic metabolites, and produced external calibrations for them, to enable quantitative single cell analysis. Absolute concentrations of 15 analytes in R2 neurons isolated from *Aplysia* CNS were determined (Table 3). Only FAD was below the LOD. Expectedly, ATP concentration was the highest (74.1 pmol) among the detected analytes. ATP is a ubiquitous metabolite playing important structural, energetic and signaling functions. Previously, the ATP content of the R2 neuron was measured by Ambron and Stein,^{47,48} who demonstrated that the endogenous concentration ranged between 24–100 pmols and depended on animal size. Our results are also in good agreement with the published concentration of cAMP in R2 neurons (0.6 pmol); cAMP is a well-known second messenger responding to different stimuli, including electrical stimulation.⁴⁹

Having knowledge of the concentrations of molecules such as AMP, ADP and ATP allows us to determine the energy status of these cells. In 1967, Atkinson and Walton⁵⁰ introduced the concept of energy charge, which summarizes basic metabolic parameters ($[\text{ATP}] + 0.5 \times [\text{ADP}] / ([\text{AMP}] + [\text{ADP}] + [\text{ATP}])$). We calculated the energy charge for the R2 neuron as 0.81, which is on the lower side for live cells, with the value normally between 0.8 and 0.95. The CE-ESI-MS approach used in this work measured endogenous concentrations of metabolites, producing results in agreement with previously published data.

Large-volume sample stacking enhancement of anionic analyte detection using CE-ESI-MS

Many on-column analyte concentration techniques have been developed for electrophoretic separations that facilitate the analysis of low abundance analytes.⁵¹⁻⁵³ Sample stacking encompasses a range of on-line analyte preconcentration methods to improve LODs in CE by injecting larger volumes; these stacking techniques counter the typical volume limitations of CE.^{54,55} Among them, large-volume sample stacking (LVSS)⁵⁶⁻⁵⁸ is an appropriate method for analyzing volume-limited samples such as individual neurons.

To validate the LVSS performance in our single cell CE-MS analysis, we assayed the contents of <60- μm diameter *Aplysia* sensory neurons, which are more than 100-fold smaller than R2 neurons, and hence, contain much lower amounts of most compounds. To simplify our setup and operation, only a reversed separation polarity was used for the process of sample stacking in these experiments. Fig. 3 is a schema of the LVSS technique used here to analyze anions with CE-nanospray-MS. The standards were diluted with pure water to maintain a lower conductivity than in the BGE. Sample injection was carried out by elevating the sample vial and capillary inlet 15 cm above the capillary outlet for 10 min. About 103 nL of sample was injected, filling around 14% (82 mm plug length) of the capillary (Fig. 3A). After the capillary inlet is placed back into buffer vial, a -3 kV potential was applied for 3 min to reverse the EOF in the bare-fused silica capillary after sample injection (Fig. 3B). Driven by high electric field strength in the sample zone with low conductivity, the anions migrated with higher electrophoretic velocities in a counter direction to the bulk solution's flow, and were stacking in a narrower sample zone with lower relative content of positive ions and neutral species. Similar to the CE-nanospray-MS approach described above, the capillary outlet was surrounded by the SL. Application of a reverse voltage drives sample buffer out of the capillary and results in formation of bubbles (air mixed with SL), which build up at the capillary outlet and may interrupt the CE electric circuit. The bubbles lead to an increase of resistance in the circuit, and therefore, affect the electric current, which cannot be used as an indicator of success of the sample stacking procedure. After stacking, a positive voltage (10 kV) was applied to the capillary for analyte separation (Fig. 3C). The electropherograms in Fig. 3 show the CE-nanospray-MS analysis of a single 60- μm sensory neuron. The sample stacking approach allowed the detection of five endogenous metabolites (AMP, ADP, GDP, ATP and GTP). A comparative analysis of the single cell data and data acquired from a mixture of the analyte standards shows that the analytes are present in the cells at lower than 500 ng/L concentrations. Sample stacking allowed detection of 51 fg of material, a 200-fold improvement compared to the analyses of the larger R2 neurons.

Conclusions

In this study, a lab-fabricated CE-nanospray-MS platform was used to successfully characterize anionic metabolites within single neurons. The method optimization process revealed that a stable and repeatable nanospray can be achieved by using 50% IPA with 0.2 mM NH_4HCO_3 as the SL, and 20 mM NH_4HCO_3 as the separation buffer. Single cell quantitative analyses were made possible due to the high sensitivity of detection and stability of the system's performance. Utilization of a simple on-line preconcentration method and large-volume sample stacking enabled the characterization of anionic metabolites in cells on the scale of larger mammalian neurons. The CE-nanospray-MS method presented here is an effective starting point for further advancements in the analysis of anionic metabolites in single cells, which is complementary to single cell analysis of cationic metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NH_4HCO_3 as the electrolyte for both the BGE (20 mM) and SL (0.2 mM).

Fig. 1.

The influence of SL composition on the relative intensities of (A) GMP, (B) CMP, (C) UMP and (D) AMP signals acquired with direct MS ($n = 3$). Mixtures of four standards were prepared in 0.2 mM NH_4HCO_3 , 2 mM NH_4HCO_3 , 0.2 mM NH_4Ac , or 0.2 mM NH_4HCO_2 buffers, as well as 50% IPA (v/v) (labeled pure solvent) and infused at a constant rate of 0.6 $\mu\text{L}/\text{min}$ through a syringe pump.

Fig. 2.

Extracted ion electropherograms acquired from solutions containing 16 anionic analyte standards using the optimized CE-ESI-MS method. Injection volume, 10 nL; separation voltage, 10 kV. Analyzed compounds (each at 100 $\mu\text{g/L}$): 1–NAD⁺ (m/z 622.102); 2–cAMP (m/z 328.045); 3–FAD (m/z 784.150); 4–AMP (m/z 346.056); 5–CMP (m/z 322.045); 6–NADP⁺ (m/z 742.068); 7–GMP (m/z 362.058); 8–UMP (m/z 323.029); 9–ADP (m/z 426.022); 10–GDP (m/z 442.017); 11–CDP (m/z 402.011); 12–ATP (m/z 505.989); 13–GTP (m/z 521.983); 14–UDP (m/z 402.995); 15–CTP (m/z 481.977); and 16–UTP (m/z 482.961).

Fig. 3.

Scheme for analyte preconcentration using large-volume sample stacking on-column and the resulting CE-nanospray-MS analysis of individual *Aplysia californica* sensory neurons. (A) Large-volume sample injection, (B) reverse voltage-applied stacking, and (C) positive voltage-applied separation. (D) Analyte standards at 500 ng/L. Extracted ion electropherograms from (E) AMP (m/z 346.056), (F) ADP (m/z 426.022), (G) GDP (m/z 442.017), (H) ATP (m/z 505.989), and (I) GTP (m/z 521.983).

Table 1

Optimization of organic solvent types and percentages.

Organic solvent percentages in SL	Methanol		Acetonitrile		Isopropanol	
	Separation ^a	Spray ^b	Separation ^a	Spray ^b	Separation ^a	Spray ^b
100%	++	+				
85%	++	+				
70%	++	–	++	–	++	++
50%	++	–	++	–	++	++

^aSeparation refers to the performance in CE separation;

^bSpray refers to the capabilities of the solutions to form cone-jet sprays. Organic solvent content in the SL is shown in percent of total volume. 20 mM NH₄Ac was used as the BGE and the SL flow rate was held constant at 0.6 µL/min. The symbols ‘++’, ‘+’ and ‘–’ correspond to ‘best’, ‘acceptable’ and ‘bad’, respectively. The effectiveness of the crossed-out cells was not determined.

Table 2

Linearity, reproducibility and sensitivity of the CE-ESI-MS method for 16 anionic analytes.

Compound	Calibration Curve ($\log(A)=s*\log(C)+B$)		Coefficient of determination	RSDs (%) (n=3)	LOD	LOQ
	Slope (s)	Intercept (B)	R ²	Peak Area	nmol/L	nmol/L
CMP	0.874	3.700	0.989	0.8	9.4	37.4
UMP	0.875	3.746	0.998	4.2	11.9	47.0
cAMP	0.919	3.952	0.984	8.6	5.3	19.6
AMP	0.924	3.558	0.986	1.0	2.2	8.0
GMP	0.903	3.709	0.981	1.6	3.2	12.3
CDP	0.873	3.661	0.991	13.6	7.1	28.1
UDP	0.747	3.984	0.986	4.8	3.5	17.5
ADP	0.818	3.590	0.989	8.4	8.9	38.6
GDP	0.894	3.559	0.994	12.9	9.1	35.1
CTP	0.844	3.381	0.999	5.6	19.9	83.0
UTP	0.785	3.543	0.996	14.8	16.0	74.2
ATP	0.908	3.265	0.994	1.3	6.9	25.9
GTP	1.097	2.757	0.993	5.1	21.2	63.4
NAD ⁺	1.305	2.242	0.977	14.6	10.9	27.3
NADP ⁺	1.357	1.959	0.987	7.8	18.2	44.3
FAD	1.200	2.114	0.980	13.7	10.0	27.3

Table 3

Anionic metabolites detected in individual R2 neurons of the *A. californica* central nervous system (CNS).

Chemicals	R2 neuron		
	Amount (pmol ^a)	RSD (%) (n=3)	Energy Charge
CMP	2.4 ± 0.4	15	$\frac{[\text{ATP}] + 0.5 \times [\text{ADP}]}{[\text{AMP}] + [\text{ADP}] + [\text{ATP}]} = 0.81$
UMP	2.5 ± 0.3	12	
cAMP	0.5 ± 0.1	17	
AMP	8.6 ± 0.9	10	
GMP	3.7 ± 0.7	20	
CDP	4.6 ± 0.8	17	
UDP	6.3 ± 1.0	17	
ADP	24 ± 2.6	11	
GDP	13 ± 1.8	14	
CTP	0.6 ± 0.1	20	
UTP	0.2 ± 0.04	20	
ATP	74 ± 1.2	8	
GTP	17 ± 2.2	13	
NAD ⁺	3.9 ± 0.7	19	
NADP ⁺	1.4 ± 0.2	16	
FAD	not detected	-	

All data points are average values of three replicates (peak area RSDs <=20%).

^aWe calculated the volume of the R2 neuron assuming a 300-μm diameter and a spherical cell, a reasonable representation of the R2 neuron cell bodies investigated in this study.