Qualitative and quantitative metabolomic investigation of single neurons by capillary electrophoresis electrospray ionization mass spectrometry

Peter Nemes, Stanislav S. Rubakhin, Jordan T. Aerts, and Jonathan V. Sweedler

SUPPLEMENTARY METHODS

This supplement describes three methods that are useful for achieving successful experimental results using the presented protocol. The following methods help to improve the performance of ion generation, separation, and data analysis:

1. Stable ion generation

Stable and reproducible system performance is a prerequisite for successful single-cell CE-ESI-MS experiments. In a typical operation, the performance of the platform is characterized continuously through multiple channels of observation. The temporal pattern of the total ion current and spray current (capillary current) are monitored using the mass spectrometer; the electrohydrodynamic behavior of the electrospray (ES) liquid meniscus is visually observed using a microscope (**Figure 1**). Representative total ion currents and spray currents are shown for erroneous and ideal conditions for the ESI-MS and CE-ESI-MS operational modes of the CE-ESI interface in **Figure S1**. The CE-ESI-MS system, when optimized, provides reproducible and stable CE separation and efficient ion generation over an extended period of time, as demonstrated in previous studies.[1-4]

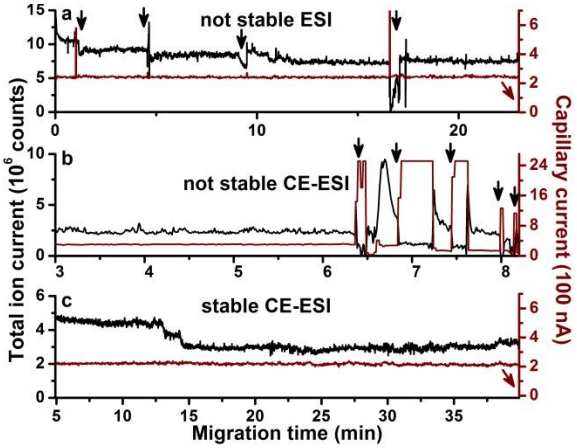

Figure S1. Troubleshooting the CE-ESI interface for robust operation. **(a)** In the ESI-only operation mode, current fluctuation (arrows in black) in combination with microscopic imaging revealed that the Taylor-cone was not anchored and was unstable on a rounded emitter tip. **(b)** In the CE-ESI operation mode, current spikes in the µA-level suggested electric breakdown (arrows in black). **(c)** Careful optimization of the experimental variables yielded reproducible CE separation and stable ion generation of high efficiency.

2. Reproducible separation

Automating the application of the CE high-voltage enhances separation reproducibility. A custom-designed system capable of remotely controlling the CE HVPS is presented in **Figure S2**. This supplement also describes operation of a custom-designed LabView-based virtual instrument, Supplementary Sequence Archive.vi (*vi*), developed in our experiments to output a time-dependent low-voltage function using a multichannel data acquisition and control (DAQ) card (model USB-6008, National Instruments). The *vi* is provided as a compressed archive file (Supplementary Sequence Archive.zip) in the Supplementary Note. The *vi*-DAQ-generated signal is directly fed into the remotely-operated CE HVPS (Bertan model 30R, Spellman High Voltage Electronics Corp.) that converts it to 20 kV. This output voltage is applied across the CE capillary to drive electrophoretic separation. Further details of the setup are presented elsewhere.[2]

Assembling the required hardware and software elements is a multistep procedure as depicted in **Figure S2** and instructed herein (see labels in **Figure S2**):

1. Install software packages on a personal computer (PC): LabView 8.5 or later (National Instruments), LabView drivers NI-DAQmx 9.5.1 or later (National Instruments), and the enclosed *vi* (label 6).
2. Connect the DAQ card to the PC.
3. Using the PC, configure the DAQ card with analog output on channel AO1 (label 4) according to the vendor's instructions.
4. Test the *vi* as follows:
   4a. Execute the *vi* (Operate/Run or "Run Continuously" commands).
   4b. Turn the CE RUN ENABELD toggle switch to ON (label 7).
   4c. Click on START RAMP (label 8).
   4d. Using a digital voltmeter, measure the output on DAQ pins GRN and AO1; the output should read stable ~3.34 V (DC). **! CAUTION** It is important to ensure proper output voltage as it will be converted to high voltage by the CE HVPS (Step 8). To adjust this low-voltage output, inspect connections, DAQ card installation, and *vi* settings (Step 10)
5. Turn off the voltage program by setting the CE RUN ENABLED toggle switch to OFF; this will gradually decrease the output voltage to ground. Use the digital multimeter to confirm 0 V between DAQ pins A01 and GND.

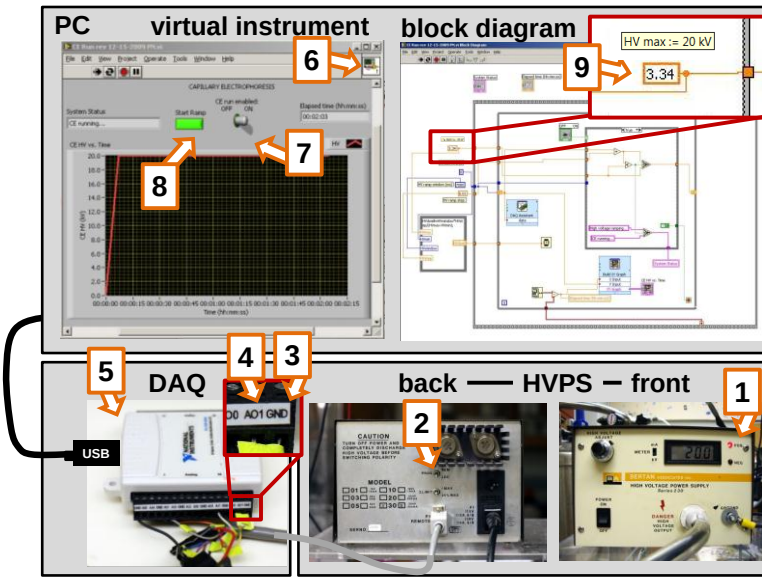

Figure S2. Setup to automate CE separation consisting of (1) a Bertan model 30R HVPS operated in (2) remote-enabled mode and connected via pins #7 and #6 to (3) GRN and (4) AO1 of (5) a USB-6008 DAQ card, respectively, controlled through a PC executing (6) our custom-written *vi*. To start separation, (7) the safety switch is engaged and (8) the run button is executed. Although this setup is designed to generate 20 kV, it can be readily modified to establish different voltage output and/or voltage functions; e.g., (9) the conversion factor from low-voltage to high-voltage may be adjusted in the *vi*.

6. Disconnect the digital multimeter, and connect pins GRN and AO1 of the DAQ to pins #7 and #6 of the HVPS, respectively.
7. Set the CE HVPS for remote access by switching the PROG toggle switch from LOC to REM on the back of the HVPS (label 2).
8. Run the *vi* by repeating steps 4a to 4c; **! CAUTION** This step generates high voltage. The HVPS output cable must be shielded from access.
9. Monitor the high voltage output on the front panel of the HVPS.
10. (Optional) Adjust the following *vi* variables to tailor the CE voltage function for a particular application:
    8a. "HV max" value (label 9) to obtain voltage output with alternate maximum.
    8b. "HP ramp window" and "HV ramp step" to modify the ramp duration of the generated voltage.
11. Stop CE separation by disengaging the CE RUN ENABLED toggle switch to OFF; this will gradually decrease the HV to ground (earth).

! CAUTION The high voltage output generated by this *vi* is not regulated. All parts of the setup should be shielded or grounded, if applicable. Post warning signs to alert of the hazard.

3. Semi-automated data analysis

Automating select steps of data analysis facilitates metabolic profiling. In this protocol, mass spectra are mass-calibrated to less than 5 ppm accuracy or better in Compass (Bruker Daltonics) and our custom-designed script (**Figure S3**) is executed to automatically generate selected-ion electropherograms for metabolites of interest

with ±5 mDa selection window. This script also smooths the resulting electropherograms via a three-point Gaussian function in preparation for peak integration. Representative metabolites and their accurate masses and typical migration types are collated in **Table S1**. Further electrophoretic reference data on more than 35 metabolites are published elsewhere[2].

Depending on the purpose of the study, the provided script may be modified to any compounds of interest by updating the accurate mass list.

Table S1. Representative metabolite identifications for automated data analysis.

Compound		Migration time (min)	*m/z* theoretical*
Name	**Formula**		
lysine	$C_6H_{14}N_2O_2$ (H^+)	14.19	147.1128
acetylcholine	$C_7H_{16}NO_2$ (+)	14.77	146.1176
histidine	$C_6H_9N_3O_2$ (H^+)	15.08	156.0765
carnitine	$C_7H_{15}NO_3$ (H^+)	17.17	162.1125
serotonin	$C_{10}H_{12}N_2O$ (H^+)	17.52	177.1022
glycine	$C_2H_5NO_2$ (H^+)	19.42	76.0393
adenosine	$C_{10}H_{13}N_5O_4$ (H^+)	20.74	268.1040
HEPES**	$C_8H_{18}N_2O_4S$ (H^+)	21.08	239.1060
valine	$C_5H_{11}NO_2$ (H^+)	24.92	118.0863
isoleucine	$C_6H_{13}NO_2$ (H^+)	25.27	132.1019
leucine	$C_6H_{13}NO_2$ (H^+)	25.62	132.1019
glutamine	$C_5H_{10}N_2O_3$ (H^+)	28.08	147.0764
tyrosine	$C_9H_{11}NO_3$ (H^+)	29.62	182.0812
proline	$C_5H_9NO_2$ (H^+)	30.06	116.0706
glycine betaine	$C_5H_{11}NO_2$ (H^+)	32.75	118.0863
proline betaine	$C_7H_{13}NO_2$ (H^+)	33.55	144.1019

*Monoisotopic accurate masses were calculated with Compass IsotopePattern (Bruker Daltonics). **HEPES is added as an internal standard and migration time marker.

```
Option Explicit
Dim i
Dim vaMyArray
vaMyArray = Array(147.1128, 146.1176, 156.0765, 162.1125,
177.1022, 76.0393, 268.1040, 239.1060, 118.0863, 132.1019,
147.0764, 182.0812, 116.0706, 118.0863, 144.1019)

Dim nArraySize
nArraySize = UBound(vaMyArray) - LBound(vaMyArray)
ReDim vaEICDefinitions(nArraySize)

For i = LBound(vaMyArray) To UBound(vaMyArray)
 Call AddEICDefinition(i, CStr(vaMyArray(i)))
Next

Analysis.Chromatograms.AddChromatograms(vaEICDefinitions)
Analysis.Chromatograms.Smooth

Form.close

Sub AddEICDefinition(i, sRange)
 Dim EIC
 Set EIC = CreateObject("DataAnalysis.EICChromatogramDefinition")
 EIC.MSFilter.Type = daMSFilterMS
 EIC.ScanMode = daScanModeFullScan
 EIC.Polarity = daPositive
 'EIC.BackgroundType = daBgrdTypeConstant
 EIC.WidthLeft = "0.005"
 EIC.WidthRight = "0.005"
 EIC.Range = sRange

 Set vaEICDefinitions(i) = EIC
End Sub
```

Figure S3. Script for semi-automating data analysis. This Compass script generates smoothed selected-ion electropherograms for the metabolite signals listed in **Table S1**. The accurate molecular mass list (see highlight in dark gray) can be modified depending on the purpose of the study.

References

1 Lapainis, T., Rubakhin, S. S. & Sweedler, J. V. Capillary electrophoresis with electrospray ionization mass spectrometric detection for single-cell metabolomics. *Anal. Chem.* **81**, 5858-5864 (2009).

2 Nemes, P., Knolhoff, A. M., Rubakhin, S. S. & Sweedler, J. V. Metabolic differentiation of neuronal phenotypes by single-cell capillary electrophoresis-electrospray ionization-mass spectrometry. *Anal. Chem.* **83**, 6810-6817 (2011).

3 Knolhoff, A. M., Nemes, P., Rubakhin, S. S. & Sweedler, J. V. in *Methodologies for metabolomics: Experimental strategies and techniques* (eds Norbert Lutz, Jonathan V. Sweedler, & Ron A. Wevers) (Cambridge University Press, 2013, in press).

4 Nemes, P., Knolhoff, A. M., Rubakhin, S. S. & Sweedler, J. V. Single-cell metabolomics: changes in the metabolome of freshly isolated and cultured neurons. *ACS Chem. Neurosci.* **3**, 782-792 (2012).