Supporting Information

Single-Photon-Induced Post-Ionization to Boost Ion Yields in MALDI Mass Spectrometry Imaging

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# Supporting Information

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Experimental Section

Chemicals. Lipid standards 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (PS(16:0/18:1)) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-phosphocholine (sodium salt) (PC(16:0/18:1)) were from Avanti Polar Lipids (Alabaster, AL), deuterated methanol (MeOD; 99% D) was from Acros Organics (Geel, Belgium). All other chemicals were purchased from Merck/Sigma-Aldrich (Steinheim, Germany).

Tissues. Mouse brain and kidney were surplus tissue dissected from 10–15-week-old female C57BL6/J mice from the work group of Prof. Dr. Nicholas Schwab (Department of Neurology, University of Muenster). Breeding and killing of animals for the purpose of scientific use was carried out with approval of the competent authorities (LANUV TVN 84-02.05.50.17.019 + 84-02.05.20.13.09) and of the ethics council of the University of Münster. Whole organs were embedded in 2-hydroxyethylcellulose polymer (M_{avg} ~1,500,000 g/mol) and then snap-frozen in liquid N\(_2\). Pig brain was from a local butchery and bulk homogenate was prepared as described. [S1] Briefly, brain tissue was snap-frozen in liquid N\(_2\) and 7 g of homogenized tissue was mixed with 3 g of aqueous 2 hydroxyethylcellulose polymer solution. Tissue sections of 10 µm thickness (mouse) or 20 µm thickness (pig brain homogenate) were produced with a cryotome (Jung Frigocut 2800E, Leica Biosystems, Jena, Germany) and thaw-mounted on histological glass slides (SuperFrost, Fisher Scientific, Schwerte, Germany).

Lipid standards were prepared to 1 µmol/L in 70% acetonitrile and evenly coated onto histological glass slides using an ultrasonic sprayer (SimCoat, Sono-Tek, Milton, NY). 400 µL of standard solution were sprayed at a flow rate of 0.03 mL/min in meandering patterns at a distance of 1.8 mm between the lines. Tissue sections and slides with standards were stored at −78 °C until further use.

Deuterated matrix was synthesized by dissolving 0.2 g of DHAP in 30 mL of a mixture of deuterated methanol (MeOD) and acetone-d\(_6\) (50:50, v:v), followed by removing the solvents in a rotary evaporator (VV2011, Heidolph Instruments, Schwabach, Germany). To ensure a close to complete degree of deuterium exchange, the process was repeated three times so that in the later mass spectrometric analysis no protonated signal of the matrix was detected. The crystalline deuterated matrix was stored at -20 °C in a sealed glass flask until further use. During synthesis and storage, the deuterated matrix was kept under dry argon.

After removal from the freezer, tissue sections and standards were thawed under a stream of nitrogen before coating with the MALDI matrix in a home-build matrix sublimation chamber. [S2] 1.2 mL of matrix solution (DHAP: 7 mg/mL in 70% acetonitrile (ACN); deuterated DHAP: 7 mg/mL in MeOD/acetone-d\(_6\) (50:50, v:v) were filled into the matrix reservoir of the sublimation chamber and the reservoir was heated to 135 °C. The sample slide was mounted onto a cooling socket at about 3.5 °C. The sublimation chamber was evacuated to approximately 10\(^{-4}\) mbar. To stop the sublimation process, the chamber was flushed with nitrogen. [S2] MALDI samples were recrystallized for 2.5 min at 70 °C in an atmosphere containing 0.5% ethanol in H\(_2\)O.
Mass spectrometer. A Q Exactive Plus Orbitrap (Thermo Fisher Scientific, Bremen, Germany), coupled with a dual-ion funnel/dual MALDI/ESI Injector (Spectroglyph, Kennewick, WA), was used as the mass analyzer. The ion source has previously been modified to enable laser-based MALDI-2. A modified version was also used in our initial LPPI work with (s)VOCs.

A frequency-tripled q-switched Nd:YLF laser (Explorer One, Spectra-Physics, Mountain View, CA; emission wavelength: 349 nm; pulse width: 7-10 ns; pulse repetition rate $f_{rep}$, adjustable from 1 Hz up to a maximum of 5 kHz) was used as the MALDI laser and for material ablation in the MALDI-SPICI experiments. In the presented spectra, the ablation laser was operated with a repetition rate of 300 Hz and a resulting pulse width of about 10 ns.

The default lens that is used in the Spectroglyph ion source to focus the laser beam on the target was replaced by two CaF$_2$ planoconvex lenses (Thorlabs, Dachau, Germany) with focal lengths of 300 mm and 1000 mm. By this replacement, the combined focal length of the optical system fits the requirements of the source more precisely and enables the production of an effective spot size of ~9 µm (defined as the area of visible material ejection). The laser focus was kept constant at this setting for all shown measurements.

To reduce background ion signal levels, throughout our experiments the ESI inlet of the MALDI/ESI Injector was blocked with a polytetrafluoroethylene (PTFE) plug. A fine-needle valve (SS-1RS6MM, 0.37 Cv, Swagelok, Düsseldorf, Germany) was used to adjust the N$_2$ buffer gas pressure in a range of 4-12 mbar in the region of the primary ion funnel. Optionally, dopant vapor from the headspace of a sealed reservoir flask was introduced into the ionization chamber via a PTFE tubing (outer diameter, 3/8") and Swagelok® fittings. A second needle valve (SS-ORS3MM, 0.09 Cv, Swagelok, Düsseldorf, Germany) was used to manually control the gas flow of dopant into the funnel. For one experiment, another PTFE tubing connected a D$_2$O chamber with the ion source in a similar manner. The D$_2$O chamber contained a glass vial filled with D$_2$O. The chamber was evacuated, closed by another needle valve, and the vial was broken inside the closed chamber. The pure D$_2$O vapor was introduced at controlled amounts through the valve. The determination of the partial pressure of the introduced gases was based on an approximation by monitoring the Pirani pressure gauge of the Spectroglyph system.

During the experiments, three different mass resolving powers of the Orbitrap of $\text{Res}_m = 70,000, 140,000, \text{ and } 280,000$ (each defined for an $m/z$ value of 200) were used. The “injection time” was set to a fixed value of 250, 500, and 900 ms for the three resolution values, respectively, resulting in data acquisition rates of 3.7, 1.9, and 0.97 pixels per second. The “AGC target” was disabled.

For higher-collision induced dissociation (HCD) tandem MS measurements, the analytical quadrupole was set to an isolation window of 0.8 Da and the collision energy (CE) was varied between 12 and 30 eV (laboratory frame). For data-dependent acquisition (DDA) of a coronal brain section, the “dynamic exclusion” was set to 9 s (for brain homogenate to 45 s) and the ACG threshold to $2 \times 10^4$. An “exclusion list” was generated prior to each experimental run with background signals of a blank spectrum acquired at equilibrated measurement conditions.

VUV module. The principle layout of the VUV SPI module has been described in detail previously. A schematic of the design with measures is plotted in Figure S1, a condensed sketch in Figure 1 (main text). Briefly, three RF-
Kr discharge lamps (model PKR-106-6-14, Heraeus Noblelight, Hanau, Germany) were integrated into a custom-designed annular mount made of PEEK. The small size of the cylindrically shaped lamps with outer dimensions of 6 × 14 mm (diameter × length) allowed for positioning the lamps with optimized geometries in order to minimize perturbation of the electrical field in the central part of the ion funnel device. The distance of the emission side of the lamps to the MALDI sample was about 1 mm and the distance to the central axis of the ion funnel was ~5 mm. Adhesive copper band served to contact the lamps. The position of the electric contacts on the three lamp bodies – especially the distance between the two electrodes – needed to be essentially similar for all lamps in order to obtain homogeneous response characteristics upon pulsed electric excitation. A symmetric ring electrode connected the front-end (emission side) of the lamps serving as ground for excitation and ion extraction.

A custom-made class E amplifier [S5] operated the lamps via the copper electrodes at the back-end with an alternating current (AC) at 13.560 MHz and a peak-to-peak voltage of 220 V. A metal-oxide–semiconductor field-effect transistor (MOSFET; model IXZ631DF18N50, IXYS, Milpitas, CA) was triggered by an RF generator (TG2000 DDS function generator 20 MHz, Aim TTi, Huntingdon, UK) and induced a resonance circuit, which operated the lamps. Importantly, all electronic components were mounted outside the vacuum to minimize the effect of stray fields on the ion funnel. A direct current (DC) power supply (BA-315, Bertan Associates Inc., Syosset, NY) was used to supply the symmetric ground electrode at the lamp’s front-end with a variable DC voltage of 300-500 V.

As an important modification to our previous setup, [S5] the RF generator was gated by a rectangular wave function generated by the internal pulse generator of an oscilloscope (InfiniiVision DSOX3032A, 350 MHz, 4GSa/s; Keysight Technologies, Santa Rosa, CA). The repetition rate was variable between 10 to 5000 Hz. This enabled the generation of pulsed VUV light with electric pulse widths between 70 µs and the continuous wave (cw) mode without modification of the in-source hardware; the minimum pulse width was found to ignite the lamps. The function generator also served for synchronization of ablation and VUV pulses with variable repetition rates up to 5 kHz; the latter corresponds to the maximum pulse repetition rate of the laser; we note, the VUV lamps themselves could be operated with a frequency up to the continuous wave mode. A custom-made delay generator served for adjusting the delay between the actively q-switched Nd:YLF laser and the VUV pulses.

Another key feature for achieving constant signal intensities is the symmetric design of the central ring electrode (see Figure S1 for details), which connects the VUV lamp’s tips. By applying a constant DC voltage to the part of the lamps facing the plume, the electrodynamic field of the ion funnel device is affected. With a controlled symmetric “offset” being added, reproducible ion trajectories can effectively be realized. Generally, the applied DC voltage $U_{\text{extr}}$ as well as the total gas pressure $P_{\text{total}}$ should be kept as constant as possible to ensure controlled and reproducible ion trajectories and thus to avoid variations in the ion counts or ion profiles. As shown in Figure 2d (main text), a slight variation of $U_{\text{extr}}$ and $P_{\text{total}}$ has only little effect on the observed ion counts and thus the exact optimization of these values plays only a minor role in the successful MALDI-SPICI experiment.

MALDI-2 was performed for comparison measurements. The laser-induced postionization was achieved with a frequency-quadrupled (266 nm) Nd:YAG laser (PL2231-100-SH/THPRETRIG, Ekspla, Vilnius, Lithuania) as described before. [S4] The laser beam of the PI laser was focused to a beam waist of ~50-100 µm in diameter and
about 400 μm above the sample surface to intersect with the MALDI plume. The delay between primary, actively Q-switched ablation laser and mode-locked PI laser pulses was set to 10 μs by an internal pre-trigger signal of the PI laser system. In the MALDI-2 experiments both lasers were operated at the maximum pulse repetition rate of the PI laser of 100 Hz.

Data analysis. Xcalibur (vs. 4.0, Thermo Fisher Scientific) was used for the initial data examination and optionally for off-line recalibration of mass spectra. MSI data sets were converted to imzML-files by using ProteoWizard software [56] and uploaded to LipostarMSI (vs. 1.1; Molecular Horizon, Bettona, Perugia, Italy) for further graphical analyses. Throughout this work all full scan ion images are presented using the viridis false-color scheme. The mass traces in the full scans of the DDA images were visualized in the rainbow false-color scheme and extrapolated to a five-pixel wide block by manually copying the information of signal intensities from the full scan pixels to the subsequent four pixels in the MSI picture file. MS/MS data were automatically annotated in LipostarMSI based on the DB manager – a complementary tool to LipoStar used to generate tandem MS data lists based on proprietary fragmentation rules. [57] Data sets of brain homogenate were uploaded to MZmine 2 software [58] for signal annotation of aligned lists with the tool "lipid search" and the integrated theoretical database [59]. Lipid identification was furthermore conducted via the online classification system of LIPID MAPS® [510] and by comparison with literature data. [S11-S15] For comparison of spectra, aligned data lists were uploaded to the MetaboAnalyst platform (http://www.metaboanalyst.ca). [S16] For the case of comparing MALDI-SPICI-MS with MALDI-2-MS data, the signal intensities were “normalized to the sum” for each m/z signal. Fold change and volcano plots were generated from these cured lists. All assignments are based upon using a mass tolerance of 1.5 ppm for R = 280,000 and 2 ppm for the lower mass resolution settings and were in several cases further confirmed by DDA.

Statistics and reproducibility. We recorded multiple measurements of different sample sections in both positive and negative ion mode within a period of about 12 months and could achieve a high degree of reproducibility among the different measurements.

MS measurements from brain homogenate in both ion modes were conducted with at least three independently prepared samples, which were yielding comparable results; e.g. a relative standard deviation (RSD) of ~20 % in the intensities of annotated signals between measurements within 6 months and an according RSD <3.5 % within one set of measurements. For MSI experiments from brain tissue, three tissue sections from three different animals were analyzed in the positive ion mode yielding comparable results. Kidney was taken from one animal, only. Thus, in this case, only technical replicates were produced, however, again yielding very comparable results. Lipid annotation was performed from averaged mass spectra of at least 10,000 pixels of the respective tissue sample and processed further as described above.

Data availability. The data that support the findings of this study, in particular all MSI data as presented in the main text, are available to download in the vendor-neutral imzML format from the corresponding author upon request.
Supporting Results

Figure S1: Schematic of the VUV module mounted into the higher-pressure ion funnel of the Spectroglyph MALDI/ESI Injector. The modular setup can easily be dissembled. Due to the symmetric ring electrodes, several modes of ionization such as regular MALDI, ESI or LPPI can be conducted next to MALDI-SPICI-MSI with different dopants without hardware modification.
Figure S2: Comparison of MALDI-SPICI with MALDI-2. **Left:** Mass spectra recorded of porcine brain homogenate in both PI modes. **Right:** Volcano plot displaying the 500 signals with highest intensity of MALDI-SPICI against MALDI-2. Data were acquired with a mass resolving power Res_m of 280,000 in the positive ion mode and the spectra averaged over 300 scans. Signals labeled in blue are detected with significantly higher intensity in SPICI, signals labeled in red show significantly higher intensity using MALDI2. Annotations are based on accurate mass and on literature data. The two different MALDI-PI methods were conducted on the same instrument on the same tissue section. However, due to the current geometries of the VUV prototype PI source, the laser pathway had to be slightly altered and the laser refocused. For this reason, a quantitative comparison between MALDI-2 and MALDI-SPICI is limited. Exemplarily annotated signals are based on accurate mass and on literature data: ▼ = PC, ▼ = Alkylacyl-PC, ▲ = PE, △ = Alkylacyl-PE, ● = PS, ▼ = PI, ▴ = HexCer;O2, ◆ = HexCer;O3, ■ = SM, for tentative [M+H]^+ species.

Figure S3: MALDI-SPICI-MS of porcine brain homogenate acquired with use of different dopants. Data were acquired with a mass resolving power Res_m of 280,000 in the positive ion mode and the spectra averaged over 300 scans. Mass spectra are shown only in a small excerpt. Exemplarily annotated signals for tentative [M+H]^+ are based on accurate mass and on literature data: ▼ = PC, ▼ = Alkylacyl-PC, ▲ = PE, △ = Alkylacyl-PE, ● = PS, ▼ = PI, ▴ = HexCer;O2, ◆ = HexCer;O3, ■ = SM; x = chemical background.
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Figure S4: LPPI mass spectrum obtained of the background in the positive ion mode with acetone as a dopant. The data was acquired with a mass resolving power Res\textsubscript{m} of 280,000 and by averaging 300 scans. Acetone was introduced at a low level of P\textsubscript{partial} = 0.08 mbar. Signal annotation by the exact mass based on \([S17]\).

Figure S5: MALDI-SPICI-MSI of a mouse kidney section recorded in the positive ion mode. The data were acquired at a pixel size of 16 µm with a mass resolving power Res\textsubscript{m} of 70,000 and over a total measurement time of 20 h. Tentative annotations are based on accurate mass and literature data; Cf. Table S2 for further signal annotation. Ion images are displayed without normalization. L = lyso-form (mono-acyl) of the according GPL, PI = phosphatidylinositol, CL = Cardiolipin.
Figure S6: MALDI-SPICI mass spectrum of porcine brain homogenate recorded in the negative ion mode. Data were acquired with a mass resolving power $R_{m}$ of 280,000 and the spectra averaged over 300 scans. Annotations are based on accurate mass, on literature data, and on DDA experiments. ST = (3'-sulfo)Hex-Ceramide.
Figure S7: Zoom-in spectrum of semi-volatile free fatty acids recorded in a (--)MALDI-SPICI-MS experiment of pig brain homogenate. Data were acquired with a mass resolving power $R_{m}$ of 280,000 and the spectra averaged over 300 scans. Annotations are based on accurate mass and on literature data. The recorded fatty acids are possibly the product of fragmentation processes, e.g., the loss of the corresponding acyl chains from a precursor phospholipid.

Figure S8: MALDI-SPICI-MSI of a coronal section of mouse brain recorded in the negative ion mode. The data were acquired at a pixel size of 20 µm with a mass resolving power $R_{m}$ of 70,000, and over a total measurement time of 10 h. Ion images are displayed without normalization. Annotations are based on accurate mass, on literature data, and on DDA experiments; cf. Table S1 for further signal annotation. cLPA = cyclic lyso-phosphatidic acid, PG = phosphatidylglycerol, GM1 = monosialotetrahexosylganglioside.
Figure S9: Investigation on tissue degradation and signal alteration of a freshly prepared sample of porcine brain homogenate. **Left**: Mass spectra: Upper spectra show MALDI mass spectra before and after 30 min of tissue irradiation with the pulsed VUV lamps, lower spectra show MALDI-SPICI mass spectra at the beginning and after 30 minutes of constant tissue illumination. **Right**: Fold change plots displaying the 300 signals with highest intensity of early against late spectra of regular MALDI (top) respective MALDI-SPICI (bottom). Data were acquired with a mass resolving power $R_{\text{m}}$ of 280,000 and a laser/VUV pulse repetition rate of 300 Hz.

Figure S10: LPPI mass spectra of acetone and acetone-$d_6$. Spectra show averages of 60 pixels. The headspace of the dopant reservoirs were introduced at a relative gas-phase pressure $P_{\text{partial}}$(acetone, acetone-$d_6$) of 0.16 mbar at an total in-source pressure $P_{\text{total}}$ of 8.5 mbar.
Figure S11: Deuteration and fragmentation experiments with PC(16:0/18:1) to reveal details of the ionization pathways. Spectra show averages of 60 pixels. a1,a2: Mass spectra obtained with regular MALDI-MS and MALDI-SPICI-MS, respectively, using the deuterated DHAP matrix; b1,b2: mass spectra obtained with regular MALDI-MS and MALDI-SPICI-MS, respectively, using a D₂O-enriched atmosphere; c1,c2: full scan mass spectra obtained with regular MALDI-MS and MALDI-SPICI-MS, respectively, without deuterated components; HG = amine head group (N(CH₃)₃), BG = background signal (polysiloxane).
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Author Contributions

C.B., K.D., and J.S. conceived the experiments; K.S. and J.S. acquired funding; C.B. and J.S. designed the VUV-based ion source; U.R. designed the class E amplifier to drive the VUV lamps; C.B., U.R., and J.S. adjusted the class E amplifier for pulsed use within the dual-ion funnel injector; C.B. conducted the experiments and performed the data analysis; C.B., K.D., and J.S. wrote the manuscript.