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Supporting Information

Direct Mapping of Phospholipid Ferroptotic Death Signals in Cells and Tissues by Gas Cluster Ion Beam Secondary Ion Mass Spectrometry (GCIB-SIMS)

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Supporting information

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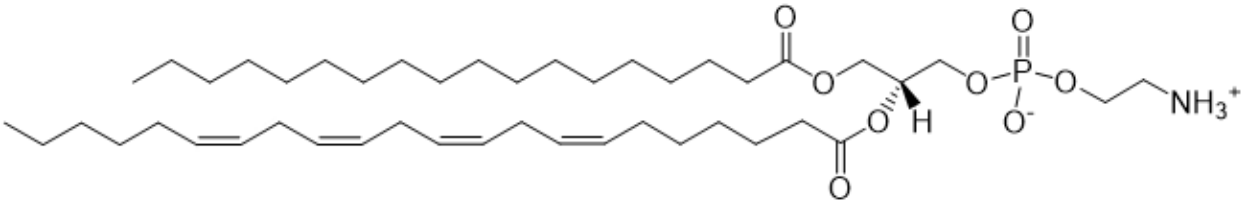
Lipid Nomenclature



Membrane phospholipids are composed of aliphatic chains (usually esterified fatty acids, as above), a central glycerolphosphate backbone, and a head group that faces the aqueous side of the membrane (e.g., ethanolamine, as in phosphatidylethanolamine, above). Shorthand notations such as PE(38:4) are noted by the headgroup abbreviation (PE for phosphatidylethanolamine) and the total number of fatty acid carbons (38) and double bonds (4). Oxidative modifications to the fatty acids (see scheme 1) are noted in shorthand such that "PE(38:4)-OH" and "PE(38:4)-OOH" are hydroxy- and hydroperoxy-modifications, respectively. Oxidatively modified chains (as part of a phospholipid or as a free fatty acid) are sometimes abbreviated as “HpETE” for a **h**ydro**p**eroxy**e**icosa**t**etra**e**noyl chain, FA(20:4)-OOH. See www.lipidmaps.org for the full lipid nomenclature system.

Oxidized phosphatidylethanolamine and fatty acid species mentioned in the text:

1-Stearoyl-2-15(S)-HpETE-sn-glycero-3-phosphoethanolamine = 1-Stearoyl-2-15(S)-Hydroperoxyeicosatetraenoyl-sn-glycero-3-phosphoethanolamine

1-Stearoyl-2-15(S)-HETE-sn-glycero-3-phosphoethanolamine = 1-Stearoyl-2-15(S)-Hydoxyeicosatetraenoyl-sn-glycero-3-phosphoethanolamine

1-Stearoyl-2-17(S)-HpDTE-sn-glycero-3-phosphoethanolamine = 1-Stearoyl-2-17(S)-Hydroperoxydocosatetraenoyl-sn-glycero-3-phosphoethanolamine

1-Stearoyl-2-17(S)-HDTE-sn-glycero-3-phosphoethanolamine = 1-Stearoyl-2-17(S)-Hydoxydocosatetraenoyl-sn-glycero-3-phosphoethanolamine

HpETE = hydroperoxyeicosatetraenoic acid

HETE = hydroxyeicosatetraenoic acid

HpDTE = hydroperoxydocosatetraenoic acid

HDTE = hydroxydocosatetraenoic acid

For species with both OH and OOH groups:

1-O-Stearoyl-2-5(S)-Hydroxy-15(S)-Hydroperoxyeicosatetraenoyl-sn-glycero-3-phosphoethanolamine

1-O-Stearoyl-2-5(S)-Hydroperoxy-15(S)-Hydroxyeicosatetraenoyl-sn-glycero-3-phosphoethanolamine

Experimental Methods

Reagents

LC-MS solvents (2-propanol, hexanes, methanol, water) were purchased from Fisher Scientific (Pittsburgh, PA, USA) and were all Optima-LCMS grade except for hexanes which were Optima™ for HPLC and GC grade. Ammonium formate (MS grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (Acros Omnisolv HPLC-Mass Spectrometry grade) and chloroform (HPLC grade with 0.75 % Ethanol) were also purchased from Fisher. RSL3 and Ferrostatin-1 were purchased from R&D Systems, Inc. and Cayman Chemical (Ann Arbor, MI, USA), respectively. Lipid standards of 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine, L-1-Stearoyl-2-15(S)-HpETE-sn-glycero-3-PE, and 1-Stearoyl-2-15(S)-HETE-sn-glycero-3-PE were purchased from Cayman Chemical.

Cell culture

Si wafers (5 x 5 x 0.5 mm^3, Agar Scientific Ltd, Stansted, UK) were cleaned by successive 5 min sonication in chloroform, water, and ethanol. The wafers were stored in the 70% ethanol until use for cell culture. The wafers were then dried in a cell culture hood before being placed into 6 well cell culture plates.

H9c2 cells (American Type Culture Collection (ATCC), part# ATCC CRL-1446) were pelleted and resuspended (2000 cells per ml) in media containing High glucose DMEM with L-Glutamine (ATCC part# 30-2002, lot# 80306190) with 10 % FBS (Fisher Scientific, Part# Gibco-A31606-01, Lot# 1992275) and 1 % Penicillin Streptomycin solution (Penn-Strep) also from Fisher Scientific (Part# Gibco-15140-122, Lot# 1333919). 1 ml of cells in media was added to each well (containing one Si wafer) and the cells were allowed to adhere overnight at 37 °C with 5 % CO_2. Once the confluence reached approximately 60%, the media was removed and replaced with 1 ml of either fresh media (control), media with 500 nM RSL3, or media with 500 nM RSL3 and 1 μM Fer-1. Additional H9c2 cultures were prepared under the same conditions for total lipid extraction and cell death

measurements. Cell death was determined by measuring released lactate dehydrogenase (LDH) activity. LDH activity was quantified using the Cytotoxicity Detection Kit (LDH) according to the manufacturer's instructions (Promega Corporation, Madison, WI). At the given time point, the wafers containing adhered H9c2 cells were removed for frozen-hydrated preparation for cold-stage GCIB-H2O-SIMS and the cultures for total lipid extraction were pelleted and frozen as previously shown[1]. Each wafer was briefly immersed once in PBS (Corning Cellgro) then twice in 0.15 M ammonium formate (Sigma-Aldrich). The wafers were plunge-frozen for 20 s in a bath of liquid ethane (UHP, Praxair) that was chilled by liquid N2 (LN_2), followed by transfer to a pre-chilled SIMS sample holder immersed in LN_2.

Animals and TBI Tissue Sections

All procedures were pre-approved and performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. TBI was induced by CCI to the left parietal cortex in two 17 day old male Sprague Dawley rats (Harlan, Indianapolis, IN, USA) with a pneumatically-driven 6 mm metal impactor tip (impact velocity 4.0 ± 0.2 m/s, penetration depth 2.5 mm) as described previously[2] . At 3 h post-injury the brains were harvested and frozen immediately in liquid nitrogen (LN_2) with neither fixation nor embedding, and stored at -80 ^{o}C until sectioning.

The CCI brain tissue was sectioned using a cryomicrotome (Leica CM 1950, US). Brain tissue was affixed to the cryotome block with minimal Tissue-Tek OCT (Sakura FineTek USA, Torrance CA, USA) to avoid contamination. The blade and working surfaces of the cryomicrotome were cleaned with 95 % ethanol and the blade did not come into contact with the OCT. Consecutive 6 µm coronal brain sections were cut at -21 ^{o}C and placed on ITO (Indium tin oxide) glass histology slides (Delta Technologies LTD) for GCIB-SIMS imaging with semi-serial sections on plain glass for subsequent total lipid extraction and LCMS. The section on the ITO slide for GCIB-SIMS imaging was immediately plunged into LN_2 and mounted onto a prechilled copper sample holder under LN_2, and followed by insertion onto the precooled sample stage in the SIMS instrument.

GCIB-SIMS Imaging

Instrumentation

SIMS imaging was performed on a quasi-DC beam buncher-ToF SIMS, J105 3D Chemical Imager [3]. The system was modified with a 70 keV GCIB that is capable of using a H_2O cluster ion beam and also is equipped with an LN_2 sample cooling stage. Due to the preferred ionization of our lipids of interest, all GCIB-SIMS scans were acquired in negative ion mode.

H9c2 Cells

Cell cultures under three conditions (no treatment, RSL3 treatment, or RSL3 and Fer-1 treatment) were subject to quick GCIB-SIMS depth profiling at a spatial resolution of 3.1 µm. The depth profiling was performed by continuous 2D imaging using 70 keV $(H_2O)_{30,000}^+$ at a primary ion dose of 1.1×10^{12} ions/cm^2. Ten imaging layers were acquired, each composed of 128×128 imaging locations (pixels) over a 400 µm ×400 µm area. Due to the low chemical damage of GCIB, the layers were able to be acquired via simultaneous image acquisition and sputtering, leaving no biomaterial wasted.

Higher spatial resolution imaging (1.2 µm) was conducted on cells which were either untreated or treated with RSL3 for 2.5 h to map the targeted lipid peroxides. Three layers of depth profiling were performed using 70 keV $(H_2O)_{30,000}^+$ at a primary ion dose of 1.2×10^{13} ions/cm^2 over 300×300 µm^2 with 256×256 pixels.

TBI Tissue sections.

A primary ion beam of 70 keV $(H_2O)_{29,000}^+$ was defocused to a larger pixel size of 6 µm in order to reduce analysis time and increase our possibility to directly image the low abundant PE/PEox species in the contusional cortex and the surrounding hippocampal and pericontusional cortical regions on the ipsilateral hemisphere. Corresponding regions of the contralateral cortex were also imaged under the same conditions. This resulted in two images of 4×4 mm^2 each. The images were produced by stitching 10 × 10 tiles of 2D SIMS scans (64 x 64 pixels each), each over a 400×400 µm^2 area with an ion dose of 4×10^{11} ions/cm^2.

PE lipid standards

In order to examine the extent of analyte fragmentation, lipid standards were spotted onto gold-coated Si wafers for cold stage H_2O-GCIB-SIMS using 70 keV $(H_2O)_{28000}^+$. Aliquots of 0.2 µL for PE(38:4), PE(38:4)OH and PE(38:4)OOH were added to pre-cleaned gold coated Si wafers and then plunged into the LN_2 bath. The samples were mounted onto the sample holder, which was also submerged in the LN_2, and transferred to the precooled sample preparation stage through the glove box (purged with N_2 gas) on the J105. The SIMS measurements were performed at 100 K and the ion dose for each spectrum acquisition was 6.55×10^{11} ions/cm^2. Five replicates were run for PE(38:4), four of PE(38:4)OH and three for PE(38:4)OOH.

IHC of Rat TBI Tissue sections

Tissue sections used for GCIB-SIMS were subsequently labeled with primary antibodies for the neuronal marker NeuN (Invitrogen cat#711054,1:200), and the appropriate secondary antibody from Jackson ImmunoResearch (a Cy3, goat anti-rabbit IgG antibody, cat#111-165-144), along with Hoescht dye (Sigma-Aldrich cat#B2883, 1 mg per 100 ml d$H_2$0). Sections were scanned using an automated, confocal microscope with a motorized stage (Nikon A1).

LCMS analysis of phospholipids for H9c2 cells and TBI tissue sections

Because GCIB SIMS MSI permits only comparative quantitative analysis of the analytes, we were further interested in performing more quantitatively accurate LC-MS analysis. To this end, we performed a Folch total lipid extraction[4] on H9c2 cells and semi-serial ipsilateral/contralateral brain tissue sections for subsequent LC-MS analysis as per our previous imaging and LC-MS work[1, 2]. Total lipid phosphorous were determined by the micro-determination method. Calibration curves (Fig S6C) were generated using mixtures of PE(18:0/20:4), PE(18:0/20:4)OH and PE(18:0/20:4)OOH (Cayman Chemical, Ann Arbor, MI). Total lipids were separated on a normal phase column (Luna Silica, 1.0 mm x 15 cm, 3 micron particle size). Solvent A: 2-Propanol/hexane/water (285/215/5); Solvent B: 2-Propanol/hexane/water (285/215/40). Both A and B solvents contained 10 mM

ammonium formate as a modifier. The gradient method was as follows: 0-3 min, 10-37 %B; 3-15 min, hold at 37 %B; 15-23 min, 37-100 %B; 23-75 min, hold at 100 %B; 75-76 min, 100-10 %B; 76-90 min, hold at 10 %B for equilibration. All gradients were linear and flow was maintained at 65 µl/min. The LC system was a Thermo Ultimate 3000 complete with a WPS-3000 autosampler. Column temperature was set at 35 ^{o}C. A mixture of each of the following deuterated (D) and non-deuterated internal standard compounds (Avanti Polar Lipids) was added to each sample for injection: PE(16:0/D31-18:1), PC(16:0/D31-18:1), PG(16:0/D31-18:1), PA(16:0/D31-18:1), PS(16:0/D31-18:1), PI(16:0/D31-18:1), SM(d18:1/12:0), CL(56:0). All species were detected as $[M-H]^-$ except for SM and PC which were detected as $[M+formate]^-$. 10 nmol of total lipid phosphorus was used for each sample injection with 1.0 µM internal standard in each injection.

MS and MS/MS analysis of PLs was performed on a Fusion Lumos Tribrid mass spectrometer (ThermoFisher, Inc. San Jose, CA) using Xcalibur 4.3 software. Analysis was in negative ion mode (profile) at a resolution of 120,000 for the full MS scan and 15,000 for the MS^2 scan in a data-dependent mode with an appropriate inclusion list for major phsospholipids. The scan range for MS analysis was 400-1800 m/z with a maximum injection time of 100 ms using 1 microscan and an AGC target of 2e5. A maximum injection time of 500 ms was used for MS2 (high energy collisional dissociation (HCD)) analysis with collision energy set to 24. An isolation window of 1.0 Da was set for the MS2 scans. Capillary spray voltage was set at 3.5 kV, and capillary temperature was 300 ^{o}C. Sheath and auxiliary gasses were set to 35 and 17, respectively. A second data-dependent scan was set as a priority 2 scan for all remaining masses not present on the inclusion list. These measurements not only afforded precise structural identification of the analytes of interest (by MS2 and MS3) but also permitted us to perform accurate quantitative estimates.

LCMS analysis of Fatty acids

Fatty acids were analyzed by LC-MS using a Dionex Ultimate™ 3000 HPLC system using a C18 column (Accliam PepMap RSLC, 300 µm 15 cm, Thermo Scientific) coupled on-line to a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA) using Xcalibur 4.3 software. Gradient solvents (A: Methanol (20

%) / Water (80 %) (v/v) and B: Methanol (90 %) / Water (10 %) (v/v). Both A and B solvents contained 5 mM ammonium acetate. The column was eluted at a flow rate of 12 µL/min using a linear gradient from 30 % solvent B to 95 % solvent B over 70 min, held at 95 %B from 70 to 80 min followed by a return to initial conditions by 83 min and re-equilibration for an additional 7 min. Spectra were acquired in negative ion mode. The scan range for MS analysis was 150-600 m/z with a maximum injection time of 100 ms using 1 microscan and a resolution of 140,000. An isolation window of 1.0 Da was set for the MS and MS2 scans with an inclusion list of 102 potential oxidized and non-oxidized fatty acyl products. Capillary spray voltage was set at 2.6 kV, and capillary temperature was 250 ^{o}C. The S-lens Rf level was set to 60.

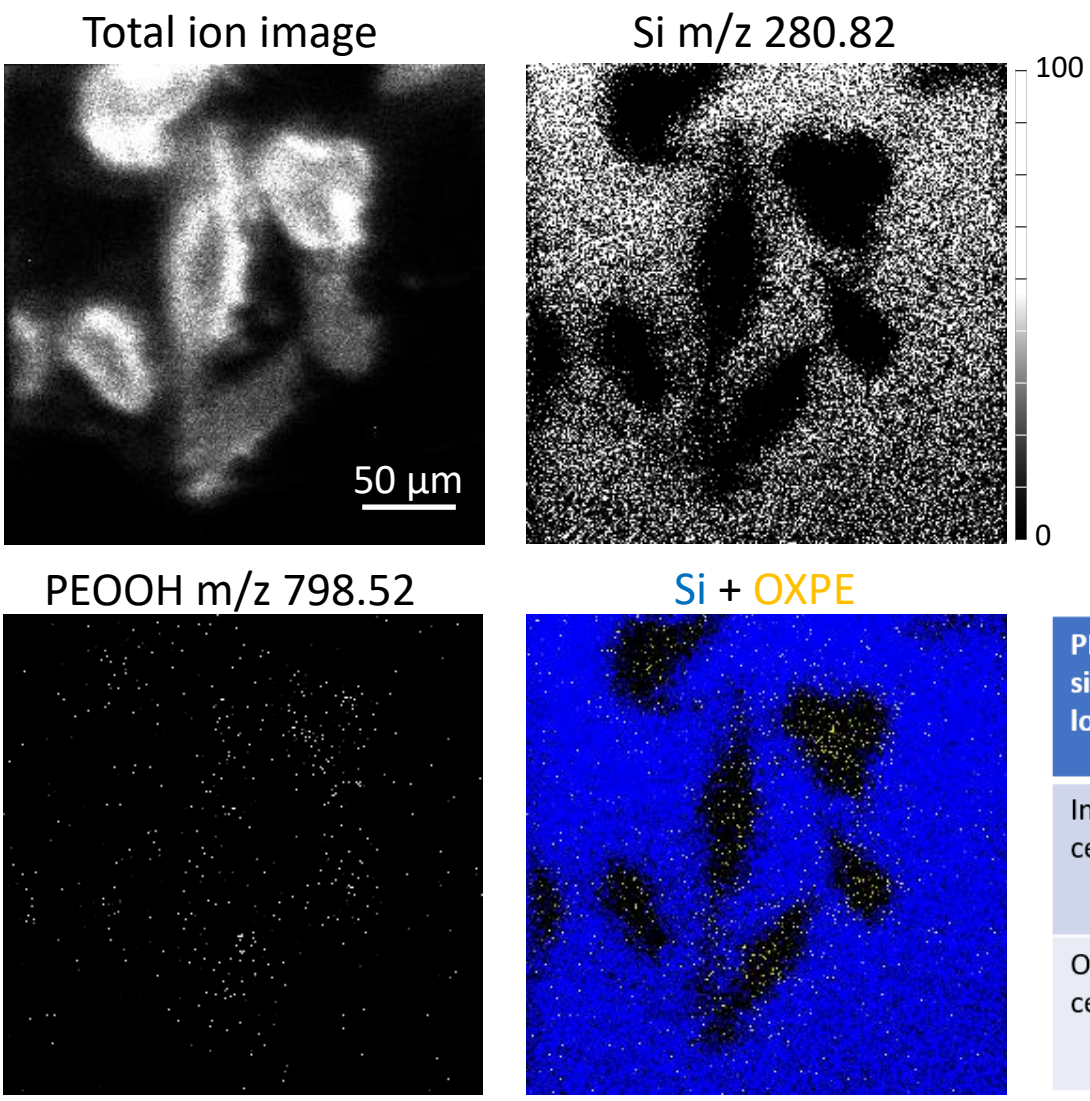
Data analysis

All GCIB-SIMS and LC-MS ions were analyzed as $[M-H]^-$ unless otherwise noted. GCIB-SIMS images and spectra were generated using Ionoptika Image Analyser (Version 2.0.2.11, Ionoptika, UK). Summation spectra from brain regions were exported to CSV files. An in-house script using Python/Anaconda/Jupyter version 6.0.1 (anaconda.com) was used to plot figure-quality spectra. GCIB-SIMS images were generated at a window of 0.05 Da centered on the exact mass and exported in BMP format. Scale bars and captions were also generated with an in-house Python script. Images are displayed in relative intensity grayscale unless noted otherwise. From the IHC binary image (NeuN), 20 pericontusional cortical neurons from the ipsilateral hemisphere were chosen along with 20 non-contusional cortical neurons from the contralateral hemisphere of the same tissue section. Regions were chosen in the same manner as previously presented[2]. In brief, the non-contusional neurons were chosen at the same anatomical location on the contralateral hemisphere relative to the hippocampus and other anatomical features. Image alignment was achieved through overlay of select background or lipid ion images that are characteristic for fiduciary markers in the brain (DG, CA1-3, L3V) with an expected accuracy within the lipid ion pixel size (6 µm). Using the IHC binary image as a mask, the GCIB-SIMS ion signals from each individual neurons were extracted from the superimposed images[1] using an in-house Python script. PCA calculations were performed using SIMCA v14.1 (Umetrics, Malmö, Sweden).

LCMS spectra and chromatograms were generated by Qual Browser (Thermo Fisher, version 4.2.28.14). Quantitative differences in ions detected in both the ipsilateral and contralateral hemispheres were assessed by Compound Discoverer (Thermo Fisher, version 2.0). P-Values were determined by a 2-tailed t-test based on duplicate runs. IHC images were thresholded into a binary layer (NIS Elements AR software version 5.02, Nikon Instruments Inc., Mellville, NY) and superimposed on relevant SIMS maps as we have shown previously [1] .

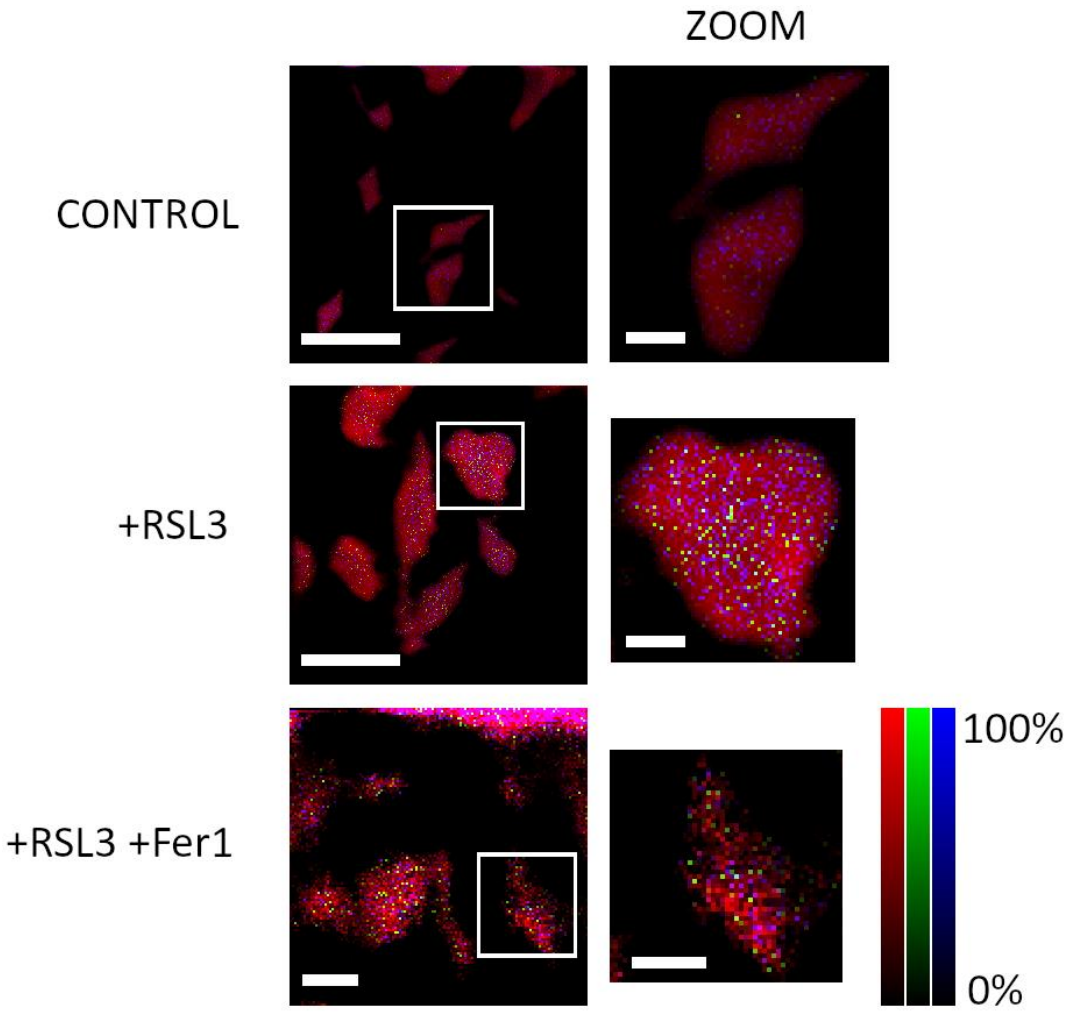
Image processing

The H9c2 cell GCIB-SIMS images obtained from Ionoptika image analyzer were processed using Cell Profiler v. 3.1.9 (Broad Institute) with custom nodes. Briefly, the cell contours are identified by using two gray scale images of the ion at m/z 280.82 (a silica specific ion Si_{10}^-) and the total negative ions. The former defines the background surface and the later defines the cell surface. The resulting image was used for the red channel. The cellular specific signals of phospholipids and phospholipid peroxides were identified by scalar multiplication of the cell surface image with the corresponding single-channel analyte image. No interpolation or deconvolution was applied. Image overlay was performed using photoshop cc.

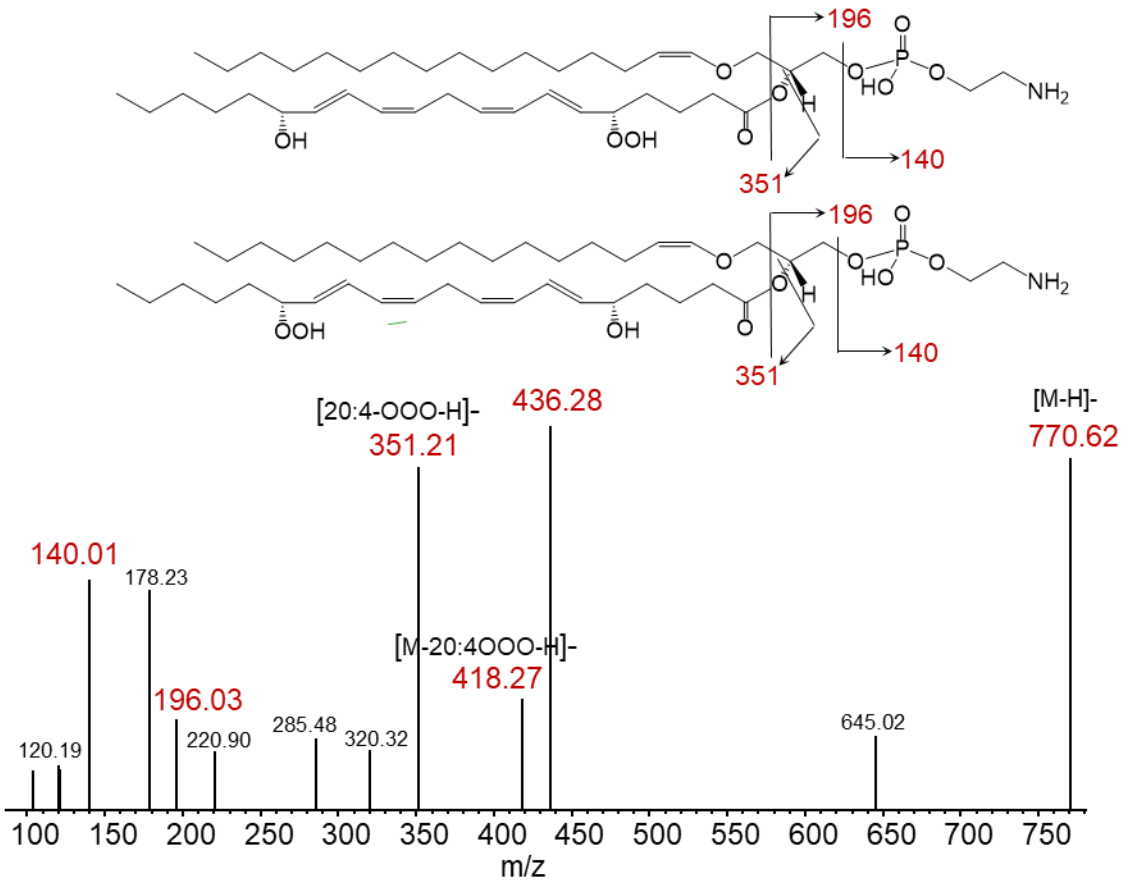


PEOOH signal location	Counts/pixel
Inside of cells	1.48
Outside of cells	0.36

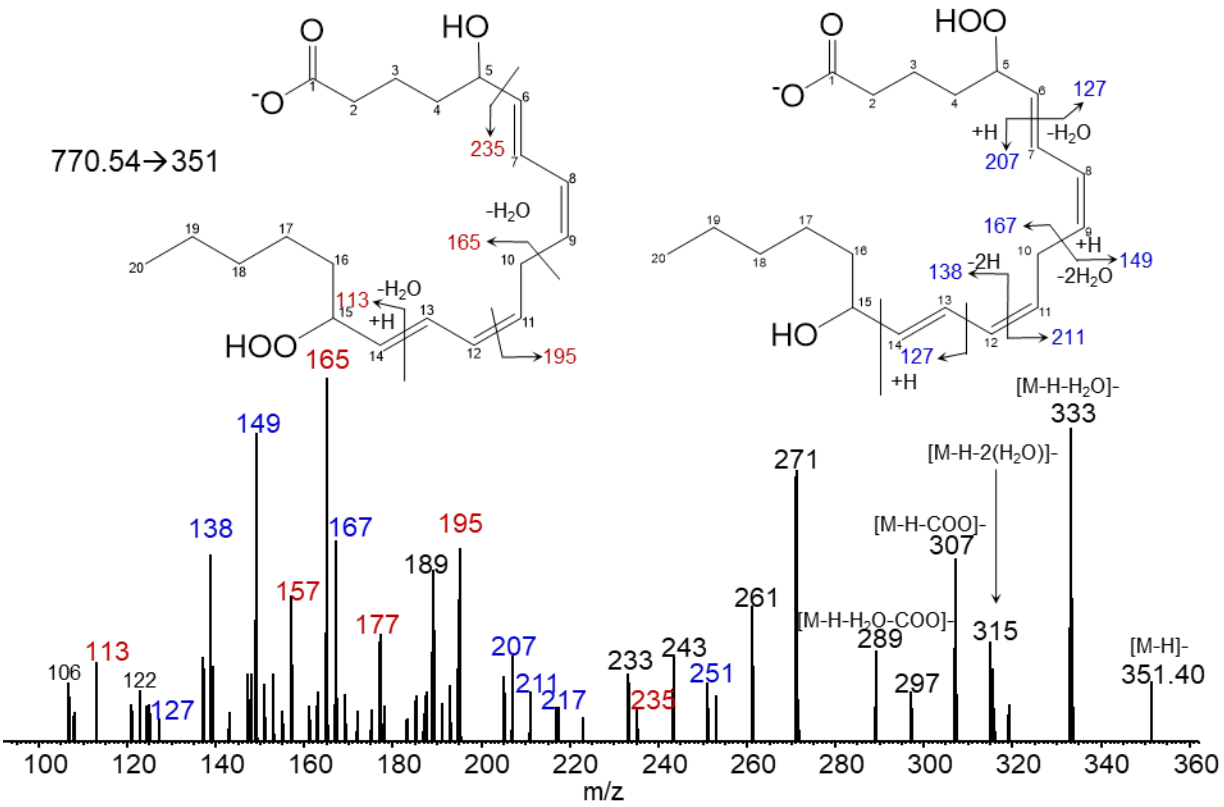
Supplementary Fig. S1. GCIB-H2O-SIMS images of RLS3-treated H9c2 cells and PEOOH signal comparison inside/outside of cells. a) Total negative ion image showing cell area. b) Image of Si_{10}^- (m/z 280.82) from substrate to confirm cell location (dark region). c) Ion image of m/z 798.52 attributed to PE(38:4)-OOH. This ion signal is much stronger within the cells, as displayed d) in the color overlay image of Si_{10}^- (blue) and PE(38:4)-OOH (yellow) and the table (e), revealing a significant difference of signal inside/outside of cells and confirming that this cell-associated. Data is from the first imaging layer. Images are prior to scalar multiplication using CellProfiler software (see Methods).



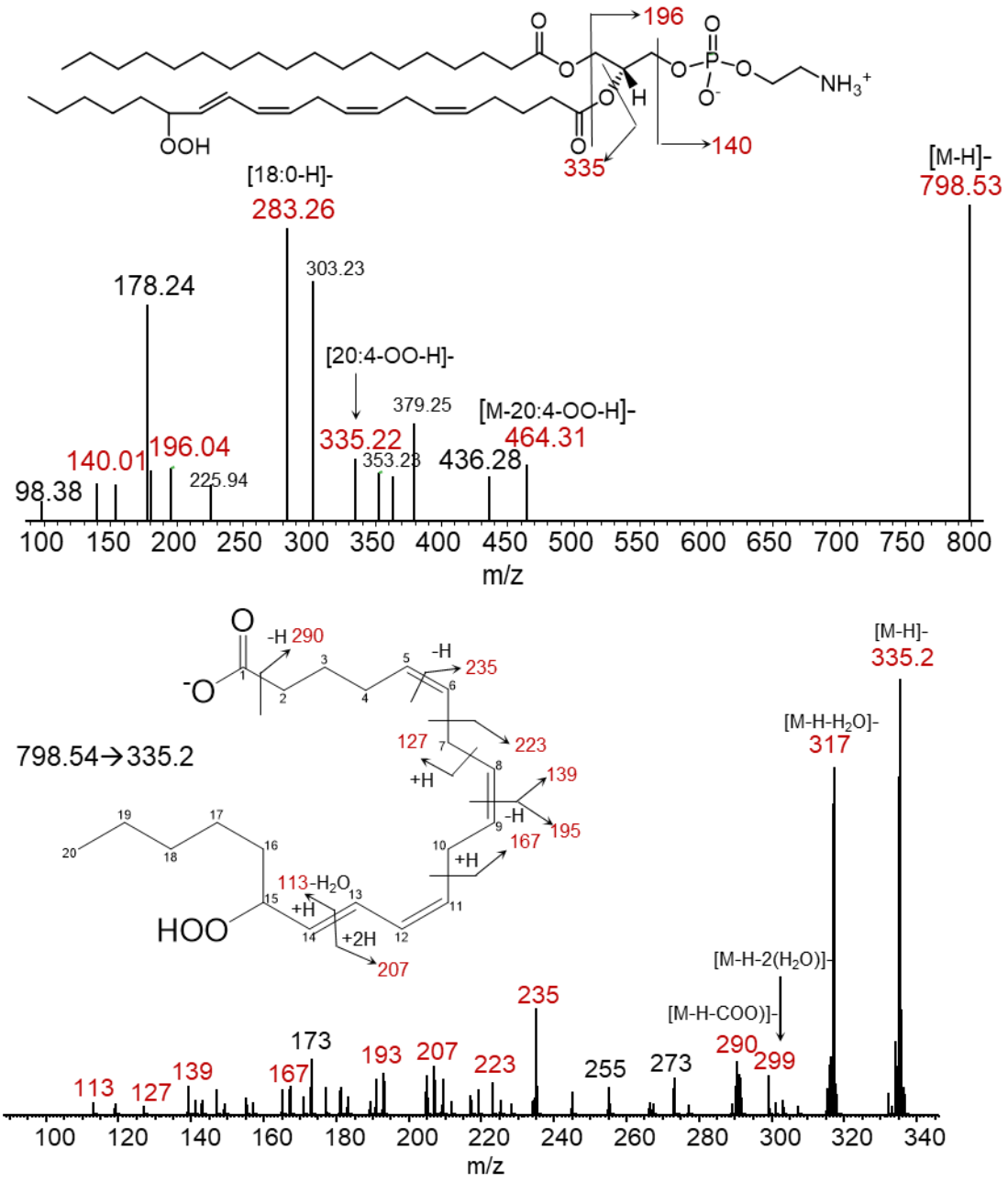
Supplementary Fig.S2. PE(38:4)-OOH is detected by GCIB-SIMS in RLS3 treated and untreated H9c2 cells. Cell borders were identified by changes in background ions and scalar intensity multiplication was performed on the non-oxidized and oxidized PE species for clarity (see Methods). Entire imaging area is in the left column with a zoomed image (marked by the white box) in the right column. Top panels: Control H9c2 cells with overlays of ions corresponding to PE(38:4) (*m/z* 766.53, blue), PE(38:4)-OOH (*m/z* 798.53, green) and cellular ions (red). Middle panels: RSL3-treated cells with overlays for PE/PEox displaying increased signals for PE(38:4)-OOH, *m/z* 798.53, green. Bottom panels: RSL3+Ferrostatin-1-treated H9c2 cells with overlays of PE/PEox displaying decreased signals for PE(38:4)-OOH (*m/z* 798.53, green) with zoom in. Data is from the first imaging layer. The control and +RSL3 were acquired at a pixel size of 1.2 microns and the +RSL3+Fer1 at 3.1 microns. The scale bars for the control and +RSL3 images are 100 microns and 20 microns in the zoomed image. The scale bars for the +RSL3+Fer1 image are 150 microns and 100 microns in the zoomed image. Intensities are relative to the maximum for each ion.



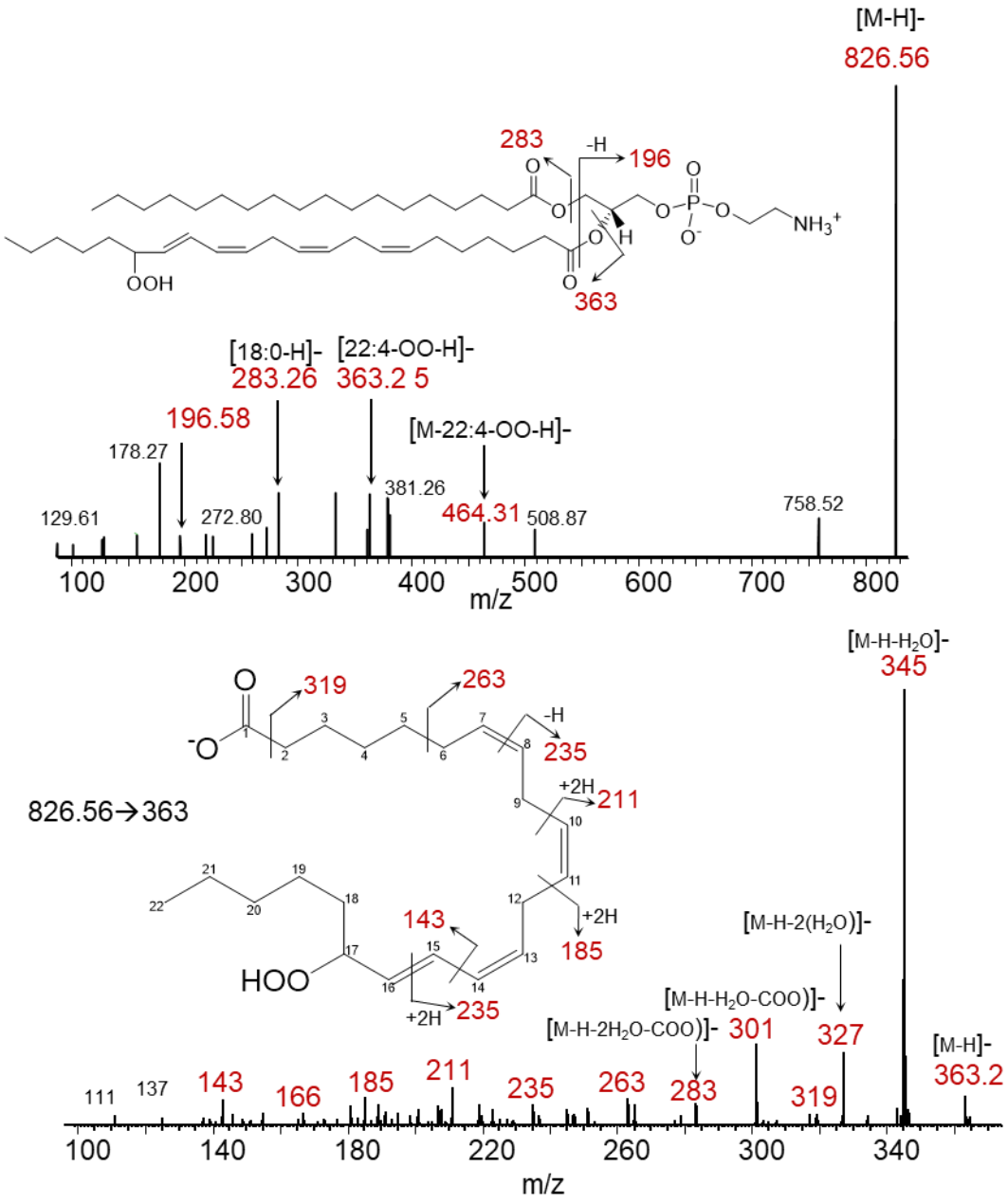
Supplementary Fig. S3A. Identification of phosphatidylethanolamine molecular species with m/z 770.5 in H9c2 cell exposed to RSL3. MS^2 spectrum of species with *m/z* 770.5; Insert: structural formulas of 1-sn-stearoyl-2-sn-15-OH-5-OOH-arachidonoyl- and 1-sn-stearoyl-2-sn-15-OOH-5-OH-arachidonoyl-phosphatidylethanolamine plasmalogens and fragments formed during MS^2 analysis.



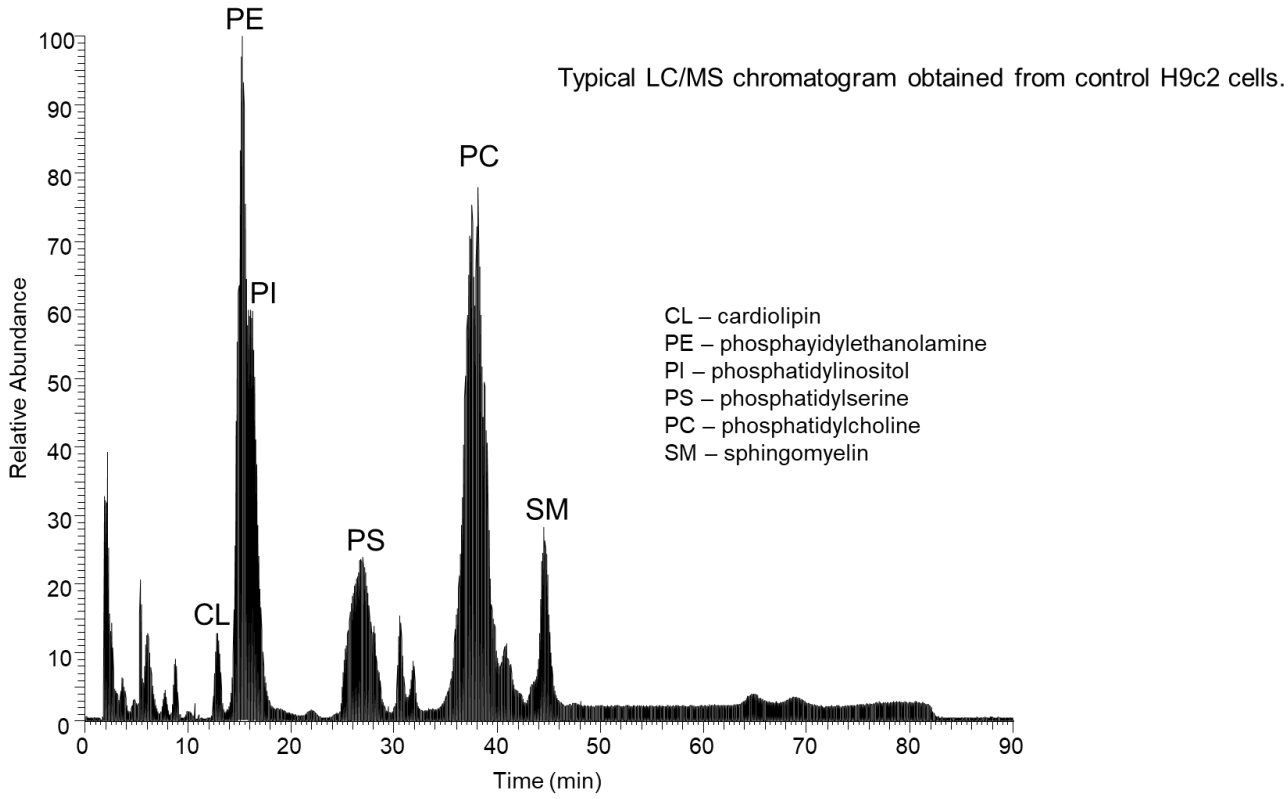
Supplementary Fig. S3B. Identification of phosphatidylethanolamine molecular species with m/z 770.5 in H9c2 cell exposed to RSL3. MS^3 spectrum of m/z 351.2. Insert: structural formulas of 15-OOH-5-OH- and 15-OH-5-OOH- arachidonoyl molecular ions and fragments formed during MS^3. The species with m/z 770 were identified as 1-sn-stearoyl-2-sn-15-OOH-5-OH-arachidonoyl- and 1-sn-stearoyl-2-sn-15-OH-5-OOH-arachidonoyl phosphatidylethanolamine plasmalogens.



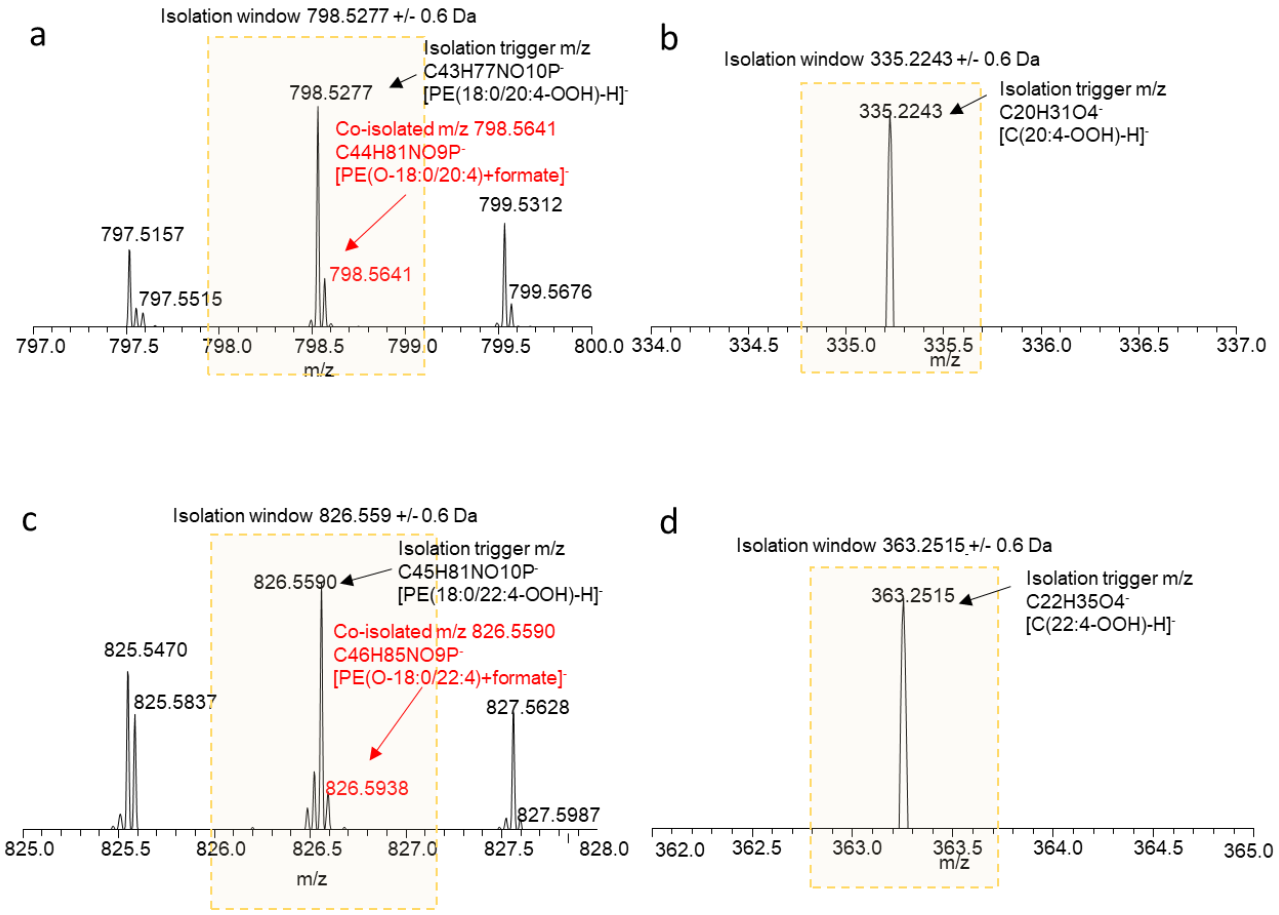
Supplementary Fig. S4. Identification of phosphatidylethanolamine molecular species with *m/z* 798.5 in H9c2 cell exposed to RSL3. A) MS^2 spectrum of species with *m/z* 798.5; Insert: structural formula of 1-sn-stearoyl-2-sn-15-OOH-arachidonoyl phosphatidylethanolamine and fragments formed during MS^2 analysis. B) MS^3 spectrum of *m/z* 335.2. Insert: structural formula of 15-OOH-arachidonoyl molecular ion and fragments formed during and MS^3. The species with *m/z* 798.5 were identified as 1-sn-stearoyl-2-sn-15-OOH-arachidonoyl-phosphatidylethanolamine.



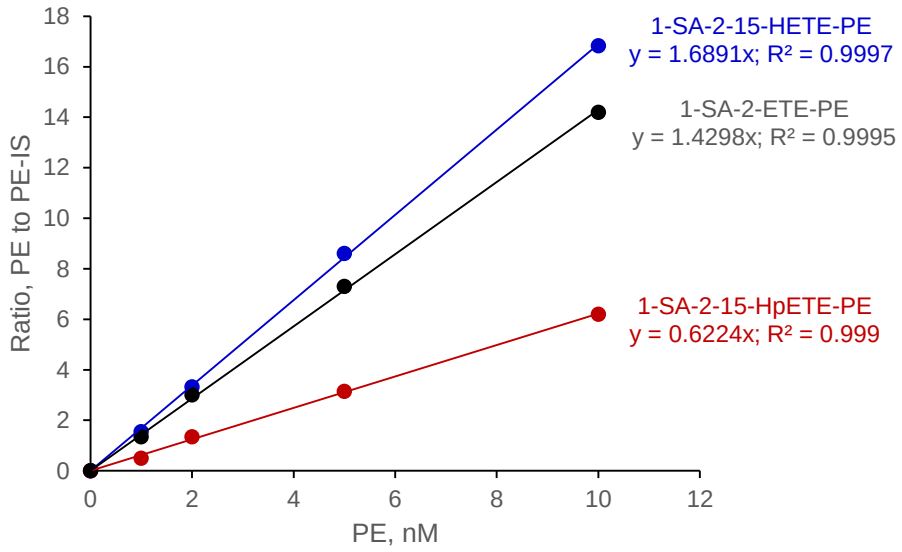
Supplementary Figure S5. Identification of phosphatidylethanolamine molecular species with m/z 826.5 in H9c2 cell exposed to RSL3. A) MS^2 spectrum of species with *m/z* 826.5; Insert: structural formula of 1-sn-stearoyl-2-sn-17-OOH-adrenoyl phosphatidylethanolamine and fragments formed during MS^2 analysis. B) MS^3 spectrum of *m/z* 363.2. Insert: structural formula of 17-OOH-adrenoyl molecular ion and fragments formed during and MS^3. The species with *m/z* 826.5 were identified as 1-sn-stearoyl-2-sn-17-OOH-adrenoyl-phosphatidylethanolamine.



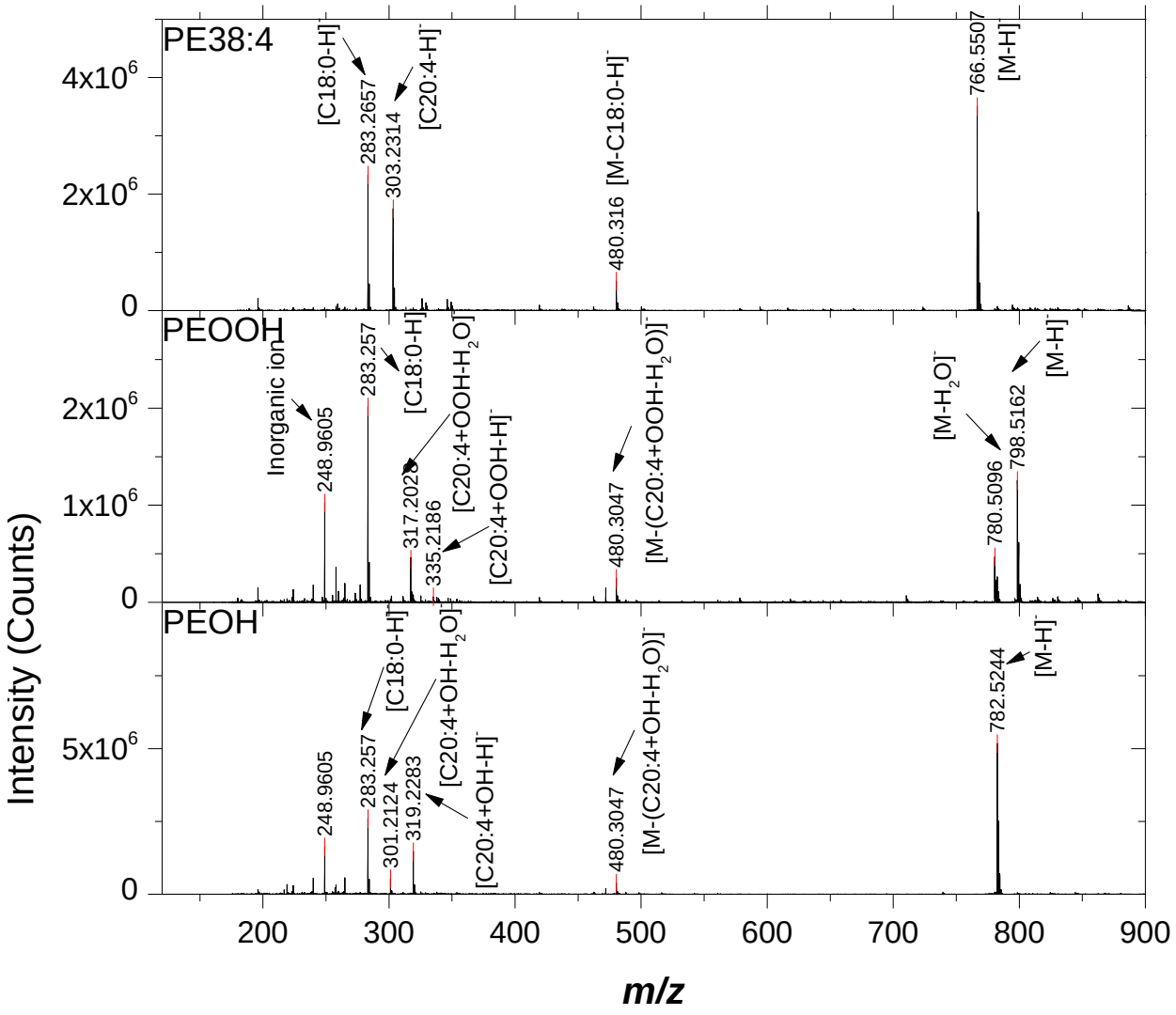
Supplementary Figure S6A. Typical LC/MS chromatogram obtained from control H9c2 cells. Total ion chromatogram (TIC) from normal phase silica column LC-MS (see Methods) with major classes of lipids indicated.



Supplementary Figure S6B. Formate adduct co-elution and co-isolation in H9c2 lipid extract LC-MS and MS/MS. Panels A and C: Zoom-in of the LC-MS spectra of a total lipid extract of H9c2 cells treated with RSL3. MS2 is triggered by Hydroperoxy PE $[M-H]^-$ species PE(18:0/20:4)OOH and PE(18:0/22:4)OOH at m/z 798.5277 and 826.5590, respectively. In each case a different non-oxidized PE co-elutes as a formate adduct: (PE(O-18:0/20:4) and PE(O-18:0/20:4) at m/z 798.5641 and 826.559, respectively (in red in the figure). In both cases, the other PE species is within the isolation window (+ or - 0.6 Da, highlighted in figure). Panel B: MS2 spectrum zoom-in corresponding to the fragmentation of m/z 798.5277 and 798.5641 in panel A. The oxidized FFA(20:4)OOH triggers subsequent MS3, but in this case there is no other ion within the isolation window. Corresponding MS2 from panel C is given in panel D, showing isolation of FFA(22:4)OOH alone for MS3.



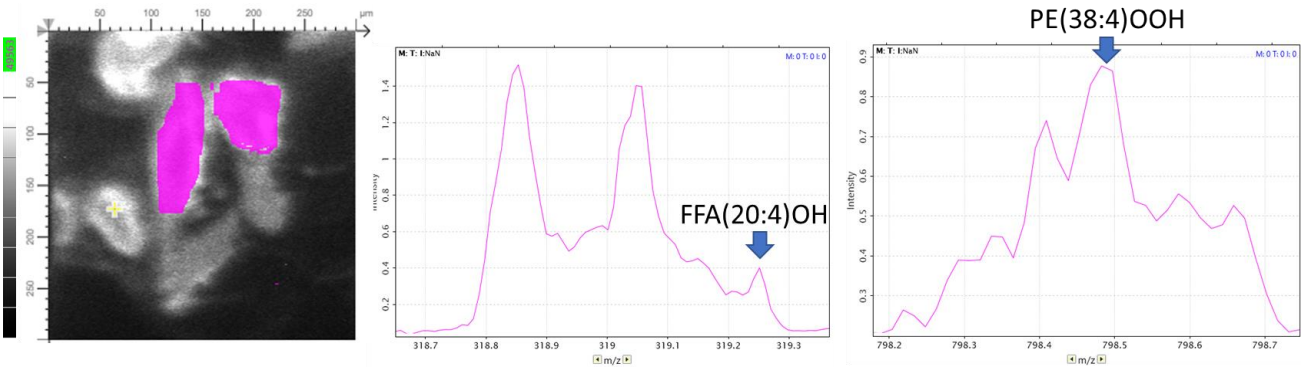
Supplementary Figure S6C. LCMS normal phase calibration curves. Quantitative assessment of 1-stearoyl-2-eicosatetraenoyl-phosphatidylethanolamine (PE(38:4), 1-SA-2-ETE-PE), 1-stearoyl-2-15-hydroperoxy-eicosatetraenoyl-phosphatidylethanolamine (PE(38:4)OH, 1-SA-2-15-HpETE-PE) and 1-stearoyl-2-15-hydroxy-eicosatetraenoyl-phosphatidylethanolamine (PE(38:4)OOH, 1-SA-2-15-HETE-PE) was performed. Deuterated phosphatidylethanolamine (PE-16:0/D31-18:1) was used as internal standard.



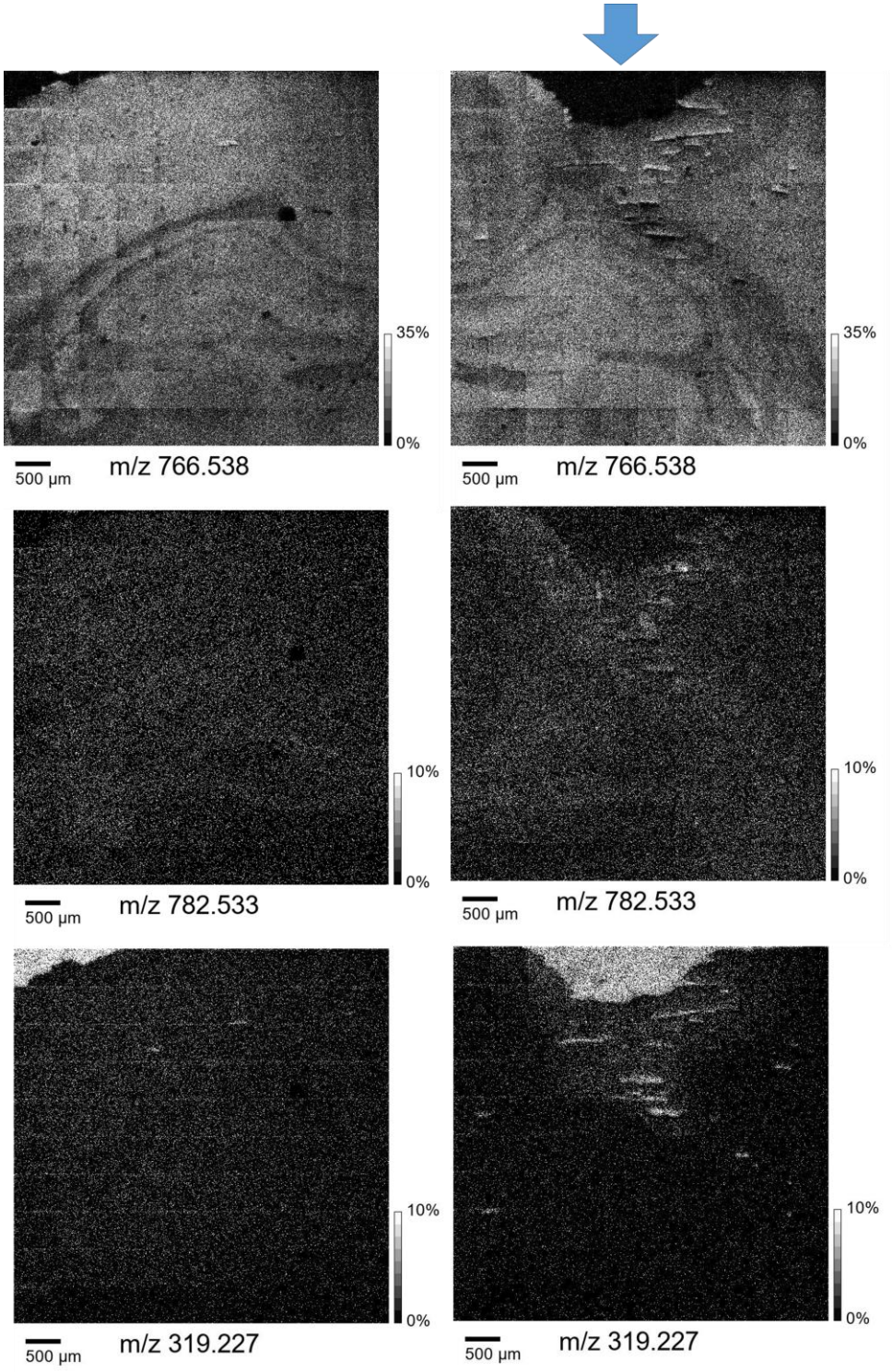
	[M-H]/total fatty acids	[M-H]/ fatty acid oxide	[M-H]/[M-H_2O-H]
PE	1.0±0.2	NA	NA
PEOOH	0.69±0.03	24.0±1.0	2.7±0.1
PEOH	1.4±0.1	4.1±0.1	NA
	Ratios are intensities of molecular ion to given analyte(s). Total fatty acids for PEOOH are summed intensity from the ions at m/z 283.26+317.21+319.23+335.22, for PEOH are m/z 283.26+301.21+319.23 and for PE are m/z 283.26+303.23. The oxide fatty acid fragment for PEOOH is m/z 335.23, for PEOH is m/z 319.23.		

Supplementary Fig S6D. Cold stage 70 keV $(H_2O)_{28000}^+$ H_2O-GCIB-SIMS detects molecular ions of PEOH and PEOOH. Representative summation spectra from lipid standards of PE(38:4) (top), PE(38:4)OOH (middle) and PE(38:4)OH (bottom) are displayed with fragment ions labeled. The

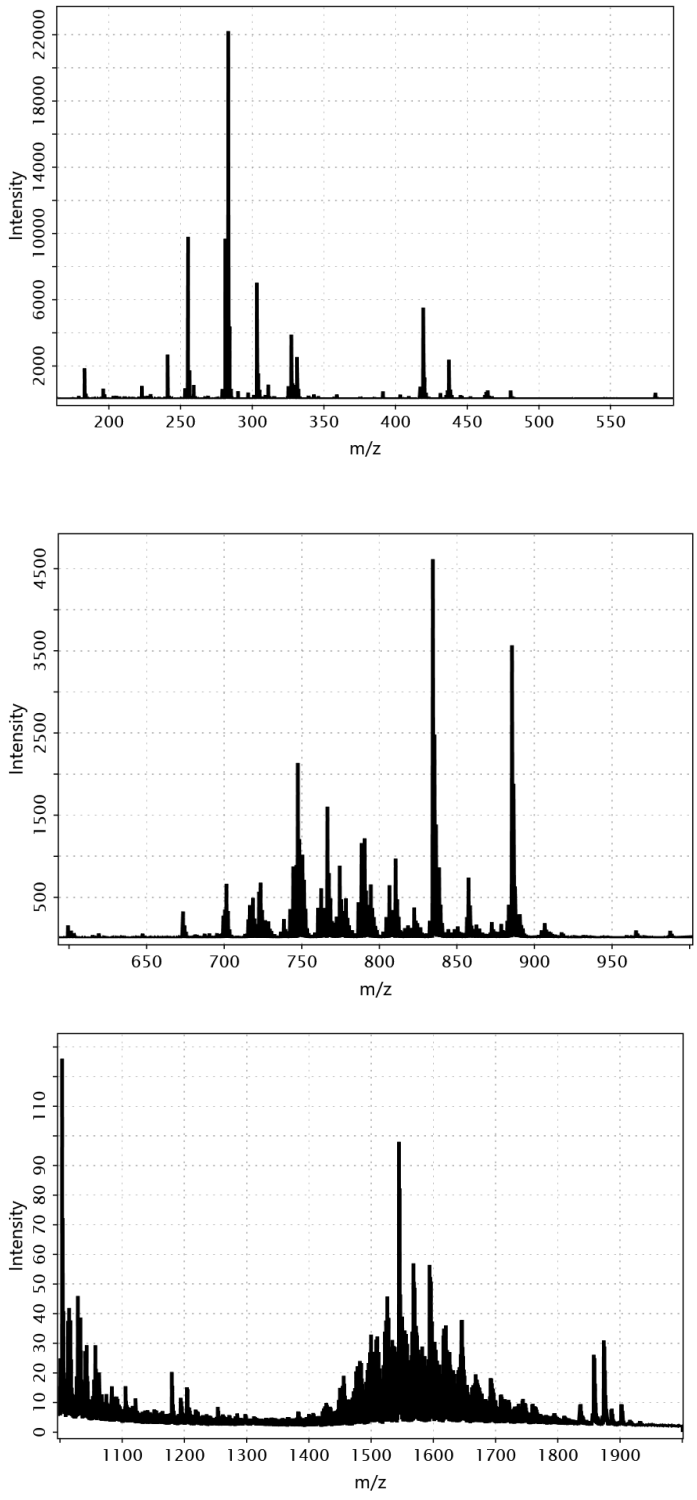
inset table shows the intensity ratios of the molecular ion the total intensity of its fatty acid fragment ions and to its FFA(20:4)OH or FFA(20:4)OOH fatty acid fragment ion (for PEOH and PEOOH standards, respectively) and the ratio of $[M-H]^-/[M-H_2O-H]^-$ for PEOOH.



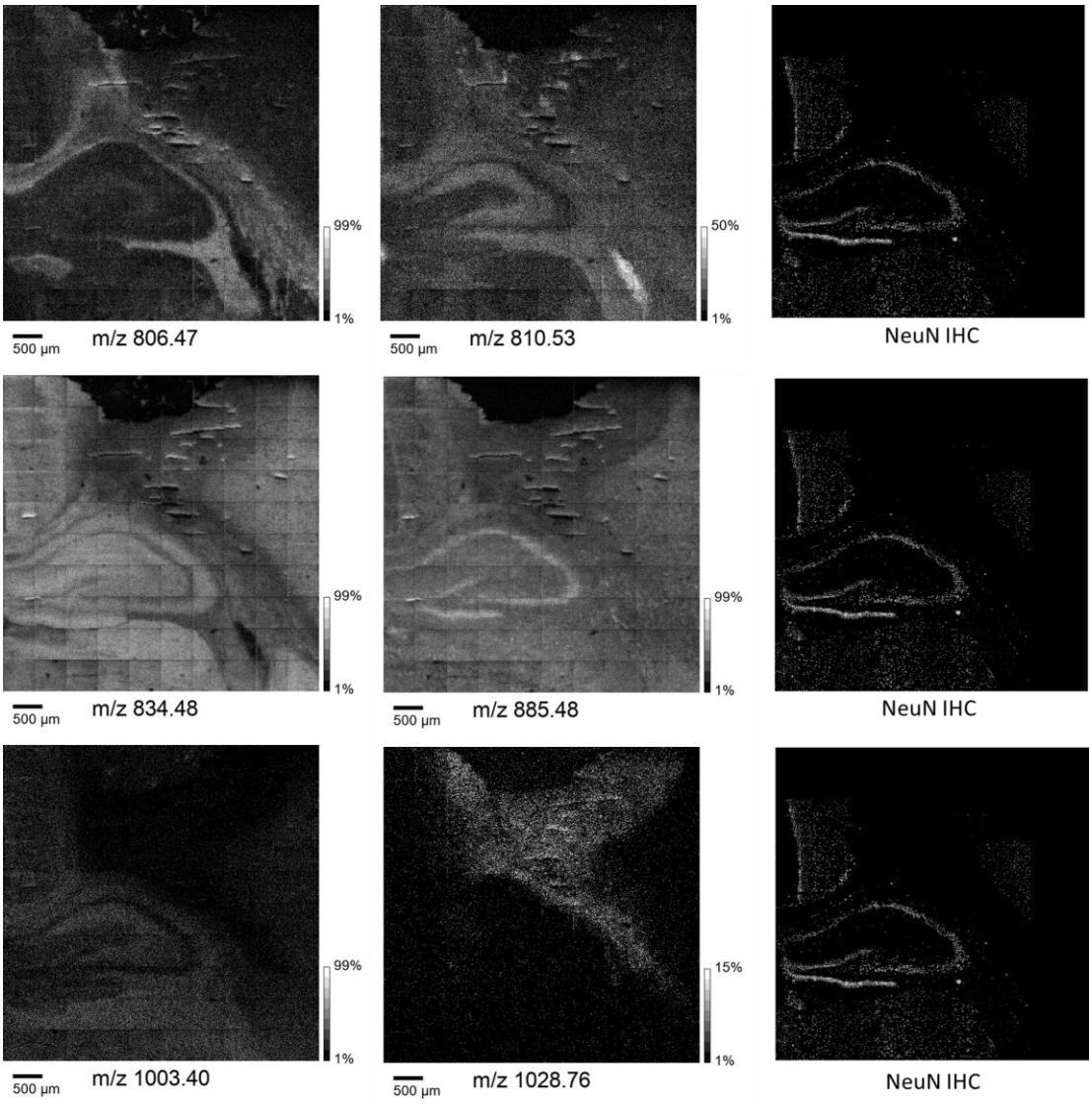
Supplementary Fig. S6E. Cellular regions and corresponding spectra from H2O-GCIB-SIMS of RSL3 treated H9c2. Left: H2O-GCIB-SIMS total ion image of H9c2 cells treated with RSL3 for 2.5 h (pixel size = 1.2 micron, scale indicated on axes). Representative cellular regions are indicated in magenta, grayscale intensity indicated at far left. Summation spectra from these regions is displayed for the mass range near FFA(20:4)OH (middle) and PE(38:4)OH (right). An isolation window of Δ 0.02 u was used to generate GCIB-SIMS ion images in order to remove interference from signals not resolvable due to the TOF MS analyzer system on the GCIB-SIMS.



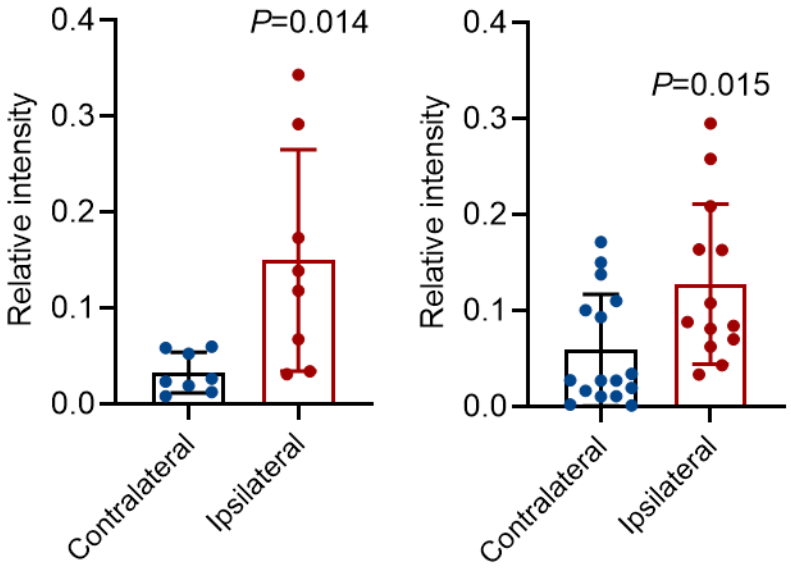
Supplementary Fig. S7A. Grayscale GCIB-H2O-SIMS ion images and spectra of TBI brain. Left column is the contralateral imaging area, right is the ipsilateral. TOP: PE(38:4) m/z 766.538, MIDDLE: FFA(20:4)-OH m/z 319.227, BOTTOM: PE(38:4)OH m/z 782.533. CCI injured area is marked with the blue arrow. Grayscale intensity is relative for each ion. Scale bar=500 microns.



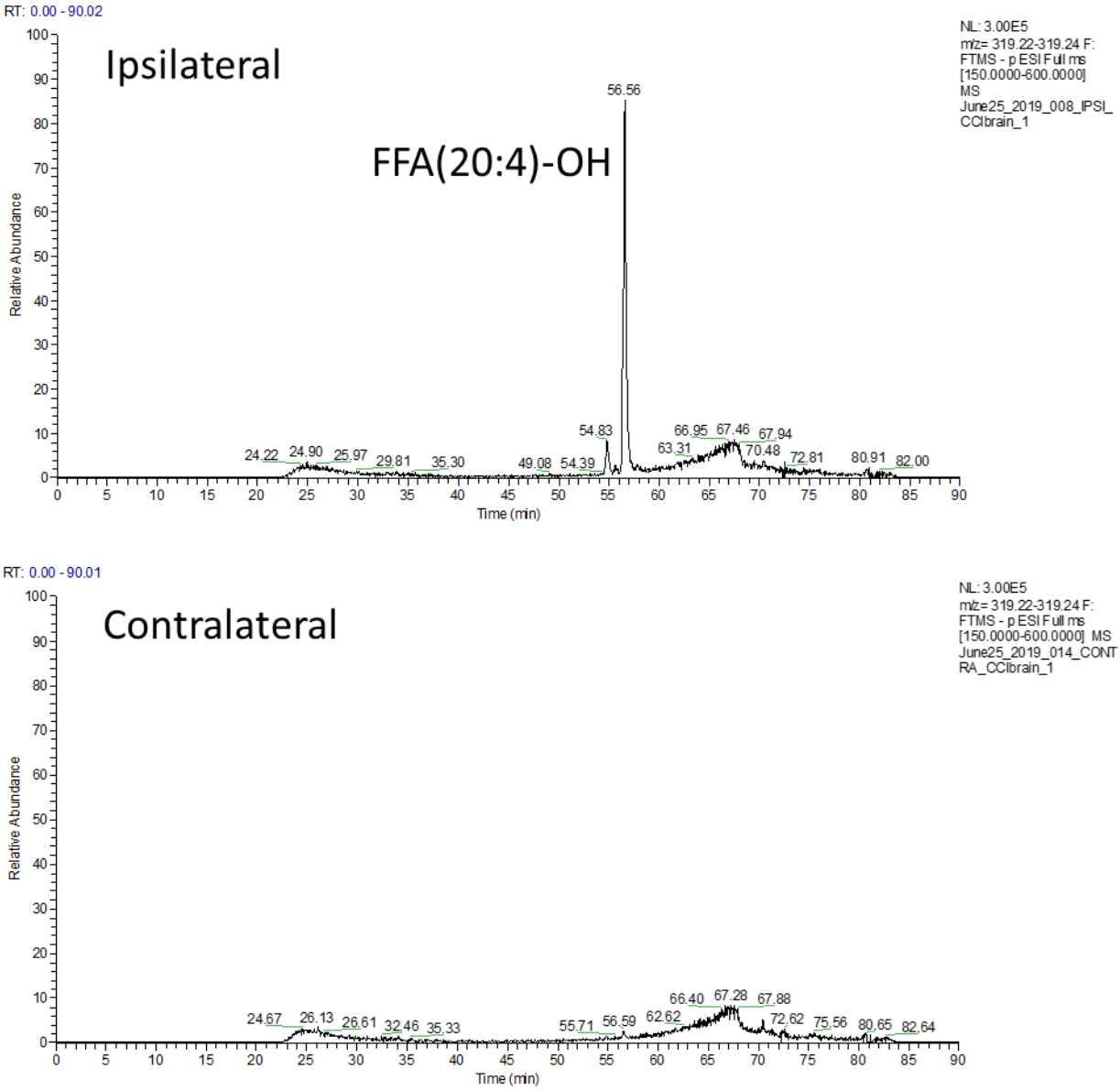
Supplementary Fig. S7B. GCIB-H2O-SIMS summation spectra of TBI brain. TOP: m/z 150-600, MIDDLE: m/z 600-1000, BOTTOM: m/z 1000-2000.



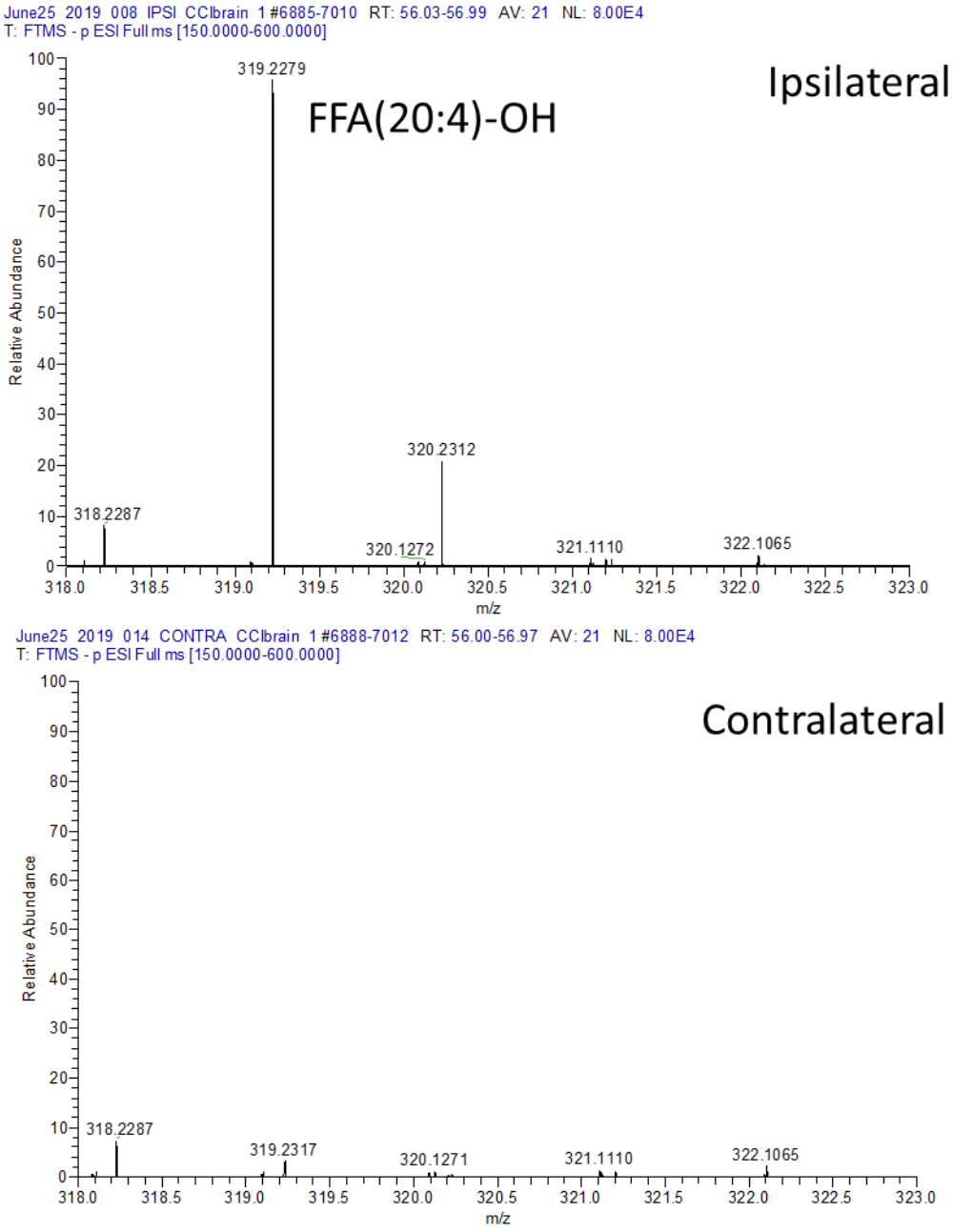
Supplementary Figure S7C. Alignment of GCIB-SIMS lipid images with NeuH IHC. GCIB-SIMS images (left and middle rows) and NeuN IHC binary image (right rows) showing alignment of tissue lipids (GCIB-SIMS) and neurons (NeuN IHC). Scale bar = 500 microns, grayscale intensities as shown relative to the maximum signal of the ion. ST(d18:1/16:0) (top left) and PS(38:4) (top middle) were used for alignment of the hippocampal cell layers and the lateral 3rd ventricle (L3V), PS(40:6) (middle left) and PE(38:4) (center) were used for the dentate gyrus (DG) and Cornu Ammonis 3 (CA3), and PIP(38:4) (bottom left) and NAPE(56:6) (bottom middle) were used for the pericontusional region,



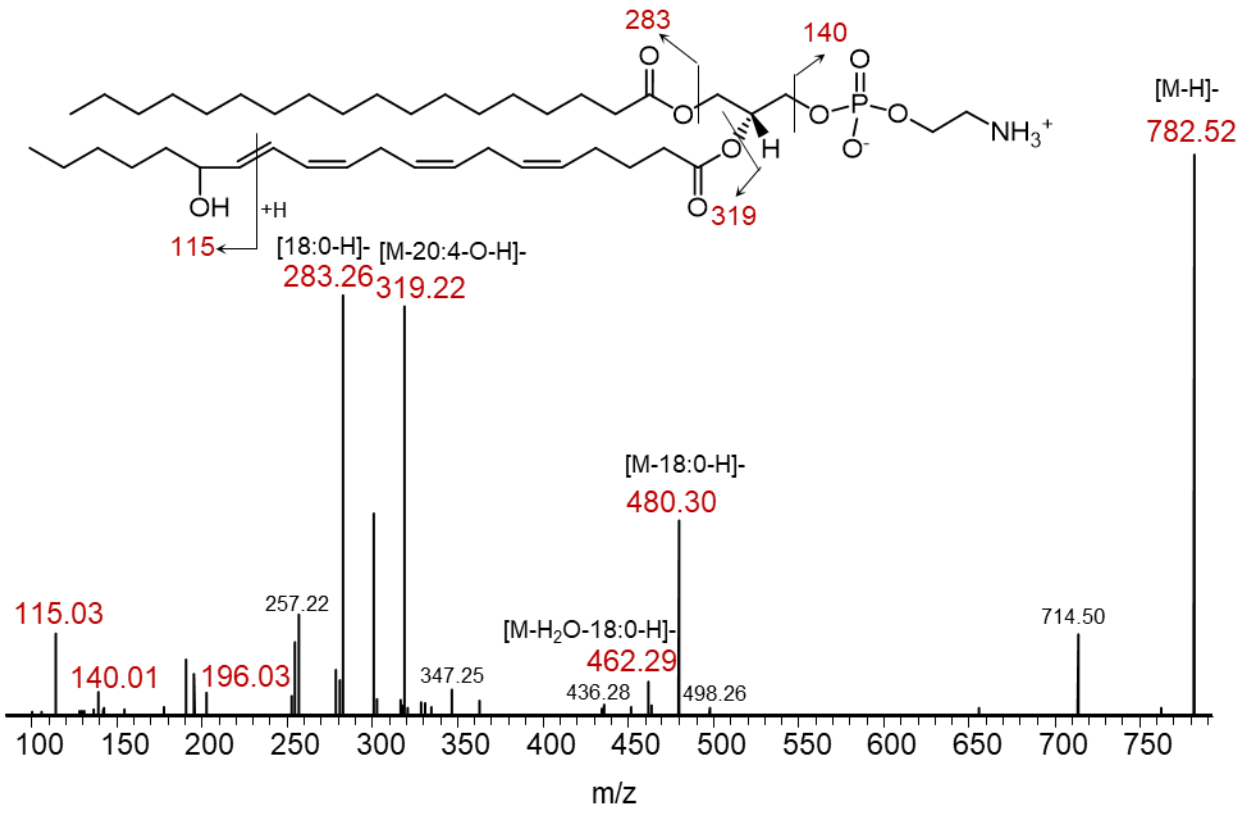
Supplementary Fig. S8. Statistical analysis of oxidized lipids in TBI rat brain neurons in the ipsilateral pericontusional region and the corresponding contralateral region. GCIB-SIMS signal intensities from 20 NeuN cell regions in each of the ipsilateral and contralateral are displayed. FFA(20:4)+1O (left) and PE(38:4)+1O (right) are significantly increased in ipsilateral pericontusional region compared to the corresponding contralateral region. *n*=20, student's *t*-test.



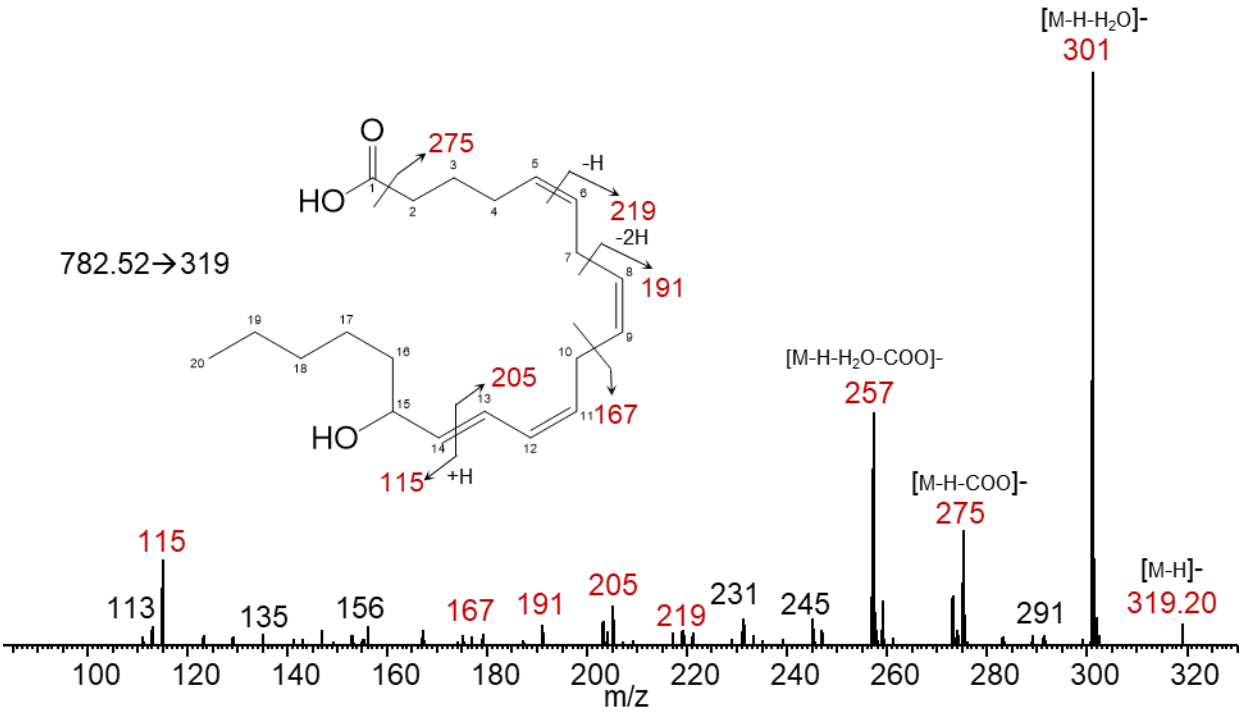
Supplementary Fig. S9. LCMS C18 (FFA) method ion chromatograms for FFA(20:4)OH. TBI brain total lipid extracts were analyzed with a C18 LCMS method for detection of FFA. Chromatograms are for m/z 319.22-319.24 from the ipsilateral hemisphere (top) and contralateral hemisphere (bottom) with a fixed intensity scale of 3e5. FFA(20:4)OH (m/z 319.22) is detected in the ipsilateral hemisphere but not the contralateral.



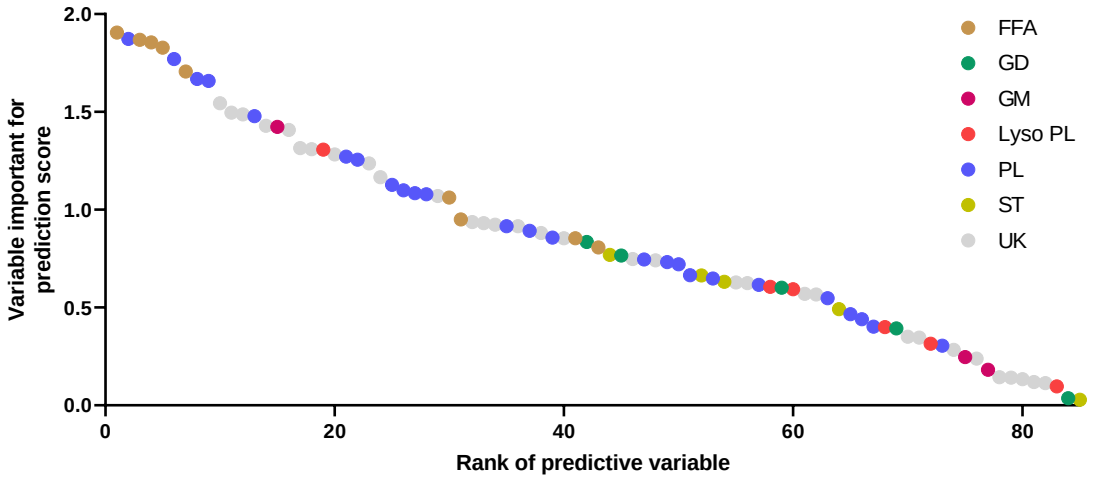
Supplementary Fig. S10. LCMS C18 (FFA) method summation spectra of FFA(20:4)OH. TBI brain total lipid extracts were analyzed with a C18 LCMS method for detection of FFA. Summation spectra from all MS1 scans is displayed for 56-57 min. Window from m/z 318-323 is displayed with a fixed intensity scale of 8e4. FFA(20:4)OH (m/z 319.22) is significantly more abundant in the ipsilateral hemisphere (top) than the contralateral hemisphere (bottom).



Supplementary Fig. S11A. Identification of phosphatidylethanolamine molecular species with *m/z* 782.5 in rat brain after traumatic brain injury. A) MS^2 spectrum of species with *m/z* 782.5; Insert: structural formula of 1-sn-stearoyl-2-sn-15-OH-arachidonoyl phosphatidylethanolamine and fragments formed during MS^2 analysis. B) MS^3 spectrum of *m/z* 319.2. Insert: structural formula of 15-OH-arachidonoyl molecular ion and fragments formed during and MS^3. The species with *m/z* 782.5 were identified as 1-sn-stearoyl-2-sn-15-OH-arachidonoyl-phosphatidylethanolamine.



Supplementary Fig. S11B. Identification of phosphatidylethanolamine molecular species with m/z 782.5 in rat brain after traumatic brain injury. A) MS^2 spectrum of species with *m/z* 782.5; Insert: structural formula of 1-sn-stearoyl-2-sn-15-OH-arachidonoyl phosphatidylethanolamine and fragments formed during MS^2 analysis. B) MS^3 spectrum of *m/z* 319.2. Insert: structural formula of 15-OH-arachidonoyl molecular ion and fragments formed during MS^3. The species with *m/z* 782.5 were identified as 1-sn-stearoyl-2-sn-15-OH-arachidonoyl-phosphatidylethanolamine.



Supplementary Fig. S12. Dot plot showing variable importance for prediction (VIP) score and their rank for individual lipids. VIP scores were obtained from OPLS-DA analysis. Lipid species were classified according to the classes.

Supplementary Table S1. Comparison of GCIB-SIMS signal intensities for lipid peroxides and GSH in H9c2 cells undergoing various treatments. Data is counts/pixel, summation of all imaging layers of a representative cell.

Treatment	PEp(36:4)-OH/OOH m/z 770.50	PE(38:4)-OOH m/z 798.53	PE(40:4)-OOH m/z 826.56	GSH m/z 306.08
Control	14.13	14.24	23.45	798.56
RSL3	47.19	80.64	90.71	324.16
RSL3+ Fer-1	33.78	35.69	78.24	495.82

Supplementary Table S2. List of retention times for oxidized PE species and their non-oxidized precursors from RSL3-treated H9c2 cells

Lipid species	m/z	Retention time
PE(p36:4)	738.5102	16.41 min
PE(38:4)	766.5408	15.90 min
PE(40:4)	794.5700	15.74 min
PE(p36:4)-OH/OOH	770.4978	17.09 min
PE(38:4)-OOH	798.5291	17.61 min
PE(40:4)-OOH	826.5603	18.16 min

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