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Lipid Analysis of 30 000 Individual Rodent Cerebellar Cells using High-Resolution Mass Spectrometry

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Abstract

Single cell measurements aid our understanding of chemically heterogeneous systems such as the brain. Lipids are one of the least studied chemical classes and their cell-to-cell heterogeneity remains largely unexplored. We adapted microscopy guided single cell profiling using matrix assisted laser desorption / ionization ion cyclotron resonance mass spectrometry to profile the lipid composition of over 30,000 individual rat cerebellar cells. We detected 520 lipid features, many of which were found in subsets of cells; Louvain clustering identified 101 distinct groups that can be correlated to neuronal and astrocytic classifications and lipid classes. Overall, the two most common lipids found were $[PC(32:0)+H]^+$ and $[PC(34:1)+H]^+$, which were present within 98.9% and 89.5% of cells, respectively; lipid signals present in <1% of cells were also detected, including $[PC(34:1)+K]^+$ and $[PG(40:2(OH))+Na]^+$. These results illustrate the vast lipid heterogeneity found within rodent cerebellar cells and hint at the distinct functional consequences of this heterogeneity.

Graphical Abstract

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Author Contributions

J.V.S., S.S.R and E.K.N. conceived the study, S.S.R. performed animal dissection and single cell dissociation, E.K.N. and A.E.T. performed microscopy and matrix deposition, E.K.N performed the MS experiments and lipid assignments, J.F.E. developed and implemented the data analysis software, E.K.N. and J.F.E. analyzed the data, E.K.N. and J.V.S. wrote the manuscript with input from all authors. J.V.S. obtained funding for the research.

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Supporting Information

Table S1 listing lipid assignments and Figures S1–S3 showing additional lipid analysis details (PDF); custom scripts (zip folder).

The authors declare no competing interest.

Single cell measurements have advanced our understanding of the function and microstructure of highly complex biological systems. However, representative single cell analysis within such complex systems is complicated by the sheer number of cells; the human brain, for example, contains over a trillion cells.¹ These cells are often morphologically, functionally, and/or chemically defined and can be categorized into cell classes and subclasses.^{2–9}

Robust classification and sub-classification of individual cells in large cellular populations that extend beyond immunocytochemical (ICC) and electrophysiological profiles have recently been spearheaded by the single cell transcriptomics community, and the resulting data has helped to uncover aspects of brain health and disease.¹⁰ However, the presence of specific lipids within a cell depends on the complex interplay between the cellular enzymes encoded by the cell transcriptome,¹¹ the cellular environment,¹² and the organismal metabolic state.^{13–15} The ability to probe the heterogeneity of lipids within the brain has lagged behind the characterization of other molecular classes at the single cell level. This is partly because of their inherent complexity and the difficulty in creating immunohistochemical and fluorescent probes that are selective for specific lipids.¹⁵

Single cell mass spectrometry (MS) has been at the forefront of chemical analysis for decades, helping to decipher the roles of molecular classes such as neuropeptides.^{16–18} MS complements transcriptomics by detecting the actual presence of endogenous metabolites, peptides, and proteins rather than their transcripts. Many different types of MS approaches have been recently reported for single cell analysis: matrix-assisted laser desorption/ionization (MALDI),¹⁹ near field desorption,²⁰ desorption electrospray ionization,²¹ T-probe,²² microextraction,^{23–24} vacuum ultraviolet laser desorption,²⁵ capillary electrophoresis (CE)- electrospray ionization (ESI),²⁶ and others.^{27–33} In particular, high-throughput single cell MALDI MS approaches have been developed to characterize the chemical heterogeneity of large cellular populations by detecting tens to hundreds of chemicals within a single cell. The fast acquisition rate (on the order of seconds) potentially allows for thousands of cells to be profiled in a day, suggesting the method is capable of multiplexed, chemical investigation of complex and abundant biological systems.^{34–37} Because of the nature of MALDI MS, much of the cellular content remains after analysis,

allowing other techniques to be easily integrated after MALDI MS, expanding the information garnered from an individual cell.^{37–41}

Brain lipids are critical molecules that are difficult to characterize and most research efforts are performed using liquid chromatography (LC)-MS-based lipidomics.^{42–43} While these methods have revolutionized the characterization of lipids in the brain, there are some limitations; they require samples that are orders of magnitude larger than a cell, do not provide information on cellular heterogeneity, and may miss lipids that are in a small subset of cells within the system. Moreover, unlike most molecular classes, probes are not generally available for specific lipids, reducing the number of techniques that can be used to ascertain cellular localization. MS imaging (MSI) is well suited for lipid analysis on a pixel-by-pixel basis, and recent advances have allowed MSI to reach cellular resolutions.^{44–45} However, even when MSI obtains a pixel size at the level of (or even better than) single cells, more than one cell is often interrogated due to the highly intertwined nature of brain cells (e.g., neurons and astrocytes). Nonetheless, by using well-established dissociation methods and depositing cells diffusely, we have the ability to ensure that only individual cells are sampled.^{36–37}

For this work, we performed microscopy-guided profiling to assay over 30,000 cells of the rat cerebellum with MALDI Fourier transform-ion cyclotron resonance (FT-ICR) MS with the goal of exploring the lipid heterogeneity within these cells. This may be the largest number of cells profiled in a single cell MS experiment. Additionally, for the first time, we used FT-ICR MS to provide high-resolution and high-mass accuracy measurements, allowing us to putatively assign 500 lipid spectral features without fragmentation. The largest issue with the single cell approach is that performing tandem MS of the compounds within each cell is problematic given the small amounts present within the cell, especially when identifying low-abundance, rare chemical species that are not present in a majority of samples. Therefore, lipid peaks were assigned according to the available LC-MS databases and MS-based data repositories of brain lipids.

EXPERIMENTAL SECTION

Chemicals.

Chemicals were purchased from Millipore Sigma (St. Louis, MO) and used without further purification unless otherwise specified.

Animals.

Fifteen, 2–2.5-month-old, male Sprague Dawley outbred rats (*Rattus norvegicus*) (www.envigo.com) were housed on a 12-h light cycle and fed *ad libitum*. Animal euthanasia was performed in accordance with the appropriate institutional animal care guidelines (the Illinois Institutional Animal Care and Use Committee), and in full compliance with both federal and ARRIVE guidelines for the humane care and treatment of animals.

Tissue Dissociation and Preparation of Individual Cell Populations.

Dissected cerebellar tissues were treated with the papain dissociation system (Worthington Biochemical, Lakewood, NJ). Briefly, tissues were incubated with an oxygenated solution of 20 units/mL of papain, 1 mM L-cysteine, and 0.5 mM ethylenediaminetetraacetic acid dissolved in Earle's balanced salt solution for 80 min at 34 °C. Tissue was then mechanically dissociated in modified Gey's balanced salt solution (mGBSS) containing (in mM): 1.5 CaCl₂, 5 KCl, 0.2 KH₂PO₄, 11 MgCl₂, 0.3 MgSO₄, 138 NaCl, 28 NaHCO₃, and 0.8 Na₂HPO₄, and 25 HEPES, pH 7.2 and supplemented with 0.04% paraformaldehyde for cell stabilization through mechanical dissociation. Following dissociation, glycerol was added to a final concentration of 40% (v/v) and 50 µL of cell suspension was transferred onto non-coated microscopy glass slides (Lab Med, Ft. Lauderdale, FL) that had been etched with fiducial marks and washed with ethanol prior to plating.

Each slide held cells from three biological replicates plated in duplicate in a random coordinate on a grid pattern. This experimental design helps control batch variation and possible position-dependent artifacts. After ~18 h, cells were stained with Hoechst 33342 (1 µg/mL in mGBSS) for 15 min before quickly rinsing twice with 500 µL of 150 mM ammonium acetate. In total, 15 animals across 15 slides were analyzed for approximately 35,000 cells. Five thousand objects were later removed because they had insufficient lipid signal and were assumed to be cellular debris and other non-cellular fluorescent objects.

Optical Imaging.

Brightfield and fluorescence images were acquired on a Zeiss Axio M2 microscope (Zeiss, Jena, Germany) equipped with an Ab cam Icc5 camera, X-cite Series 120 Q mercury lamp (Lumen Dynamics, Mississauga, Canada), and a HAL 100 halogen illuminator (Zeiss). The DAPI (ex. 335–383 nm; em. 420–470 nm) dichroic filter was used for fluorescence excitation. The images were acquired with a 10× objective (1 pixel width = 0.55 µm) with a 13% overlap produced during image tiling. Images were processed and exported as big tiff files using ZEN software version 2 blue edition (Zeiss).

Sample Preparation, MALDI Matrix Application, and MS Analysis.

Slides were coated with a 50 mg/mL 2,5-dihydroxybenzoic acid (DHB) solution dissolved in 1:1 ethanol: water with 0.1% trifluoroacetic acid (TFA) using an automatic sprayer as described previously.³⁵ The matrix solution was nebulized at 10 mL/h using N₂ gas at 50 psi with 100 passes. Samples were taped to a rotating plate and the spray was placed 3 cm above the samples. The total amount of matrix applied was between 0.1 and 0.2 mg/cm².

Single cell MALDI mass spectra were acquired on a solariX XR 7T FT-ICR mass spectrometer (Bruker Corp., Billerica, MA) with a mass window of 150–3000, yielding a transient length of 2.94 sec. The ultra large laser setting was used with a laser footprint of approximately 100 µm.; 50 laser shots were accumulated with a frequency of 1000 Hz at 50% of the maximum power settings. Some additional key instrumental parameters include a MALDI plate offset of 100 V, an ICR excitation power of 8.8 dB, and 1 ICR cell fill per acquisition. Single cell coordinates and a custom geometry file were ascertained using micro MS, as previously described,³⁷ as well as an Excel file for the target automation function on

ftms Control (v. 2.1.0, Bruker Corp.). Cells were filtered by distance (to remove cells that were not 100 μm apart) and size to remove cell nuclei. The acquisition was randomized across all three animals on the slide. The instrumental parameters are provided in the Supporting Information (Scripts.zip; file name: apex Acquisition. method). Other types of mass analyzers are applicable to single cell MALDI MS analysis and are often faster than FT-ICR analysis. For this work we used a MALDI FT-ICR mass analyzer instead of a time-of-flight or linear ion trap mass spectrometer because of the higher mass accuracy and mass resolution afforded by FT-ICR measurements.

In addition to individual cells, cerebellar tissue was homogenized within a solution of 1:2 chloroform: methanol (v:v). Contaminants were removed by centrifuging with water and the organic layer was dried down. Lipid extract was re suspended in a 1:1 ratio of chloroform to a solution of MALDI matrix (50 mg/mL DHB solution dissolved in 1:1 ethanol: water with 0.1% TFA) and spotted onto a Bruker 364-spot target where the lipids were characterized and identified as previously reported.³⁸ Briefly, MALDI MS of the lipid extract was acquired using the same mass window, laser size, and frequency; 150 laser shots were accumulated at 60% of the maximum power settings. We used a higher number of laser shots on extracts in comparison to single cells to maximize the number of detected lipids within the extracts while reducing laser power to enable multimodal analysis.³⁸ Direct infusion ESI of a cerebellar lipid extract was performed for comparison with the single cell results. The ESI flow rate was 150 $\mu\text{L}/\text{h}$ and a capillary voltage of 3900 V.

Data Analysis and Statistics.

We used 34,585 cell-like features from 15 animals in this study, as detailed below. Single cell mass spectra were converted from Bruker.d into.xml files using a custom script and the Automation Engine within the Data Analysis software (v. 4.4, build 200.55.2969, Bruker Corp.). Data for peaks with a signal-to-noise ratio above three were then imported into MATLAB 2017A (The Math Works, Inc., Natick, MA) using custom scripts (provided as Supporting Information) to parse individual xml files for m/z value, intensity, and resolution. Data for each cell were combined into a single dataset. Each slide was independently recalibrated with $[\text{PC}(32:0)+\text{H}]^+$, $[\text{PC}(34:3)+\text{H}]^+$, and $[\text{PC}(34:1)+\text{H}]^+$ at m/z 734.5694, 756.5538, or 760.5842, respectively, to correct for mass shifts. Cells had to contain at least one of these lipids to be included in further analysis. Because mass resolution changes as a function of m/z value for FT-ICR MS, the single cell mass spectra were aligned with non-uniform piecemeal bin widths. For example, the bin width at m/z 150 was 0.001 and 0.05 Da at m/z 2500, with 10 additional divisions in between. Bin widths were estimated as the average peak width at m/z values and constructed as piecewise linear divisions over the entire spectral range. All intensities were root-mean-square (RMS) normalized after alignment.

Peaks between m/z values of 700–900 with mass defects greater than 0.3 Da or less than 0.8 Da were kept for multivariate statistical analysis. These peaks are assumed to be lipids because the ions were detected with mass defects consistent with lipid ions and were within the appropriate mass range for positive mode. The intensity matrix from the selected lipid range was imported into R,⁴⁶ where the matrix was mean-centered and z-scored prior to

dimensionality reduction using principal component analysis (PCA). Two-dimensional visualization was performed using the first 100 principal component (PC) scores for t-distributed stochastic neighbor embedding (t-SNE) with a perplexity of 30, initial dimensions of 30, and max iterations of 2500.⁴⁷ Clustering was performed on the t-SNE Euclidean coordinates using the Louvain clustering method, weighting each edge in the graph by the Jaccard overlap index. R scripts for this method were obtained and modified from Shekhar et al. 2016.^{48–49}

To determine which t-SNE clusters were more similar to neurons or astrocytes, we incorporated a neuron/astrocyte relative enrichment spectrum as a training set published previously (mass match < 3 ppm error).³⁸ A Pearson pairwise linear correlation was used to compare the reduced spectrum to the relative enrichment training spectrum and the correlation matrix was subsequently mean-centered and plotted with a cutoff similarity of 5%. Cells previously classified by t-SNE and Louvain clustering were recolored in our figures to indicate their correlation with either astrocytes or neurons.

Lipid class information was derived by concatenating the assigned lipids into a single matrix containing all lipids within that class and then summing the intensities after normalizing to the maximum lipid intensity within that class. The relative abundance of each respective lipid class was plotted on the t-SNE plot using the “Hot” color map (MATLAB) between the normalized minimum (0) and maximum intensity (64).

Rare cell finding methods were based on work by Ong et al.,³⁴ where lower PCs were used to target unique populations that contributed to lower degrees of variance. In MATLAB, the RMS-normalized intensity matrix for the lipid range was mean-centered for PCA. Using only the PCs contributing to the top 90% of the total variance, the PC scores were back-projected and subtracted from the preprocessed spectra resulting in a ‘difference mass spectrum’.

Lipids were subsequently assigned using high mass accuracy, the LIPID MAPS structural computational database,⁵⁰ previously published biological data (i.e., lipids found only within bacterial systems were excluded), and for intense lipids in the extracts, tandem MS.³⁸ All m/z values containing less than 10 cells were removed from selection. The 10 m/z values contributing most to the difference mass spectrum were selected as rare lipids and labeled with their putative lipid identity from LIPID MAPS. Lipid assignments all consisted of errors less than 5 ppm, although most assignments consisted of errors less than 3 ppm. Lipids found in less than 10 cells were removed from the study because these spectral features were likely a result of a small mass shift or contamination and, typically, not found between more than one biological replicate.

RESULTS AND DISCUSSION

Single Cell MALDI FT-ICR Analysis.

Cells were dissociated onto a slide, and features of interest located and then probed via MALDI MS using the cell finding and analysis software microMS.³⁷ Next, the slide was imaged to determine the locations of the cell nuclei; the fluorescent nuclei were filtered to

exclude structures located less than 100 μm apart and objects that appeared non-cellular by shape and size (e.g., debris, broken cells, and bare nuclei). Filtering by distance and size reduces contamination of the single cell spectra from other nearby cells. Mass spectra were then acquired from each of the thousands of remaining cells. An advantage of this approach is that only cell-like features are measured; in a typical MSI experiment, much of the time is spent sampling the space between dispersed cells or probing only a portion of a cell, depending on where the cell is within the MSI raster pattern. Here, once the spectra were obtained, spectral features with mass defects between 0.3 and 0.8 m/z values were used to find lipid candidates. Each selected m/z value was then matched against the LIPID MAPS structural computational database^{50–51} to assign putative identities for each spectral feature. Although tandem MS is the standard for chemical identification, the small amount of cellular material and matrix present in the individual cell locations precluded effective tandem MS. While tissues can be homogenized and tandem MS performed on the homogenates and the lipids mass matched to the individual cell spectral features, this has yet to be found effective, particularly for rare ions only detected in a small number of cells spread across multiple slides; future work will address this issue. For our mass matching approach, only candidates with mass errors below 5 ppm were considered, although most errors were below a 3 ppm threshold.

Using this procedure, we detected 520 distinct spectral features within the lipid mass region (m/z 700 to 900) of ~30,000 rat cerebellar cells (Table S1), with the individual spectra available at the Illinois databank.⁵² The number of detected features is an order of magnitude higher than the number of lipid features detected within a lipid extract analyzed by either MALDI FT-ICR MS (34 lipid features) or ESI-FT-ICR MS (52 lipid features; Figure S1). Typical lipidomics experiments survey the lipid content through comprehensive lipid extractions followed by LC-MS.⁴² While assaying lipid extracts from larger structures allows one to characterize hundreds of lipids, not only are details on cell heterogeneity lost, the approach does not detect lipids found only in the occasional cell, even if a lipid is at high levels in that cell. Single cell analysis circumvents these issues, especially for low-abundance compounds found at higher levels within a subpopulation of cells. The largest limitation to the single cell approach is that identifications can only be made according to other studies such as LC-MS-based databases because, as noted above, quality tandem MS is difficult to achieve at the single cell level. Future work is aimed at developing methods for more robust chemical identification, such as pooling together cells containing a rare chemical feature with our liquid micro junction probe post-MALDI MS analysis.³⁹

On average, we detected 34 lipids within each cell (ranging between 1 and 116) from the following lipid classes: phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), triglyceride (TG), diglyceride (DG), hexagonal ceramide (HexCer), and phosphatidic acid (PA), demonstrating the capacity to detect many types of molecules using single cell MALDI MS. In total, 297 of the filtered spectral features were matched to known lipids within the LIPID MAPS database using the aforementioned criteria.

As expected for positive-mode MALDI MS, most of the assigned lipids were phospholipids, likely originating from the outer plasma membrane. Differences in the abundance of these

lipids impact membrane fluidity and curvature; the lipid composition can be correlated to cellular outgrowth and morphology. Other lipids are involved in cell-to-cell signaling or cellular division. The rich diversity of lipids has been noted within the literature,^{53–55} and our capacity to detect many different types of lipids is necessary for beginning to understand lipid heterogeneity at cellular resolutions.

Lipid Clustering and Classification.

We used the detected lipid features to cluster individual cells using t-SNE with e subsequent Louvain-Jaccard clustering.^{48–49} Overall, 101 distinct clusters were identified using the Louvain-Jaccard clustering criteria (Figure 1A). To our knowledge, this is the largest number of clusters determined using any single cell MS approach^{36,56–58} and rivals that of single cell transcriptomic measurements,⁵⁹ demonstrating the rich information garnered using our high throughput lipid profiling approach. Cluster 1 was the largest and contained 882 cells; clusters 100 and 101 were the smallest with 31 cells each. Because there are less than 30 canonical cell types within the cerebellum,^{60–61} obtaining 101 clusters suggests functional / cell state heterogeneity within known cell types. We expect that these distinct clusters may correspond to different functional / activity states of the canonical cell types rather than new cell types; however, verifying this postulate requires more research. We expect that the clusters identified within this dataset are not from animal-to-animal differences or sample preparation because neither of these experimental variables explain the extent of clustering, further demonstrating the ability of MALDI MS for monitoring biologically derived chemical differences between cells as opposed to sampling artifacts (Figure S1). For instance, many of the clusters contain cells that were sampled from different animals or different days, instead of cells clustering based on the sampling date or animal of origin.

Recently we profiled several hundred rat hippocampal cells with both ICC and single cell MS, enabling us to determine the lipids found in astrocytes (GFAP-positive cells) and neurons (neuro filament light chain-positive cells) using a lower resolution MALDI time-of-flight MS dataset.³⁸ Here we used these lipid profiles and ICC-derived cell types to determine how neuron-like or astrocyte-like each cluster is within our t-SNE plot (Figure 1B). Even though the lipid profiles were determined using different instrumentation and animals over a year apart, we found clusters that correlate well with neurons or astrocytes, such as clusters 11 and 39. Even with ICC/MS data from different cells and instruments, robust and conserved MS lipid profiles were obtained, suggesting that these profiles enable independent classification of canonical cell types within the brain without the use of antibodies. Moreover, it demonstrates that specific lipids may be conserved within individual cell types. While the clusters can be grouped in these broad classes, additional differences between the clusters are observed. For example, the neuron-like clusters represent subclasses that differ by their lipid constituents (e.g., clusters 39 and 72), and the same is true for astrocyte-like clusters. These subclasses of astrocytes and neurons can be confidently determined because we are capable of measuring tens of thousands of cells from multiple animals with enough resolving power to distinguish different types of lipids at similar m/z values. For instance, if too few cells are profiled, smaller clusters would be difficult to separate from the larger clusters and essentially masked, while unresolved lipids would, additionally, mask the heterogeneity found within the sample. Because the MALDI MS-ICC

protocol can only stain a small number of profiled cells, it became imperative to remove the staining step to assay large numbers of cells to determine subclasses of astrocytes and neurons, as demonstrated here. Also interesting are the cell clusters that have lipid profiles that do not correlate with either neurons or astrocytes (perhaps these are other glia, excluding astrocytes, and other cell types). We will perform additional MS training from other ICC-based classifications in follow-up studies to determine if these additional clusters belong to other canonical cell types such as microglia. We can then find subclasses and different chemical states of these other cell types as well. Using this information, we can also determine what aspects of cell biology MALDI MS is sensitive to and capable of measuring.

We can visualize the distribution of specific lipid classes and use this information to parse out what distinguishes each cluster. For example, PA lipids are in higher relative abundance within cluster 78 compared to all other lipid classes, and TG lipids are in higher relative abundance within clusters 99 and 49 compared to DG and PS lipids (Figure S3). We show the recolored t-SNE plot for PC (Figure 2A), SM (Figure 2B), and PG (Figure 2C) lipids for clarity; the other classes are shown in Figure S3. PC lipids appear ubiquitous within the profiled cells (Figure 2A), which is in good agreement with their common and relatively high abundance within cell membranes, as well as their high ionization efficiency.^{62–64} The overlap between neuron-like cells and clusters containing a high abundance of SM lipids (e.g., clusters 11, 62, and 69; Figure 2B) helps validate our assignment of these as neurons; SM lipids are well known to compose neurons and have been implicated in diseases that affect neurons.^{65–68} PG lipids, however, are not as widespread as either PC or SM lipids, and are localized to only a few clusters, such as clusters 5, 25, and 68 (Figure 2C). Because none of the lipid classes have identical distributions within cells and their subsequent clusters, t-SNE allows us to visualize this heterogeneity within the profiled cells and their relative abundance, which can be linked to biological function, as is the demonstrated case correlating neurons and the SM lipids—observed to be essential in neuron metabolism and survival. It also allows us to quickly compare which (if any) lipid classes correlate to cell types.

Average Cluster Spectra and Rare Lipid Analysis.

We further discriminate the lipid profile of each cluster by averaging the spectra acquired from each corresponding cell (Figure 3 and Figure S2). Of the 520 spectral features detected within the cells, 57% of the compounds could not be matched to known lipids and will be the focus of follow-up studies. $[\text{PC}(32:0)+\text{H}]^+$ ($m/z = 734.5712$) and $[\text{PC}(34:1)+\text{H}]^+$ ($m/z = 760.5845$) correspond to spectral features that are present within 98.9% and 89.5% of cells, respectively, indicating that these are highly conserved lipids, as has previously been shown.^{69–71} Moreover, the ~1% of cells that do not contain PC(32:0), contain PC(34:1) or PC(34:3), indicating that they are indeed cells rather than debris with an undetectable level of PC(32:0). Because PC(32:0) is such a ubiquitous lipid, the subset of cells without detectable amounts could be of interest in future studies. There are others, such as putative $[\text{PC}(34:1)+\text{K}]^+$ and $[\text{PG}(40:2(\text{OH}))+\text{Na}]^+$, which are only observed in a small number of cells and hence, in a few clusters. Each cluster has a distinct lipid profile, with some having large differences between them, such as clusters 28 and 91, which clearly differ in the

number of lipid signatures and their relative intensities (Figure 3), especially with [HexCer(t34:2)+K]⁺ (*m/z* 752.5074) and [PC(34:1)+Na]⁺ (*m/z* 782.5652). While the average mass spectra for only eight clusters are displayed in Figure 3 for clarity, similar results were obtained for the other clusters and are shown in Figure S2.

One advantage of this dataset is that it provides the ability to discover lipids that are uncommon and detected within a small number of cells and subsequently find the cell they were detected in for further analysis. Ten spectral features were detected in less than 6.5% of cells, with one unknown compound present in as little as 0.1% of the cells (Figure 4A). Because of their relatively low abundance, we assume that these lipids are often overlooked using traditional visualization and analytical methods. Moreover, by using PCA, as compared to listing lipids in a small number of cells, we can ascertain how much these rare lipids contribute to the overall variance within the dataset. A peak for putative [PC(34:1)+K]⁺ at an *m/z* value of 798.5414 is shown along with its C¹³ peak (*m/z* 799.5453), supporting that this is a lipid rather than a spectral artifact (Figure 4B, top), and putative [PG(40:2(OH))+Na]⁺ at an *m/z* value of 869.5807, with the exception that its C¹³ peak is not completely resolved from the peak at *m/z* 870.5856 (Figure 4B, bottom). Nonetheless, this single cell MALDI MS method can be used to uncover unusual lipid features and locate these interesting cells for follow-up analysis with other analytical approaches.^{36,38–39,41}

CONCLUSIONS

We have demonstrated the utility of high-throughput single cell MALDI MS for in-depth chemical characterization of single mammalian cells. In sum, we detected 520 lipid features within 30,000 individual cells using MALDI FT-ICR MS. From the information gained here, we were able to determine 101 significantly different clusters, ranging in size, using a combination of t-SNE and Louvain-Jaccard clustering, correlate some of the resulting clusters to previously ascertained astrocytic and neuronal lipid markers, and visualize the localization of different lipid classes. Each cluster is represented by a different lipid profile while still having some conserved lipid features, such as PC(32:0). Some of the spectral features were located only in a small number of cells, perhaps relating to rare functions possessed by specific brain cells.

We focused on profiling lipids in this study because of their functional importance within the brain, and also to address the significant technical difficulties surrounding lipidomics, particularly at individual cellular resolutions.^{72–74} Our ability to characterize thousands of cells for their lipid contents enables lipid heterogeneity to be examined at an unprecedented level, and facilitates follow-up studies to link the cellular phenotype to the transcriptome and organismal state. Of course, these approaches can be expanded to include additional molecular classes, such as metabolites, peptides, and proteins. A last exciting aspect of this work is the ability to target the rare cells highlighted here for follow-up analysis using other analytical approaches, such as CE-MS³⁹ and vibrational spectroscopy.⁴¹ This multimodal analysis is made possible because MALDI MS leaves enough sample material behind to perform these enhanced classification schemes.⁴⁰ While the described approach can easily be optimized for other brain regions and animal models, the future of these experiments lies in the quality and abundance of the chemical information held within each single cell that

can be acquired and used to tackle the poorly understood lipidome. Using the data obtained, we can relate lipid profiles to known cellular functional and morphological differences among canonical cell types that have yet to be understood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Visualization of lipid diversity via t-SNE analysis. **A)** Single cells within the rat cerebellum can be classified into 101 clusters based on their spectral features within the lipid mass range. The clustering is based on lipid content and/or relative signal intensity. Information from ~30K single cells is visualized using a t-SNE plot and clustered using the Louvain-Jaccard method. Each cluster is colored and numbered. Cluster 1 contains the most cells (882 cells); clusters 100 and 101 contain the least (31 cells in each). **B)** t-SNE plot recolored to show the correlation of individual cells to neuronal and astrocytic lipid profiles that have been previously determined.³⁸

Figure 2.

Visualization of the abundance of different lipid classes via t-SNE analysis. **A)** t-SNE recolored by the relative amounts of the phosphatidylcholine, **B)** sphingomyelin, and **C)** phosphatidylglycerol lipid species to visualize the localizations of lipid classes.

Figure 3.

Average spectra of all cells located within their respective clusters. Visual observation of the clusters shows detectable spectral diversity that can be extended to all 101 clusters. Spectral assignments are provided in Table S1 in m/z order, as well as averages for each cluster (Figure S3). Only eight are shown here for clarity.

Figure 4.

Analysis of thousands of cells uncovers rare cells with unusual endogenous lipids. **A)** Spectral features/lipids that are detected within less than 6.5% of the analyzed cerebellar cells as determined by reverse principal component analysis. A variety of lipid classes are represented within the plot, including PE, PGs, and HexCer. **B)** Two single cell mass spectra that highlight two of the rare spectral features, PC(34:1) and PG(40:2(OH)), within two cells from the dataset.