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Towards Understanding Region-Specificity of Triplet Repeat Diseases: Coupled Immunohistology and Mass Spectrometry Imaging

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Summary

Many trinucleotide repeat disorders exhibit region-specific toxicity within tissues, the basis of which cannot be explained by traditional methods. For example, in HD, the toxic disease-causing protein is ubiquitously expressed. However, only the medium spiny neurons in the striatum are initially targeted for death. Many changes are likely to initiate in these cells at an intracellular and microstructural level long before there is a measureable phenotype, but why some regions of the brain are more susceptible to death is unknown. This chapter describes a method to detect functional changes among brain regions and cell types, and link them directly with region-specific physiology. Due to the neurodegeneration that accompanies many of triplet repeat disorders, we focus on the brain, although the methods described in this chapter can be translated to other tissue types. We integrate immunohistology and traditional mass spectrometry with a novel mass spectrometry imaging technique, called nanostructure initiated mass spectrometry (NIMS). When used together, these tools offer unique insights into region-specific physiology of the brain, and a basis for understanding the region-specific toxicity associated with triplet repeat disorders.

Keywords

Mass Spectrometry Imaging; Immunohistology; Cryosectioning; Multivariate Analysis; Metabolite; Brain; Mouse; Anatomy

1. Introduction

Regional toxicity remains a puzzling and poorly understood feature of neurodegenerative diseases, including Huntington's, Alzheimer's and Parkinson's (1–6). Defining the

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functional basis for region-specific toxicity holds the key to understanding why some cells live and others die (7, 8). Three substantial barriers have made this approach difficult. First, cell death in many neuropathologies are limited to certain cell-types among surrounding unaffected cells within specific brain regions (9). Without methods for spatial resolution, the properties of susceptible cells in specific regions of the brain cannot be distinguished from unaffected cells, whose cell metabolism, composition, and functions are likely to differ. Second, the progression towards cell death is often accompanied by structural changes to the microenvironment including gliosis, an overcompensation of astrocyte growth in wound areas (10, 11). In addition, stem cells may migrate to replace dying neurons (12). Thus, it is unknown whether cellular dysfunction can be meaningfully measured amongst the changing environmental profile of cell-types with age or disease progression. What might possibly be interpreted as defects in mixed populations may reflect natural variations among cell-types as the tissue ages. Third, cells *in vivo* operate within the context of the local microenvironment. Yet, most information about dysfunctional cells has been derived from the properties of dispersed cells (13). Conventional methods require the pooling of millions of cells, which “averages out” regional or cell-type specific differences, and separate a particular cell from its neighbors (i.e. FACS, Western Blotting and PCR) (13). Thus, isolating cells separates them from the very cell-cell communication to which they must respond. High-resolution spatial methods are needed to dissect the integrated dynamics among cells.

Nanostructure-initiator Mass Spectrometry (NIMS) can spatially resolve the metabolite distribution in an intact tissue slice without disruption of the tissue architecture (14). NIMS works by absorbing metabolites onto a vacuum compatible ‘initiator’ liquid in the pores of a nanostructured surface (15). The surface, when irradiated with a laser, vaporizes the pore-trapped initiator and triggers desorption/ionization of surface absorbed analytes in the tissue. As the laser rasters across the tissue surface, m/z ions are collected at each point and the complete mass spectrum is archived for retrieval (*see* Fig. 1). Aligning each pixel in a two-dimensional, x-y coordinate position along the entire tissue creates a metabolic tissue image. Any ion of interest can be viewed later for imaging and analysis. Thus, NIMS provides region-specific ion composition (14–16). By aligning the mass spectrometry images with histological maps, NIMS profiling can form the basis from which we define functional changes associated with region-specific toxicity and lead the way to therapeutics. NIMS can be applied to any disease state, to any tissue section, and is ideal to sort out complex changes. Collectively, the visualization and computational methods described in the following chapter can yield the underpinnings of region- and cell-type specific metabolic changes. The methods discussed in this chapter couple traditional immunohistochemistry with mass spectrometry methods, including NIMS, to create a highly correlated metabolic/microstructure map of a disease state.

2. Materials

2.1 Tissue Dissection and Preparation

1. Guillotine (Antron Small Animal Guillotine – VWR 5000–450)
2. DecapiCone (Braintree Scientific - MDC-200)

3. Scissors (ToughCut Straight/Sharp-Sharp/9 cm Fine Science Tools 14058–09; Dissector Scissors Heavy Blades/Angled to Side/ Sharp-Sharp/10.5 cm 14082–09)
4. Forceps (FST by Dumont #7 curved forceps)
5. Dissection Scope (Olympus SZ61)
6. Sagittal Brain Matrix (Zivic Instruments BSMAS005–2)
7. Double Edge Razor Blades (Zivic Instruments BLADE010-D)
8. Leica CM 1950 Cryostat with Vacuum attachment (Leica 141950OC3US)
9. Large Well Mounting Block (Leica Embedding well bar – 14020139120)
10. Dispensing Slides (Leica 14020139127)
11. Mounting Chuck (Leica Specimen Disc 37×37 – 14047743763, 28×28 – 14047742604)
12. Heat Extractor (Leica Dr. Peter's Assembly, 14047741338)
13. O.C.T. Compound (Tissue-Tek 4583)
14. Glass Histoslides (VWR Microscope Slides, 16004–408)
15. Glass Insert (Leica 70 mm, 14047742497)
16. Anti-roll plate (Leica 50 um, Leica 14047742491)
17. Finger rest assembly (Leica 14047740387)
18. Specimen Stage (Leica 14047742604)
19. High Profile Microtome Blades (Leica 818 – 14035838926)
20. Brushes (fine – 14018328642, large – 140183 40426)

2.2 Histology and Imaging

1. PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 adjusted to pH 7.4
2. PBSTx: PBS + 0.1% Triton $\times 100$
3. Blocking medium: PBSTx+ 5% Horse Serum
4. Antibodies (Ms $\times$ MAP-2 Millipore MAB3418l; Alexa Fluor 633 goat anti-mouse Invitrogen A21050; Ms $\times$ Neuronal Nuclei Alexa Fluor 488 Millipore MAB377X; Ms GFAP-Cy3 abcam ab49874)
5. Vectashield (Vector with DAPI, H-1200)
6. Zeiss 710 Microscope
7. Cresyl Violet acetate (Sigma C5042)

2.3 NIMS and MSI Imaging

1. Absciex 5800 with 4800 Imaging Tool Software
2. NIMS Chips (Etched as described below, Siliconquest 708-029INV)
3. Diamond-tip scribe (Techni-Tool, cat. no. 758IE222)
4. Stainless steel tweezer (Techni-Tool, cat. no. 758TW106)
5. Stainless steel ruler (Techni-Tool, cat. no. 400PR124)
6. DC Power supply, 0 – 15V, 3 A (HQ Power Model No. PS1503SBU)
7. Nanoinitiator Substrate (bis(heptadecafluoro-1,1,2,2-tetrahydrodecyl) tetramethyl-disiloxane, Gelest Morrisville, PA)
8. Modified MALDI Well Insert (ABSciex Opti-Toff 384-Well Insert 123×81mm 1016491)
9. Custom-made Teflon etching cell (as described) with gold foil (ESPI, 0.004" thick, 3N5 purity) and platinum wire (ESPI, 0.02" diameter, 3N5 purity)

2.4 Traditional MS

1. Microcentrifuge Tubes (MBP 3458)
2. Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA)
3. SpeedVac (Thermo Fisher, Waltham, MA, USA)
4. MSFTA and TMCS (ThermSci TS-48915)
5. Gerstel automatic liner exchange system
6. Gerstel CIS cold injection system (Gerstel, Muehlheim, Germany)
7. Agilent 6890 gas chromatograph (Santa Clara, CA)
8. Rtx5Sil-MS column (Restek, Bellefonte PA, 0.25 µm 5% diphenyl film and additional 10 m integrated guard column)
9. Leco Pegasus IV time of flight mass spectrometer (St. Joseph, MI)

2.5 Data Analysis

1. MATLAB (The MathWorks; Natick, MA)
2. R (S.Urbaneck and S.M.Iacus, R Foundation for Statistical Computing)

3. Methods

3.1 Tissue Preparation for Coupled MSI and Histology: Segmentation, Sectioning and Transfer

Analysis of metabolites requires precise and rapid handling of tissue. Storing harvested samples for later use depletes sensitive metabolites and decreases spatial resolution. Thus, our laboratory focuses on a 10 minute maximum time window from animal sacrifice to

‘cryostat frozen’ sample, and we image the NIMS mounted tissue section the same day. Immunohistology can be performed on appropriately stored samples for up to 3 months. To attain samples appropriate for immunohistology, chemical staining, and MSI (mass spectrometry imaging), we use fresh, unfixed tissue.

3.1.1. Tissue Segmentation—For the most humane, rapid sacrifice, we restrain animals in a DecapiCone disposable mouse restrainer and rapidly decapitate the mouse using a small animal guillotine (*see* Note 1). Brains are placed on a pre-chilled sagittal brain matrix (*see* Fig. 2A), oriented for linearity along the midline axis, and segmented ± 2 mm from the midline (*see* Fig. 2B). Segmentation in the sagittal orientation provides representation from 4 regions of interest in our laboratory: the cortex, hippocampus, striatum, and cerebellum. Cortical or axial matrices are also commercially available; alternative orientations or tissue types do not critically impact the procedure. The range from 2mm inward towards the midline yields a volume in which a trained cryostat user can calibrate slicing for optimal representation from each of these regions without encroaching upon the midline. This thickness also yields enough residual material (post sectioning) for further mass spectrometry analysis (*see* section 3.4).

3.1.2. Tissue Sectioning—In accordance with Dr. Peter’s method (17), tissue segments (2 mm thick) are transferred to a dispensing slide lightly coated in OCT with the midline facing upwards (*see* Fig. 2C). Tissue is then transferred to a chilled (-15°C) well chuck with the midline facing upwards (*see* Fig. 2D). The well is then filled with OCT, covered with a chuck (*see* Fig. 2E), and placed in the cryostat chamber to solidify for 15–30 minutes (with the heat extractor mounted on top of the chuck). The chuck-mounted tissue section is then extracted from the well using the heat extractor and surface defects corrected with a thin coating of OCT. The chuck is allowed to equilibrate for a minimum of 30 minutes before sectioning (*see* Fig. 2F).

During this equilibration period, the cryostat is arranged. For $5\mu\text{m}$ sections, a $50\mu\text{m}$ anti-roll plate is used in place of the $70\mu\text{m}$ plate. A fresh blade is placed in the blade mount and covered with either the blade protector or the anti-roll plate. The vacuum attachment is optional; some users find it helpful while others prefer not to use it. The anti-roll plate will have to be adjusted frequently whether or not the vacuum attachment is in place (*see* Note 2).

Because NIMS utilizes a technique in which the laser must pass through the tissue to the underlying nanostructure, $5\mu\text{m}$ sections are an absolute necessity (*see* Note 3). To attain these sections, adjust the anti-roll plate and note the temperatures of the chamber and the chuck mount (for brain, we have had success with -15°C and -12°C respectively). At least one section is used for histology and the immediately contiguous section is used for NIMS (*see* Fig. 2G–J). Approximately 7 – 10 slides (with 2 brain sections per slide) are collected on either side of the chip-transferred section for extra histology samples. This yields approximately $100\mu\text{m}$ of tissue on either side of the NIMS slice. NIMS chips are taken for immediate analysis a glass slides are dried at room temperature overnight or for a minimum of 3 hours at 37°C (*see* Note 4).

3.2. Histology and Imaging

We utilize traditional immunohistochemistry and chemical staining techniques to show gross morphology. These methods can be applied to most antibodies and chemical stains.

3.2.1. Immunohistology—To dehydrate the tissue and remove OCT, immerse the dried slides in cold (−20 °C) acetone for approximately 10 minutes. OCT can then be carefully removed with tweezers, leaving only slide-affixed tissue. Tissues are then rehydrated in PBS for 5 minutes at room temperature and blocked in PBS containing 0.1% Triton-X 100 (PBSTx) and 5% Horse Serum for 1 hour at room temperature. As an example, antibodies against glial projections (anti-Glial fibrillary acidic protein, anti-GFAP-Cy3), neuronal nuclei (anti-neuronal nuclei, anti-NeuN-Alexa 488) or neuronal projections (anti-microtubule-associated protein 2, anti-MAP2) are used to visualize brain regions. For 4-color stains containing MAP2, NeuN, GFAP and DAPI (a fluorescent chromatin stain), sections are incubated with anti-MAP2 (diluted 1:200 in PBSTx) for 30 minutes, washed 3 times in PBSTx for 10 minutes each, and incubated with goat anti-mouse-Alexa 633 secondary antibody for 1 hour at room temperature. Secondary antibody is rinsed out with an overnight wash in PBSTx. The tissue is then stained with anti-GFAP (1:400 in PBSTx) and anti-NeuN (1:300 in PBSTx) antibodies for 30 minutes. After washing (3 times for 5 minutes in PBS), slides are mounted in Vectashield containing DAPI and imaged on a Zeiss LSM 710 confocal microscope. For whole brain images, z-stacked, tiled images were obtained with a Plan-Apochroma 20x/0.8NA objective, and a maximum intensity projection of the z-stacks was isolated to adjust for focal plane shift over the sample. If immunohistology staining in which only a 3-color staining is employed eliminate the anti-MAP2 and secondary antibodies; the remaining protocol is the same. When stained for neurons and glial with NeuN, GFAP and DAPI (as above) the intricate microstructures in whole brain (*see* Fig. 3C) and the hippocampus (*see* Fig. 3D–F) are apparent.

3.2.2. Chemical Methods—Cresyl violet may be used to quickly stain brain to show general morphology based on cell density (*see* Note 5). Cresyl violet is diluted to 1% w/v in water containing 1% glacial acidic acid; for staining, OCT from slide-mounted samples is removed with acetone as described above and the slide is air-dried for 15 minutes. A dried slide is placed in a 50 mL falcon tube containing 20 mL cresyl violet solution. Invert the tube 3 times, wick off additional cresyl violet solution with a Kim-wipe and transfer the slide to a primary wash bath (20 mL water in a 50 mL falcon tube) followed by a secondary wash bath (30 mL water in a 50 mL falcon tube). To wash, invert the tubes 3 – 5 times until excess stain is fully removed. To destain, transfer slides to a 50 mL falcon tube containing 70% ethanol, invert the tube twice and transfer the slide to a 50 mL falcon tube containing 100% ethanol (optional). Dry the slides and image. We use a simple 5 megapixel camera (Canon Rebel T1i) attached to a dissection microscope. H&E staining, according to generally recognized protocols, uses more toxic solutions but can give more information about inflammation; this staining method can be employed for certain disease states.

The position and morphology of brain regions are apparent based on cell density, as visualized by cresyl violet (*see* Fig. 3B). Areas of high neuronal density are particularly prominent (dark purple) (*see* Fig. 3B), while areas rich in glia are lightly stained. Specific

antibodies and Nissl staining are highly effective in distinguishing glial or neuronal patterning and cell density in specific brain regions. The region-specific structures highlighted by our stains correlate well with the Mouse Brain Atlas (*see* Fig. 3A) (18).

3.3. NIMS and MSI Imaging

3.3.1. Chip Preparation—The preparation of NIMS chips has been described in detail elsewhere (19). Cut 4'' silicon wafers (single-sided polished, p-type, Boron-doped, orientation <1-0-0>, resistivity 0.01–0.02 Ω cm, thickness 525 ± 25 μ m) into a 70 $\times$ 70 mm square. Prior to etching and coating, wash the chips with 100% methanol solution. Etch the chips with 25% hydrofluoric acid in ethanol using a customized Teflon etching chamber under constant current of 2.4 amps for 15 minutes (*see* Note N). Coat the etched chips with 200 μ l of initiator liquid (bis(heptadecafluoro-1,1,2,2-tetrahydrodecyl) tetramethyl-disiloxane). Alternative initiator liquids can be substituted to alter the profile of ionized metabolites (19). The initiator mentioned above preferentially ionizes brain lipids of particular interest to our lab (20). Once prepared, chips can be stored at room temperature for several months without signal degradation.

3.3.2. Imaging mass spectrometry—Once tissue transfer to the chip is completed, NIMS mass spectrometry imaging can be performed on any mass analyzer system. We use a 5800 TOF/TOF mass analyzer system in positive reflector mode. Spots of calibrant material (containing either a known range of lipids or other appropriate metabolites) are arranged around the corners of the tissue slice. This arrangement allows for chip flattening in which the spots allow for mass adjustment and calibration. We apply the third harmonic generation of a Nd:YAG laser (355 nm) at a repetition rate of 200 Hz with 25 shots per spot. Collect a full mass spectrum ranging from 50 to 2000 m/z for every pixel at an x-y step-size of 50 μ m. Imaging data is stored in the Analyze 7.5 data format (Mayo Foundation; Rochester, MN) by the Absciex MS software, which is a binary format described below.

3.4. Coupled Traditional MS Analysis

NIMS provides the spatial variation of ions over tissue, however it has limited sensitivity for certain ions and reports a lower diversity of ions than more traditional methods. Tissue remaining from the sectioning can be truncated into regions to perform more traditional metabolite profiling, such as GC- and LC-MS (*see* Note 7).

3.4.1. Sample preparation—Collect microsections of the remaining tissue (approximately 1 mm thick) using a scalpel, transfer each section to pre-weighed microtubes, and record the tissue weight. Lyophilize wet tissue until dry, record the dry weight, and homogenize the tissues using a Mini-Beadbeater. Metabolites, including lipids from these brain regions, can be extracted with a pre-cooled methanol:isopropanol:water (3:3:2 v/v) mixture. Collect the supernatant and dry using a SpeedVac.

3.4.2. Derivatization—Derivatize the extracts with 10 μ l methoxyamine in pyridine for 1.5 hours at 30 $^{\circ}$ C, at 850 rpm in a Thermomixer. To each tube add 90 μ l MSTFA (N-Methyl-N-trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) and 1 μ l FAME (fatty acid methyl esters- 0.8 mg/ml for C8 - C16 and 0.4 mg/ml for C18 - C30).

3.4.3. GC-MS Analysis—We inject 0.5 μ l of each sample into an automatic liner exchange system with multipurpose sample MPS2 dual rail using splitless mode into a cold injection. Injected analytes are separated using an Agilent 6890 gas chromatograph, with a 30 m long, 0.25 mm i.d. Rtx5Sil-MS column. Our mass spectrometry information is collected with by a Leco Pegasus IV time of flight mass spectrometer with 280 °C transfer line temperature, an ion source temperature of 250 °C, and electron ionization at -70 eV, although other systems can also be employed. We collect mass spectra from m/z 85 to 500 at 17 spectra s^{-1} with a detector voltage of 1850 V detector. (*see* Note 8)

3.4.3. Data Processing—Result files should be preprocessed after data acquisition and stored as ChromaTOFspecific *.peg files, as generic *.txt result files, and additionally as generic ANDI MS *.cdf files. We typically process these files using a metabolomics BinBase database (21). All database entries in BinBase are matched against the Fiehn mass spectral library of $\sim 1,200$ authentic metabolite spectra using retention index and mass spectrum information or against the NIST05 commercial library (*see* Note 9). We only report identified metabolites if they are present within at least 50 % of the samples per study design group (as defined in the SetupX database) (22). Alternatively, open-source software is available to partially or completely produce the data processing capabilities mentioned above. Deconvolution and peak alignment is performed using AMDIS and Spectconnect (*see* Note 10). For a more general approach, XCMS (23) and MZmine (24) cover a broad range of data processing methods, including data import and export, irrespective of the instrumental platform that is being used.

3.5 MSI Data Analysis

3.5.1. 2D Image Construction and Statistical Analysis—For simple display of a single m/z image, the Analyze 7.5 format can be opened either on TissueView, an ABSciex proprietary program, or BioMap, which runs on an IDL virtual machine. Because these data sets are large, programs often bin data to save computational time. Ions have to be visualized manually in either of the above programs and simple images (*see* Fig. 4A) can be exported as *.tiff files for later use. For example, the complex microarchitecture of the cerebellum and hippocampus can be roughly visualized with single m/z peaks (*see* Fig. 4B,C).

The m/z data-set for a given point or a region of interest can be exported as an image in *.tiff or as a *.txt, which is compatible most plotting software, such as Excel, or data analysis programs such as MeV. For example, to compare brain regions within a single brain slice image, the m/z values for points within each of regions (cortex, hippocampus, striatum, and cerebellum) can be exported and used to visualize statistical significance. This type of analysis can compare regions within a single slice or between slices, for example between regions within the brain of Huntington's Disease model animals and breed-matched control animals.

3.5.2 Multivariate Analysis Techniques—For more complex multivariate analysis using the entire MSI data set, the data must be imported into an applicable analysis program. We chose to use R, an open-source programming tool with many available statistics packages. All programming scripts are open-source and available either as downloadable

packages for R in the CRAN source-base or on the McMurray Lab website. One challenge in utilizing the open-source R program is its limited memory processing abilities; it runs slowly for large data sets. Thus, we use a combination of MATLAB (for initial data manipulation) and R (for statistical evaluation). Similar methods can be used in other programming languages.

An R script package is available online, which allows read-in of the three files (*.hdr, *.img, and *.t2m) to yield the total image information. The header file (.hdr) yields the dimensions of the image given in the .img file (*see* Note 11). The *.img represents a three dimensional binary matrix ($x \cdot y \cdot m/z$), where the third dimension is the intensity for each m/z value. The values of each m/z are stored in the .t2m file.

To identify peaks, we first imported the large dataset into MATLAB. An open-source MATLAB script reads the spectra at each x-y coordinate of each position and stores the intensity information in a 2D array (*see* Note 12). One dimension of the array represents all the pixels and the other dimension represents all the m/z intensities. As mentioned above, we acquire the m/z length from the .t2m file. We read in the image file and resample it, allowing us to bin the data if desired and set limits (in m/z) for the metabolic region of interest (*see* Note 13). Following a baseline correction, we smooth the peaks using a default MATLAB protocol with mslowess. Peaks are chosen using MATLAB's peak finding algorithm. MATLAB returns 2 arrays in sparse format. A script reconstitutes this data into binary file that corresponds to the data type of the original file.

The reduced dataset can be imported into R using the readBin command (with a little endian and a single size of 4 for the .t2m and a integer size 2 for the .img) (*see* Note 14). Once MSI data is appropriately loaded, library packages can be used for a variety of multivariate statistics. As an example, we briefly describe here analysis of a single brain slice using non-negative matrix factorization (NMF), k-means clustering, and image principle component analysis (iPCA).

PCA and k-means clustering analysis are built-in to the R stats package and have been described for MSI extensively (25, 26) (*see* Note 15). For either method, arrange the data set in a matrix in which the dimensions are the number of m/z points by the number of x-y points in the image. In the k-means cluster analysis (27), the data is partitioned into k groups, defined by the user, in a way that minimizes the sum of squares from points to their cluster centers. The default algorithm is based on that of Wong (28), and other algorithms are available. Pixels can then be pseudo-colored based on their assigned clusters and the average m/z plotted using a short, user written summary script (*see* Fig. 5). Pixels can be similarly clustered based on PCA scores using the prcomp script (27). NMF is a script package that solves a set of matrixes (W and H) iteratively to reduce the value of an objective function. Various NMF algorithms (such as the brunet algorithm (29)) are available in the NMF package. Each algorithm utilizes specific optimization techniques and seeding methods that are described in the package manual. An NMF image can be created based on the pixel loading (in H) of each particular component (in W) and requires loading an additional graphics package into the R workspace. More elaborate clustering algorithms,

which take spatial similarity into account, are also available (30) but do not have open-source packages.

4. Notes

1. For animal handling, the brain is harvested via decapitation and removed for preliminary segmentation. Animals are sacrificed without anesthesia to prevent chemical alterations of metabolic profiles. Thus, this method must be completed only by a trained professional, extensively evaluated, and approved by your local IUCAC. The mice are decapitated 2.5 mm distal to the ears. Brains are removed by cutting through the occipital, post-glenoid foramen, basisphenoid, and zygomatic bones.
2. For tissue sectioning, the glass insert must be checked for damage before each use of the cryostat. It may be advantageous for frequent users to maintain a personal glass insert. Often, newer cryostat operators use the anti-roll plate as a hand rest while collecting samples. This activity bends the anti-roll plate arm and pushes the glass insert against the blade ejector (often chipping the glass). Although the best solution is to avoid use as a hand rest, placing electrical tape around the blade ejector can protect the glass insert but does not prevent bending of the anti-roll plate arm.
3. Sample integrity is critical. We prepare the tissue to in a manner that simultaneously optimizes for both histological and metabolic profiling.
4. Because of brain has a dynamic range of tissue sub-architecture, orientation is extremely important for our analysis. For other tissues more traditional methods for orientation could be useful. For example, creating a small box out of tinfoil and orienting the tissue within unfrozen OCT may be a simpler solution for some tissues. More structurally robust tissues, such as bone, can be attached directly to the cryostat chuck with a small amount of OCT.
5. Cresyl violet solution stains almost any surface or material dark purple, lay down absorbent material on your workspace and use dedicated or disposable containers. Although soluble in ethanol, washing cresyl violet staining can be difficult.
6. CAUTION: Hydrofluoric acid is very corrosive and poses a very real health risk. Extreme caution and care should be used when handling this chemical. Check with your EH&S regarding institutional handling and disposal regulations.
7. Should residual tissue mass from cryosectioning be insufficient, or OCT integration into the tissue reduce ionization signal, additional tissue can be dissected into regions (or used whole). This method, however, decreases the direct association with the imaging data.
8. As with all mass spectrometry, parameters will need to be optimized for the system, tissue and running protocol.

9. NIST05 Commercial Library - (<http://fiehnlab.ucdavis.edu/Metabolite-Library-2007/>). AMDIS - (www.nist.gov). Spectconnect - (<http://spectconnect.mit.edu/>).
10. While reading in the *.hdr file in R (to determine dimensions), care must be taken. If the *.img length is greater than $2^{16}-1$ the *.hdr will return an inaccurate value for the *.t2m length. Thus, it is imperative that the length of the *.t2m file be verified independently using R, MATLAB or other software.
11. We mostly follow the procedures outlined in the Preprocessing Raw Mass Spectrometry Data example (<http://www.mathworks.com/products/bioinfo/demos.html?file=/products/demos/shipping/bioinfo/mspreprodemo.html>).
12. R is an accessible statistical programming language. A basic, functional knowledge of its use can be beneficial for most biology-based researchers (19, 20). As with Biomap, R can be used to display a single m/z image or the m/z spectrum for a single point. However, R is particularly useful as a multivariate analysis tool because open-source package libraries are readily available for many statistical applications.
13. The data set is typically reduced to the range 600 – 950 m/z , a range representative of phospholipids (PE, PC, and PS), which are of interest for our research.
14. The R packages mentioned are thoroughly annotated in their respective R packages manual (NMF and the preloaded stats packages respectively); other multivariate analysis script packages are also available. Take care when loading new packages as some open-source R code is similarly robust.

5. Future Directions

The NIMS technology is on the verge of several groundbreaking advances. First, with faster computational processing and improved imaging throughput, 3 dimensional tissue maps can be constructed which correlate to histological images. This would allow for searchable mapping of entire tissue structure databases. Further, as the Mass spectrometry imaging technology improves, the option for single-cell analysis becomes a realizable possibility. Currently, the highest spatial resolution can be achieved by secondary ion MS (SIMS). However, this technique requires high-energy particles and leads to destruction of most metabolites, which causes extreme difficulty during metabolite identification. Alternatively, high-resolution mass spectrometry (e.g. the Fourier transform ion cyclotron resonance mass spectrometer) coupled to MALDI MS can generate images with a lateral resolution of $0.6 \times 0.5 \mu\text{m}$ (when coupled to a scanning microprobe MALDI source) (31). Further, ion microscopy can improve lateral image lateral resolution to $4 \mu\text{m}$ (32). Among the commercially available platforms, Bruker Daltonics Inc. has constructed new MALDI-TOF/TOF system, the UltrafleXtreme™ and the Smartbeam™ laser, which can reach $20 \mu\text{m}$ lateral resolution (33).

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Figure 1 –. Schematic for NIMS mass spectrometry imaging.

A laser rasters across a NIMS chip-mounted tissue, ionizing metabolites to create a complete m/z spectrum for each x-y position. Any given m/z value can then be visualized as intensity over x-y to produce a NIMS-MSI tissue image.

Figure 2 –. Schematic for tissue preparation and sectioning.

Brain is placed in a sagittal slicing matrix (A) and segmented ± 2 mm from the midline (B). Segmented sections are placed on a dispensing slide (C) and transferred to the well bar (D). Once covered with OCT, a mounting chuck is placed over the well (E), allowed to cool, and extracted for placement on the sectioning mount (F). Tissue sections (5 μ m) are transferred to either the NIMS chip or glass histoprep slides (G, H, respective magnifications I,J).

Figure 3 –. Brain histology reveals tissue microarchitecture.

A schematic highlighting the four brain regions of interest in our lab (A). Nissl staining (cresyl violet) showing gross tissue morphology (B) which corresponds to the immunohistochemical microstructure stained with anti-NeuN-Alexa 488, anti-GFAP-Cy3 and DAPI. A magnification of the hippocampus and corpus callosum (D) shows drastic microenvironmental changes illustrated by further magnification of this region (E, F).

Figure 4 –. MSI images correlated with immunohistochemistry.

Immunohistology corresponds to single m/z images for whole brain (A), the cerebellum (B) and the hippocampus (C).

Figure 5 –. Multivariate tissue MSI analysis.

An example of a 4 Component k-means clustering analysis performed in R on unreduced data.