Optimization of Sensitivity and Reproducibility of Mass Spectrometry Imaging and Its Application to the Study of ALS-Affected Tissue

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ABSTRACT

Optimization of Sensitivity and Reproducibility of Mass Spectrometry Imaging and Its

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Amyotrophic Lateral Sclerosis, or ALS, is the most common neuromuscular disorder, affecting approximately 5600 Americans each year. It is caused by the degeneration of both upper and lower motor neurons, and those diagnosed only have 2-3 years before death due to progressive muscle paralysis and respiratory failure. The underlying mechanism of the disease is unknown, though it is theorized to be a mixture of cellular processes such as genetics, oxidative stress and excitotoxicity, where nerve cells are damaged by excessive stimulation by neurotransmitters. There is no standardized form of diagnosis and no biomarkers for ALS. Discovery of biomarkers would help to properly diagnose those with ALS, as well as accelerate drug development. One method of finding a biomarker is through mass spectrometry, more specifically MALDI-MS imaging. MALDI-MS imaging creates a map of ion intensities across a given section of tissue based on protein concentration and spatial distribution. In this study, we optimize a set of parameters to use in the preparation of samples for MALDI-MS imaging, since there is no universal method yet developed. Optimization was performed with META (8:8:1:1 methanol, ethanol, acetonitrile and 0.1% TFA in water), a matrix solvent that fixes proteins in their respective locations in the tissue and found an optimum exists at 3 depositions at 20 mg/mL. We also discovered that the number of depositions of a given concentration of matrix solution makes a very large difference in the quality of spectra obtained, even more so than the total concentration of matrix on the tissue. This discovery was significant, and we then applied our discovery to a more general MALDI solvent – a 50/50 mixture of acetonitrile and 1% trifluoracetic acid (TFA) in water. We tested the same parameters – number of depositions and initial concentration to find that they also play a large role in the spectral quality obtained with this matrix solvent. The optimal conditions for this solvent existed at 5 depositions of 20 mg/mL. We also discovered that in addition to an effective matrix solvent, META is an excellent wash solvent. Use of a META wash before matrix application resulted in more sensitive and reproducible spectra, with higher intensity and lower variability and preserved fluorescence of transgenic YFP tissue. Optimal conditions were applied to a comparison study with G93A positive and control tissue in the motor cortex, and preliminary differences in spectra acquired were discovered. Future experiments will explore these differences further, both in the motor cortex and spinal cord to facilitate the discovery of a biomarker for ALS.

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Introduction

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Charcot's disease or Lou Gehrig's disease, is the most widespread type of motor neuron disease. It affects an estimated 5,600 people in the US per year and as many as 30,000 Americans have the disease at any given time. ALS is a neurodegenerative disorder characterized by progressive muscular paralysis as a result of the degeneration of upper and lower motor neurons. "Amyotrophy" refers to the atrophy of muscle fibers as motor neurons degenerate, whereas "lateral sclerosis" refers to hardening of corticospinal tracts as motor neurons in these areas degenerate.[2]

The upper motor neurons are located in the motor cortex or brainstem and function to relay information to the lower motor neurons. Upper motor neuron symptoms include weakness, speech impediments, overactive reflexes, spasticity, and/or inappropriate emotionality. The lower motor neurons are located in the brainstem and spinal cord, and relay impulses from the upper motor neurons to the peripheral nervous system. Lower motor neuron symptoms include weakness, decreased reflexes, cramps, twitching, and muscle wasting.[1] The mean age of onset varies between 55 and 65 years, with a median age of onset of 64 years. ALS patients usually die within 2-3 due to progressive paralysis and respiratory failure.[2] The majority of ALS cases occur sporadically, with no known cause or precursor. Only 5-10% o ALS cases are familial, meaning the disease is caused by inherited genetic mutations.[1]

ALS is more common in males, presumably due to male hormones and composition of the population as there are more male war veterans and smokers, both of which have higher reported incidence of ALS.[1] Approximately two-thirds of patients with typical ALS have a spinal

form of the disease, known as classical "Charcot ALS" or "spinal onset" with a survival rate of 3-4 years after onset. Classical Charcot ALS presents with symptoms related to localized muscle weakness in the upper and lower limbs. However, about a quarter of ALS patients experience "bulbar onset," where symptoms first present in the face, mouth/jaw and tongue muscles such as dysarthria of speech and sialorrhoea, or excessive drooling.[2] Bulbar onset is more common in women and in older age groups, and is associated with a poorer prognosis, with patients often dying within one year.[1]

There is no single test for the diagnosis of ALS, rather diagnosis must be treated more as a process of elimination to ensure the patient is not suffering from one of a myriad of different motor neuron disorders. About 10% of patients with other disorders are diagnosed incorrectly with ALS.[3] Standards to help diagnosis and classify patients, known as the "Airlie House" criteria, have been develop by The World Federation of Neurology (WFN) Research Group on Motor Neuron Diseases for research studies and drug trials. Based on these criteria, patients can be classified into "clinically definite," "clinically probable," "clinically probable-laboratory supported," and "clinically possible."[8] However, these standards are not very useful for clinical practice, and are better suited for research purposes and therapeutic trials.

Although most cases of ALS are sporadic, about 5-10% of cases are familial. Of these familial ALS (fALS) cases, 20% have a mutation in the superoxide dismutase 1 (SOD1) gene.[1] Mouse models have been developed to study this small subset of ALS instances to gain a better understanding of the mechanism of the disease. To date, more than 140 mutations in SOD1 have been identified in cases of familial ALS, most of which are point mutations.[5] SOD1 is a homodimeric metalloenzyme that catalyzes the dismutation of superoxide radicals produced by cellular metabolism to O_2 and H_2O_2.[6] SOD1 contains an active site that binds copper, a binding site for zin, an electrostatic loop that funnels in the substrate, and an intramolecular disulfide

bond between cysteines. The effects of fALS mutations on normal enzyme activity vary considerably, as mutations in SOD1 do not cause ALS as a loss of function, and they increase the tendency for protein aggregation.[7] It is hypothesized that the destabilization of the dimer causes nucleation of higher order aggregation of monomers and form inclusion bodies.[8]

The exact molecular pathway causing the motor neuron degradation seen in ALS is unknown, but it is likely to be an extremely complex mechanism that involves multiple pathogenic cellular mechanisms.[2] There are genetic factors, including mutations in numerous other than SOD1, such as OPTN, ANG, and UBQLN2. OPTN encodes optineurin, a multifunctional cytosolic ubiquitin-binding protein involved in a host of different pathways, including vesicular trafficking and autophagy.[1] A recent study has shown that about 3.5% of sporadic ALS and 1.2% of familial ALS patients had mutations in the OPTN gene.[16] Another gene implicated in ALS, ANG encodes angiogenin, a small ribonuclease A involved in angiogenesis, the process through which new blood vessels are created from pre-existing vessels. Angiogenin promotes cell survival in motor neurons and is mutated in a small number of fALS and sporadic ALS cases.[17] ANG mutations cause a loss-of-function phenotype and cause ALS patients to present with bulbar onset, a form of the disease associated with a poorer prognosis. Recently, an additional gene linked to ALS, UBQLN2 has been found to cause X-linked dominant fALS, but is not yet implicated in sporadic ALS. The encoded ubiqilin-2 protein normally tethers proteins destined for degradation to the proteasome, though mutant-UBQLN2 form inclusion and correlate with instances of dementia in 20% of the X-linked ALS patients.[1]

Oxidative stress could also play a role, as accumulation of reactive oxygen species (ROS) causes cell death. Mutations in the anti-oxidant enzyme, superoxide dismutase 1, gene has been shown to cause familial ALS.[1] It is possible that the mutations in SOD1 impair its anti-oxidant activity, causing a buildup of ROS. It is the this toxic gain of function, rather than the loss of

SOD1's normal function which is generally regarded as causing motor neuron death. Studies have also shown impaired axonal transport as a result of SOD1 mutations. There is evidence of slowed anterograde and retrograde transport in SOD1 transgenic mouse models of ALS.[9] Mutations in transport protein genes such as kinesin are known to cause neurodegenerative motor nerve diseases in humans like hereditary spastic paraplegia,[10] so it likely that other transport proteins may be implicated in ALS. In addition, other molecular pathways have been connected to ALS, such as abnormalities in mitochondrial morphology and biochemistry, which have been reported in sporadic ALS cases.[11] There is also indication of both neurofilament and protein aggregation found in axonal and intra-cytoplasmic inclusions, respectively, in both sporadic and familial ALS.[12]

Excitotoxicity is the damage of cells due to excessive stimulation of receptors by neurotransmitters such as glutamate. In ALS, it has been shown that there is neuronal injury due to excessive stimulation of postsynaptic glutamate receptors, such as cell surface NMDA and AMPA receptors. This results in a large influx of calcium into the neurons, leading to increased nitric oxide formation and thus neuronal death.[13] The irregular glutamate metabolism is targeted by riluzole, the only drug approved for the treatment of the general symptoms of ALS.[14] The drug is thought to inactivate sodium channels, inhibit glutamate release, and block postsynaptic actions of NMDA receptors. Thus the levels of glutamate will be decreased and motor neuron function will be preserved. Limb and bulbar function are partially preserved, but actual muscle strength is typically not improved. However, riluzole only adds about 2-3 months to patient survival and only increases the chance of an additional year of survival by 9%.[15]

Need For Biomarkers

Although great progress has been made in understanding the underlying mechanisms of ALS, the cause of 90% of all ALS cases is unknown and there are currently no biomarkers for the disease. Biomarkers are characteristics that can be objectively measured as indicators. Traditionally, there are two different types of biomarkers: those that signal the disease, and those used to determine whether treatment is efficacious and to what to degree. Biomarkers can range from a traceable compound injected in a patient to examine organ function, detectable compounds such as antibodies that indicate the presence of a certain disorder, or imaging studies that can discern the formation of foreign masses or monitor the spread of a disease.

There is a pressing need for both types of traditional biomarkers, first to identify those with the disease so diagnosis is relatively simple and rapid. As previously stated, there is no single test to diagnose ALS, and a biomarker for this disease could eliminate many painful and costly tests to patients and potentially catch the disease in early stages. Prognostic biomarkers, which serve as rapid proxies for a future event such as death, are also needed because they vastly accelerate drug development for the eventual treatment of ALS. It has been shown that development of treatment of diseases with known biomarkers can increase up to 20 times faster by enabling proof-of-concept studies early on in the drug discovery process and accelerating clinical trials.[18] Various studies have been conducted to search for biomarkers of ALS, including one that compared different proteins present in the cerebrospinal fluid (CSF) of ALS patients compared to healthy subjects. They used 2D electrophoresis and MALDI-TOF technology to identify Parkin-like and iron/zinc binding proteins. Identification of these proteins could be a significant step in ALS research, however, the proteins and their function is still unknown.[19] Thus, there is still a lot of improvement to be made in the search for biomarkers of ALS.

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry Imaging

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry utilizes a matrix, a small acidic aromatic molecule that absorbs energy at the same wavelength as the irradiating laser, which is mixed with the analyte to form matrix-analyte co-crystals. These crystals are then subjected to laser pulses, resulting in the desorption and ionization of the analyte.[20] The ionized molecules are then separated based on time-of-flight (TOF) and analyzed by the mass spectrometer. Unlike other mass spectrometry techniques, one can easily analyze relatively complex mixtures from samples that are not particularly pure.[21] MALDI-MS has revolutionized the study of biology and biochemistry by permitting sensitive, rapid and molecular specific analyses of peptides and proteins.[22] However, proteins must be obtained from a complex biological sample via isolation and purification procedures which can be complicated and result in significant losses of material.[23] Because of the limited dynamic range of MS, a molecule that is present in only a small part of an organism, tissue or region would not be detected in a tissue homogenate[24], which would offer little information about protein spatial localization and expression levels within the tissue.

Analytical techniques with high sensitivity and throughput are needed to discover new biomarkers and drugs. Identification of differentially expressed peptides and proteins in tissues enhances the understanding of the biological processes underlying disease.[20] Previous molecular imaging techniques, such as green fluorescent protein labeling or immunohistochemistry, have difficulties in discovering new disease-specific abnormal molecules in the tissue. Also, biopsy samples obtained from patients are usually too small to be prepared for ordinary proteomic and metabolomics analyses.[25] A relatively new technique, MALDI-MS imaging has been developed to visualize the spatial distribution of compounds across tissue sections.[24] MALDI-MS imaging allows for simultaneous mapping of hundreds of peptides and proteins present in thin tissue

sections. An array of spectra is obtained and then an ion image of particular m/z can be created from the spectra acquired. The image represents the spatial distribution and relative abundance of that particular ion. A given ion is represented by a single color and the intensity of the color represents the ion intensity at a given location.

MALDI-MS imaging is capable of discriminating healthy from diseased tissue in many systems. For example, Schwartz *et al.* compared peptide and protein expression in primary brain tumor and non-tumor tissue. Their initial results demonstrate that MALDI-MS imaging can significantly aid in the process of understanding the molecular complexities of gliomas, as it accurately and reliably identified normal and neoplastic tissues, and could be used to discriminate between tumors of increasing grades.[26] MALDI-MS imaging can also be used clinically to differentiate drugs from their metabolites, and to determine to what extent the drug has penetrated its intended target.[24] In another study, human brain tumor samples were analyzed and used to start a reference database. Recurrent and progressive meningiomas were imaged and indicated a broader use of MALDI-MS imaging for in-clinical decisions.[27]

The most commonly used matrix solvent used for MALDI-MS imaging is a 50/50 mixture of acetonitrile and aqueous 0.1% TFA. However, this solvent is thought to solubilize proteins, so the application of matrix solution to the tissue delocalizes proteins.[28] Delocalization of proteins has a deleterious effect on image quality, so a matrix solvent that minimizes protein delocalization is needed. There are many methods to apply matrix to the tissue, such as electrospray, acoustic deposition, or manual spotting, all of which form small discrete spots on the tissue. However these techniques have yielded inconsistent image quality and spatial resolution.[29] One can also immerse the entire tissue sample in matrix, but a solvent which does not delocalize proteins must be used.

Matrix solution fixation (MSF) method is a histology compatible method that simultaneously applies matrix and fixes the tissue. The preparation is quick and it has the potential to improve diagnosis of malignancies in a variety of biopsy types. Agar *et al*. tested various solvent combinations, such as 50% acetone/50% methanol and 95% ethanol/5% acetic acid. They found that acetone combinations gave good spectral data but dehydrated and deformed the tissue, and ethanol washes resulted in a decrease in overall spectral intensity and selective disappearance of peaks. The solvent mixture that maximized spectral quality and minimized diffusion of proteins and tissue deformation was found to be a 2:2:1:1 ratio of methanol/ethanol /0.1% aqueous TFA/acetonitrile (also known as "META").[29] Thus a new method was developed for the analysis of tissue via MALDI-MS imaging at a subcellular spatial resolution in prepared tissues.

Purpose of Research

Currently, the Agar Group is among the leaders in MALDI-MS imaging resolution. For the most part, the spatial resolution of MALDI-MS imaging is limited by the laser focal diameter and displacement of analytes during matrix deposition. However, due to recent advancements in laser optics and matrix deposition methods, spatial resolution has improved drastically and imaging of single eukaryotic cells is now possible.[24] Targeted proteomics can be performed on cells of interest in their native environment through in situ labeling of single cells by microinjection of matrix. In this method, cells are visualized using fluorescently labeled motor neurons to determine the location of matrix deposition. A transgenic mouse model was developed to specifically label motor neurons with yellow fluorescent protein (YFP). The combination of microinjection of matrix with the transgenic mouse model allows for targeted proteomic studies that can achieve about 20μm resolution.[30] By depositing matrix on the cells of

interest with micropipettes, analyte diffusion is restricted to the matrix spots without the need for robotics.

The combination of MALDI-MS imaging at cellular resolution with fluorescently labeled neurons is of great use in the study of neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, multiple sclerosis, and most significantly, ALS. In all of these disorders, the death of a relatively small population of neurons triggers the disease process. MALDI-MS imaging studies have been conducted on mouse models of Parkinson's disease[31] and Alzheimer's disease, though neither utilizes transgenic mice expressing fluorescent proteins such as YFP. There have also been studies done with transgenic ALS mice,[32] though analysis was performed via microscopy, not through MALDI-MS imaging. The two different technologies can be combined in order to further visualize the cells that are affected by the disease. MALDI-MS images of these animals could further inform our knowledge of neurodegenerative diseases at the cellular level. The goal of this research is to develop and improve upon the methods used for single cell imaging to create a sample preparation protocol that maintains spatial resolution, but can be applied to an entire tissue section rather than singular cells. In this way, we can better analyze what is happening macroscopically throughout the entire motor cortex throughout disease progression and potentially identify a biomarker for ALS.

To achieve this goal, we need to determine an optimal matrix solvent and at what concentration. Even though META has been shown to be a good solvent for matrix solutions with minimal movement of proteins, there is still a lot of work to be done with META. The protocol has never been optimized and the matrix concentration and number of depositions of matrix applied to the sample have been shown to be critical (data pending). A method with low variability and high intensity, that is a large number of peaks in a given spectrum, is needed to conduct comparison studies. Once an optimized tissue preparation protocol has been developed,

where the quality of spectra acquired is consistent, imaging of control and ALS-affected brain and spinal cord tissue taken from ALS-affected mice can be analyzed via MALDI-MS imaging. Hopefully through MALDI-imaging, we can discover proteins in the motor cortex and spinal cord that are affect throughout all stages of ALS, a subset of which may be biomarkers or cause-associated compounds and will help to elucidate further information regarding the mechanism of the disease.

Methods and Materials

Materials and Solutions. Sinapinic acid was purchased from Sigma-Aldrich (St. Louis, MO) and calibration standards were purchased from Bruker Daltonics (Billerica, MA). Solutions used included META (methanol/ethanol/acetonitrile/0.1% TFA in water in an 8:8:1:1 ratio), matrix solvent (50/50 acetonitrile/1% TFA in water), 70% ethanol, 95% ethanol, 100% ethanol, chloroform, and acetic acid. Matrix was prepared by dissolving sinapinic acid in the appropriate solvent to specified concentrations. The solutions were vortexed for 1 minute, sonicated for 10 minutes, and centrifuged for 2 minutes, resulting in clear pale yellow solutions.

Tissue Preparation. The mouse colony was maintained by Kristin Boggio and Joseph Salisbury at Brandeis University. All animal experiments were approved and performed in accordance with the Brandeis University Institutional Animal Care and Use Committee. Intact brains were removed from mice by Joseph Salisbury, flash frozen on liquid nitrogen, and stored in an -80°C freezer until analysis. On the day of slicing, the tissue was thawed to -18°C in the Microm HM

525 Cryostat from Mikron Instrumentals Inc. (San Marcos, CA). All tissue sections except those

for the *G93A/Control Comparison Study* were sliced in the same cryostat at -18°C, with each

brain first cut in half at the midline sagittally with a razor blade. The section was then fixed to a

cryostat mount, cut side up with optimal cutting temperature (OCT) polymer. The tissue was

sliced at 10 μm and thaw-mounted to indium tin oxide coated (ITO) glass slides. All experiments

were conducted with n=3. Slides were stored in -80°C freezer until analysis. Before application

of matrix onto the tissue sections, the slides were thawed to room temperature in a desiccator to

remove all moisture from slides. Protein calibrant standard 1 (0.5 μL) was mixed on a small spot

on the slide with 25 mg/mL matrix solution (0.5 μL) and a sample of the first META wash (1

μL) was mixed with saturated matrix solution (1 μL) on each slide to analyze wash composition.

Mass Spectrometry. All tissue sections were analyzed by Microflex LT MALDI-TOF

interfaced with FlexImaging Software (in-house version) from Bruker Daltonics (Billerica, MA)

except those in *META Optimization 2*. The spectrometer was operated in the linear mode for the

mass-to-charge ratio (*m/z*) greater than 5000 and less than 20000. The MALDI-TOF was

equipped with a standard 337 nm wavelength nitrogen laser and a maximum repetition rate of 20

Hz. A custom-made glass slide holder was produced by drilling a 25 mm x 50 mm x 1.1 m cavity

into a Bruker's Microflex stainless steel target to accommodate ITO glass slides. The slides were

held on the target by minimal conductive adhesive tape.

Spectral Peak Analysis. A computer script was written to standardize analysis of the quality of

spectra. A script was written within flexAnalysis to baseline subtract, smooth and find all peaks

that fit a set of parameters for each spectrum obtained. This data was then exported and matched

up to each individual spectrum using MATLAB, and the data was analyzed in Microsoft Excel.

This analysis was only applied to experiments performed with 50/50 acetonitrile/1% TFA in water used as the matrix solvent.

META Optimization 1. Tissue (YFP #47f) was sliced using the standard protocol previously described. Each ITO coated slide was cut in half, and three slices were mounted onto each half slide. A stock META solution was created for use in *META Optimization 1, Initial META Wash Experiment*, and *META Optimization 2* with methanol, ethanol, 1% TFA in water, and acetonitrile. The stock solution was stored in the 4°C refrigerator to preserve solvent ratios, and brought to room temperature before use. Matrix solutions with sinapinic acid in META were created at concentrations of 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL. A hydrophobic barrier about 2mm from the edge of the tissue was drawn around each section using an Immedge pen. Matrix solution (20 μL) was spread across the entire region encircled by the hydrophobic border, at varying depositions of 1, 3 or 6. All three tissue sections on a single slide were all prepared with the same concentration and same number of depositions. The matrix solution was allowed to dry completely before addition of another deposition. The spectra were analyzed by eye in flexAnalysis, by counting how many spectra out of the total spectra acquired were "good spectra." A "good spectrum" was defined as one that qualitatively had a decent number of resolved peaks (> 20 peaks) and large signal to noise ratio with resolved peaks. A MALDI-image was obtained with a raster width of 800 μm.

Initial META Wash Experiment. Tissue (YFP #47f) was sliced using the standard protocol previously described. Three samples were prepared with 3 depositions of 15 mg/mL matrix to tissue that was washed with META, and 3 samples with the same conditions to tissue that was not washed at all. The META wash (20 μL x2) was applied to the tissue via pipette, allowed to

sit for 30 seconds and then flicked off the slide. The resulting spectra were analyzed by eye in flexAnalysis using the criteria for a "good spectrum" described earlier. A MALDI-image was obtained with a raster width of 1250 μm.

META Optimization 2. Tissue (SOD1/YFP #41f) was sliced using the standard protocol previously described. ITO coated slides were kept whole and 8 tissue sections were mounted onto each slide. All slides were prepared on the same day and treated to a META wash (20 μL x2) before application of matrix solution of sinapinic acid in META (20 μL) at concentrations of 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 25 mg/mL and saturated solution at 1, 3, and 6 depositions. Saturated matrix solution was made with 52 mg sinapinic acid in 1 mL META. Slides were analyzed at Harvard Medical School with an UltrafleXtreme MALDI-TOF/TOF equipped with a 1kHz smartbeam laser from Bruker Dlatonics (Billerica, MA). The resulting spectra were analyzed by eye in flexAnalysis using the criteria for a "good spectrum" described earlier. A MALDI-image was obtained with a raster width of 1250 μm.

META Optimization 3. Tissue (SOD1/YFP #41f) was sliced using the standard protocol previously described. Each ITO coated slide was cut in half, and three slices were mounted onto each half slide. New stock META solution was made with methanol/ethanol/0.1% TFA in water/acetonitrile. All slides were treated to a META wash (20 μL x2) before application of matrix solution of sinapinic acid in META (20 μL) at concentrations of 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 25 mg/mL and saturated solution at 1, 3, and 6 depositions. The matrix solution was applied across the entire region defined by the hydrophobic border drawn around the tissue about 2 mm away from tissue. Each tissue section was divided into two parts and each part analyzed non-sequentially to minimize instrument bias. The resulting spectra were analyzed

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by eye in flexAnalysis using the criteria for a "good spectrum" described earlier. A MALDI-image was obtained with a raster width of 1250 μm.

50/50 ACN/1% TFA Optimization 1. Tissue (YFP #25f) was sliced using the standard protocol previously described. Each ITO coated slide was cut in half and three slices were mounted onto each half slide. Matrix solutions with sinapinic acid in META were created at concentrations of 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 25 mg/mL and saturated solution. One slide was treated to a META wash (20 μL x2) and the wash flicked off before application of matrix solution, while the other slide was left unwashed. All conditions were tested on one single slide. The wash solution was applied across the entire region defined by the hydrophobic barrier drawn about 2mm away around all tissue sections. A micropipette was used to create small spots of matrix on the tissue, around 300 μm in diameter, to create about 30-40 spots per tissue slice. The micropipettes were formed using program 19 on a PMP-102 micropipette puller from MicroData Instrument, Inc. (S. Plainsfield, NJ). Matrix was applied at the various concentrations listed above at 1, 3, and 6 depositions. One spectrum was obtained at each individual spot. Spectral analysis was conducted using the spectral peak analysis script.

50/50 ACN/1% TFA Optimization 2. The same preparation was followed as described in 50/50 ACN/1% TFA Optimization 1, except the number of depositions tested were 5, 6, 7, and 9 depositions of 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL matrix solution. Also, all slides were washed with META (20 μL x2) and each condition was spotted across an entire tissue section. One spectrum was obtained at each individual spot. Spectral analysis was conducted using the spectral peak analysis script.

50/50 ACN/1% TFA Image Prep. Tissue (unlabeled) was sliced using the standard protocol previously described. Each ITO coated slide was cut in half and three slices were mounted onto each half slide. Matrix solutions with sinapinic acid in META were created at concentrations of 5 mg/mL, 10 mg/mL, and 15 mg/mL. All slides were treated to a META wash (20 μL x2) and the wash flicked off before application of matrix solution. The wash solution was applied across the entire region defined by the hydrophobic barrier drawn about 2mm away around all tissue sections. Matrix was applied using an ImagePrep automated sprayer from Bruker Daltonics (Billerica, MA) at Harvard Medical School, using the instrument optimal conditions and various concentrations of matrix. MALDI-MS analysis was conducted at Brandeis University. A MALDI-image was obtained with a raster width of 800 μm. Spectral quality analysis was conducted using the spectral peak analysis script.

50/50 ACN/1% TFA Spatial Test. Tissue (unlabeled) was sliced using the standard protocol previously described. Each ITO coated slide was cut in half and three slices were mounted onto each half slide. Matrix solutions with sinapinic acid in META were created at concentrations of 15 mg/mL, 20 mg/mL, and 25 mg/mL. All slides were treated to a META wash (20 μL x2) and the wash flicked off before application of matrix solution. The wash solution was applied across the entire region defined by the hydrophobic barrier drawn about 2mm away around all tissue sections. The same concentration of matrix and number of depositions was spotted with a micropipette across an entire tissue slice. The number of depositions varied from 5 to 9. One spectrum was obtained at each individual spot. Spectral analysis was conducted using the spectral peak analysis script.

Further Wash Experiments. Tissue (YFP #83m) was sliced using the standard protocol previously described. Each ITO coated slide was cut in half, and three slices were mounted onto each half slide. Matrix solution with sinapinic acid in META was created at 20 mg/mL. Each slide was washed with a different solvent. For each wash process, the slide was dipped into a 50 mL falcon tube with 35-40 mL of the wash solution for 30 seconds x2, except for the delipidation protocol. The slides were washed with META, ethanol, repeated washes (8x) of META, and a delipidation protocol. The delipidation protocol called for 30 seconds in 70% ethanol, followed by 30 seconds in 95% ethanol, followed by 2 minutes in a 6:3:1 mixture of ethanol/chloroform/acetic acid, and a final 30 seconds in 95% ethanol. Matrix solution was applied to the tissue via micropipette to create about 30-40 spots per tissue slice. One spectrum was obtained at each individual spot. Spectral analysis was conducted using the spectral peak analysis script. Fluorescent images of the tissue sections post-wash were also taken using a Leica MZFLIII stereo-fluorescence microscope from Leica Microsystems (Buffalo Grove, IL) courtesy of the Nelson Lab at Brandeis University.

G93A/Control Comparison Study. Tissue (G93A positive and littermate control) was sliced coronally every 300 μm, with the base of the brain fixed to the cryostat mount with OTC polymer. Sectioning was performed in a Microm HM 550 Cryostat from Mikron Instrumentals Inc. (San Marcos, CA) at Harvard Medical School, and the sections were thaw mounted onto ITO coated slides for a total of 14-15 sections per glass slide. Slices were also taken for 8 other slides and for H&E staining. The slices were matched to a mouse brain atlas to determine where the motor cortex was located in each section. Matrix at a concentration of 20 mg/mL was applied to the entire tissue manually using a micropipette at 5 depositions. All spots on the tissue were analyzed via MALDI-MS, and then the spots located on the motor cortex, as determined by the

mouse brain atlas, were analyzed from both the G93A and control. The data was compared by

mass peak list and visually comparing peaks in the spectra.

Results and Discussion

META Optimization

Through the first initial optimization, the data showed that the total concentration of

matrix applied to the tissue was not the determining factor in spectral quality or consistency. A
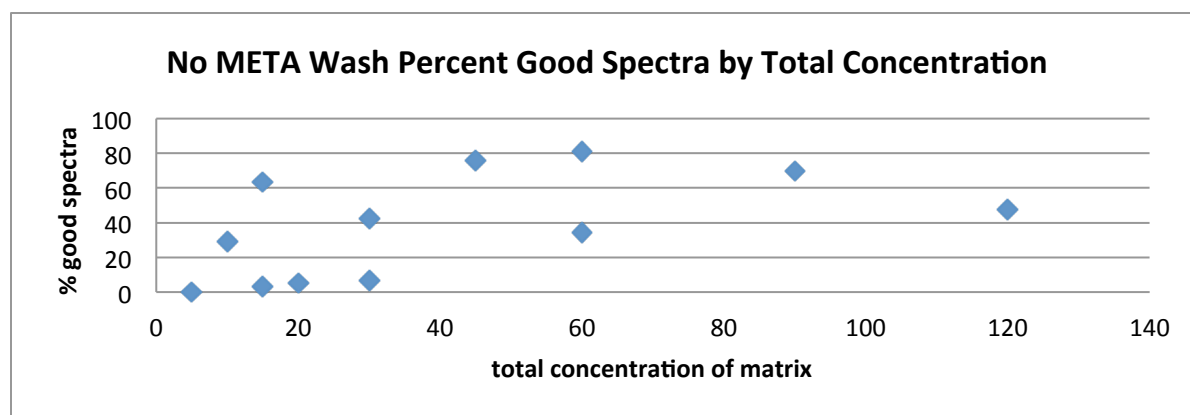

Figure 1: Graph depicting the percent of good spectra acquired at particular total concentrations. Total concentration here means the cumulative total of matrix on a given tissue section. Total concentrations were reached using 5, 10, 15, and 20 mg/mL of matrix solution at 1, 3 or 6 depositions.

general trend could be extrapolated from the data – that as the total concentration of matrix

applied to the tissue increased, the quality of the spectra increased (Figure 1). Rather, the

concentration of the matrix being applied had a much larger impact on the quality of spectra

obtained (Figure 2). A total concentration of matrix applied to a tissue section could be reached

multiple ways – 1 deposition of 15 mg/mL matrix solution has the same total concentration of

matrix (15mg/mL) as 3 depositions of 5 mg/mL matrix solution. For example, an image obtained

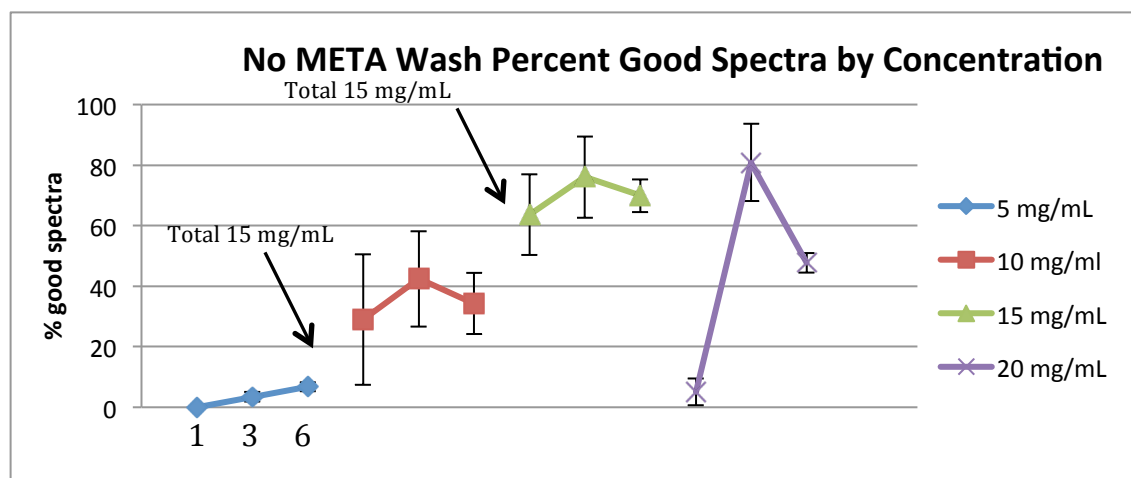**No META Wash Percent Good Spectra by Concentration**

Figure 2: Graph depicting the effect of the number of depositions on the percent of good spectra obtained at various matrix concentrations. Here in the graph, for each data set, the first data point represents 1 deposition, the second represents 3 depositions, and the third represents 6 depositions. A "good spectrum" was qualitatively determined by looking at the general quality of the spectrum, the number of peaks and signal to noise ratio by eye.

with 3 depositions of 5 mg/mL (total of 15 mg/mL) had 3.4% good spectra, whereas an image

obtained with 1 deposition of 15 mg/mL (also a total of 15 mg/mL) had 63% good spectra, a

significant increase. As the initial concentration of matrix being applied increases, the number of

good spectra for a given tissue section increases. For each given concentration of matrix solution

except 5 mg/mL, 3 depositions yielded the best spectra, with 1 deposition resulting in the least

good spectra, and 6 depositions somewhere in the middle. Through this first initial experiment, a

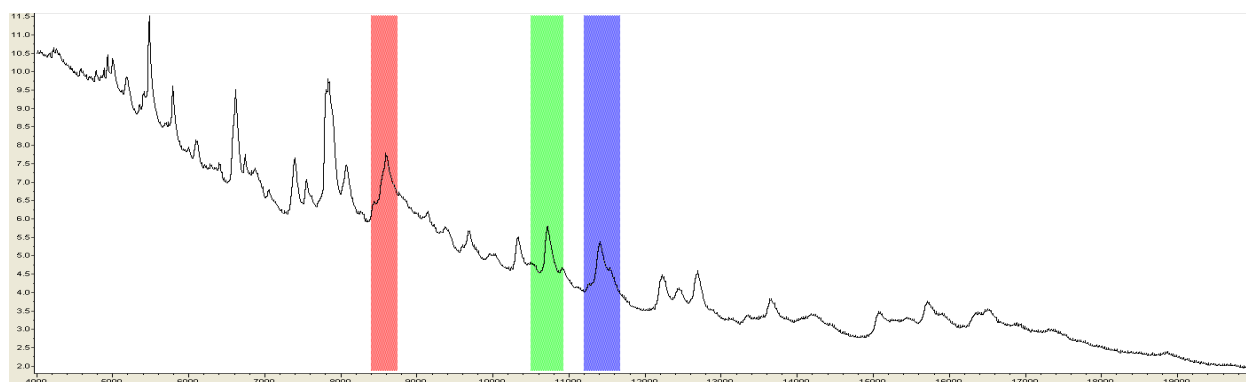maximum was observed at 20 mg/mL at 3 depositions (Figure 3).

Figure 3: Class average spectrum of a MALDI-MS image of tissue treated with 3 depositions of 20 mg/mL matrix solution. The colored bands represent the m/z analyzed in the MALDI-MS ion image.

However, there were some caveats to these discoveries. It was later discovered that the acid concentration within the META solution used to make the matrix solutions was at 1% aqueous TFA, instead of the prescribed 0.1% aqueous TFA. An increase in acid could have resulted in protein diffusion throughout the tissue. Also, the variability in this experiment was large, and the experiments done at 1 deposition of 20 mg/mL seemed anomalous. On the other hand, this seemingly erroneous result was indicative of the general observation that 1 deposition of matrix tended to yield sporadic results. This may have been due to the fact that all three duplicates of a single set of conditions were located on the same slide, and different slides were analyzed on different days. To validate and substantiate these results, further optimization experiments were performed.

A large, two day study was put together using the same conditions as the first optimization, except for the addition of a META wash and higher matrix concentrations of 25 mg/mL and saturated matrix solution. A small trial META wash experiment was performed after the first optimization study, and it was found that spectra obtained from tissue washed with META had a higher percentage of good spectra, so a META wash was adopted for all successive studies. All slides were washed and prepared on day one, and then transported to Nathalie Agar's laboratory at the Harvard Medical School and analyzed the next day. The different conditions were staggered on the slides, so the three duplicates of a single condition would be located on different slides in different positions to account for the differences in each individual slide.

Spectra were obtained for wash spots, calibrants and shot data in addition to MALDI-MS images of the tissue sections. Wash spots were samples of the META wash when applied to the tissue mixed with matrix solution to determine if any proteins leaked due to the wash procedure. Analysis of the wash spots found there was no significant loss of proteins due to the META wash. Shot data was collected to determine the persistency of signal by repeatedly irradiating one
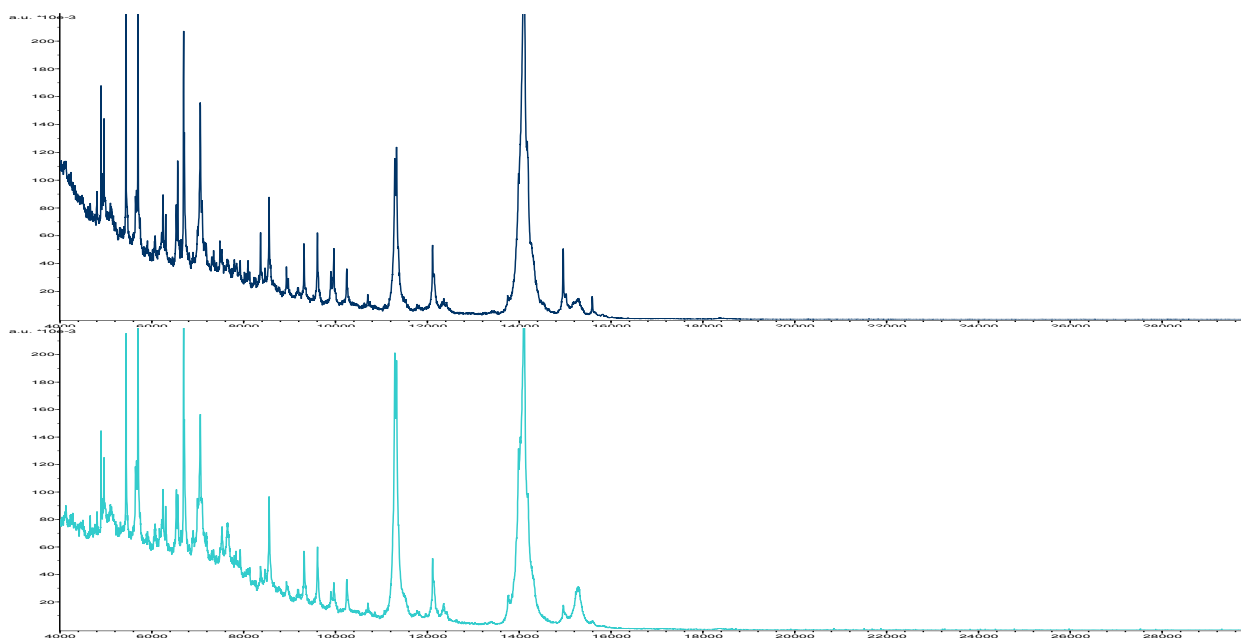
Figure 4: Class average spectra from second META optimization. The top spectrum was obtained with 1 deposition at 10 mg/mL matrix solution and the bottom spectrum was obtained with 1 deposition at 15 mg/mL matrix solution.

spot on the tissue until the signal disappeared. This allowed us to determine how many spectra to take at a given spot to maximize signal. It was found that 200 shots per spot were optimal. Class average spectra were used to compare conditions and each condition was ranked against the other conditions tested on the same slide. Due to technical issues, individual spectral data could not be accessed so class average ranking was the only point of comparison.

From this study, it was found that at 1 deposition of matrix, 15 mg/mL and 20 mg/mL produced the highest quality spectra (Figure 4). For 3 depositions, rankings were very consistent and 5 mg/mL and 10 mg/mL yielded the best results. This finding did not correlate with the conclusions made from the first study where the best spectra were obtained with 15 mg/mL and 20 mg/mL matrix. For 6 depositions, again 5 mg/mL and 10 mg/mL yielded the best results that again did not correlate with previous data. The differences between 1, 3, and 6 depositions of matrix were not very drastic from class average spectrum to class average spectrum. The number

of peaks and the resolution of the peaks were relatively similar for 1 deposition and 3 depositions of a given matrix concentration.

The conclusions drawn from this experiment differed from the results of the initial study. This could have been due to the high TFA concentration in the META solution used, both in the matrix solutions and META washes. The high acid content could have potentially removed some of the proteins from the tissue during washing, resulting in different matrix crystal formation or protein abundances. This experiment was also only evaluated by class average spectra because of lack of access to individual spectrum data, and an analysis of the individual spectra may have resulted in more similar results. Thus the results from this study were not seen as significant.

To confirm the findings from either study, another optimization study was performed, this time with a fresh META stock solution with the proper proportions of TFA. The three duplicates of each set of parameters were staggered across three slides, so each slide had the same concentration of matrix at different depositions. The tissue was washed with META, matrix was applied, and then imaged and the spectra evaluated by eye for "good spectra." From this study, it was found that 1 deposition of any given concentration of matrix resulted in the worst spectra for every concentration except with a saturated solution (Figure 5). This makes sense because at such a high initial concentration of matrix, 6 depositions would lead to an extremely high level of matrix present on the tissue section, enough so that it negatively affects the quality of spectra obtained. At concentrations of 20 mg/mL or greater, the variability of spectra obtained with 1 deposition was extremely high, and the variability of spectra with 1 deposition was generally higher than that of spectra obtained with 3 or 6 depositions at any concentration of matrix. Application of 3 depositions of matrix at any given concentration yielded a greater percentage of good spectra than 6 depositions except at 5 mg/mL, presumably
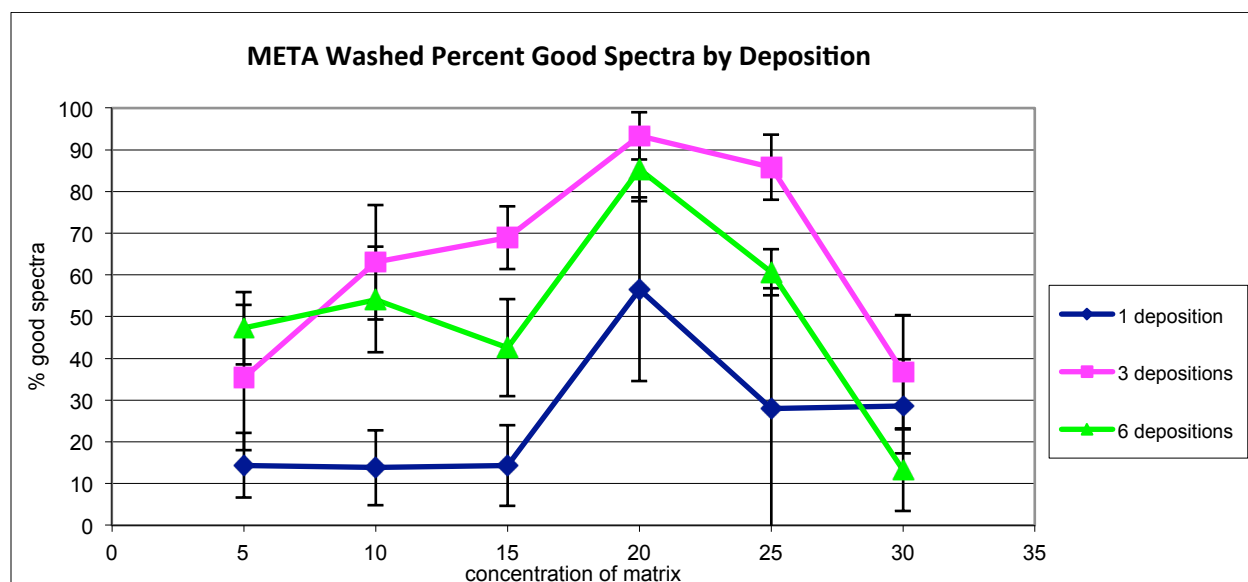
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Figure 5: Graph depicting the effect of the concentration of matrix on the percent of good spectra obtained at various numbers of depositions. Here in the graph, for each data set, the blue line represents 1 deposition, the pink line represents 3 depositions, and the green line represents 6 depositions. A "good spectrum" was qualitatively determined by looking at the general quality of the spectrum, the number of peaks and signal to noise ratio by eye.

because at such a low initial concentration, application of more matrix will increase the quality of spectra in that range.

There is also a definitive observable maximum at 3 depositions of 20 mg/mL matrix solution. These parameters produced the highest intensity with the lowest variability, perfect conditions for identifying a biomarker for ALS because the variability of the technique was below that of biological variability so it was no longer the limiting factor. Through these multiple optimization studies, a clear maximum in percentage of good spectra was found at 20 mg/mL at 3 depositions. The spectra obtained contained a good number of peaks that were well-resolved and little noise (Figure 6). This is a novel finding and a breakthrough in the MALDI-MS imaging sample preparation field. Because MALDI-MS imaging is a relatively new field, there was no universal method developed for sample preparation. However, most researchers tended to use matrix at a concentration around 20 mg/mL and applied it once to the tissue. From our data, we
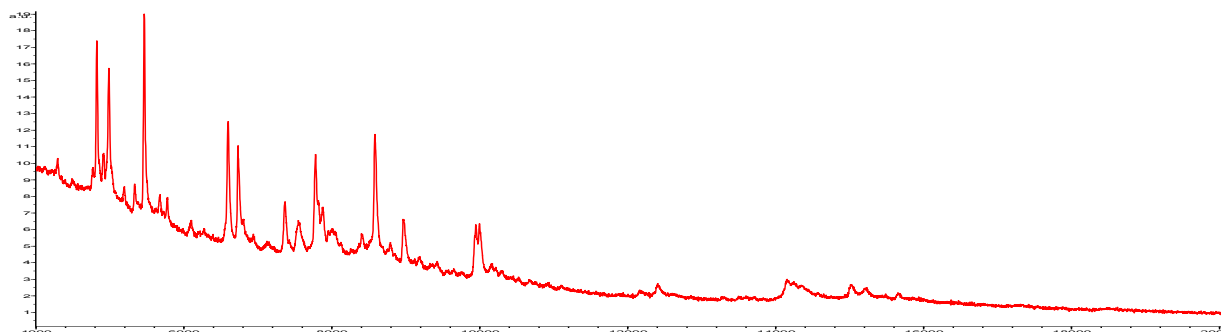
Figure 6: Class average spectrum from META optimization 3 obtained from tissue prepared with 3 depositions of 20 mg/mL matrix solution.

can clearly see almost a doubling of percentage of good spectra when the number of depositions is increased from 1 to 3, thus greatly improving upon existing methods.

Now that an optimized sample preparation protocol was developed for the use of META matrix solvent, we wanted to put our new discovery in perspective. While META is optimal for the use of matrix fixation, the majority of mass spectrometrists utilize a 50/50 mixture of acetonitrile and 1% TFA for MALDI-MS imaging. This is vestigial from previous years when MALDI-MS imaging was not yet invented and most were simply preparing spots of solution on a MALDI target. We wanted to see if what we learned from the META optimization – that the number of depositions and the initial concentration of the matrix solution being applied to the tissue were large determining factors in spectral quality – was more generally applicable. To do this, we tested some 50/50 spots using the optimized META conditions – 3 depositions of 20 mg/mL and compared the quality of spectra obtained to the spectra obtained with META. Comparing the two spectra (Figure 7), the 50/50 matrix yielded a better quality spectrum with more peaks and smaller variability. Since the 50/50 mixture was superior to the META matrix solution, we decided that 50/50 matrix solution should be used moving forward to give the highest probability of discovering a biomarker for ALS.
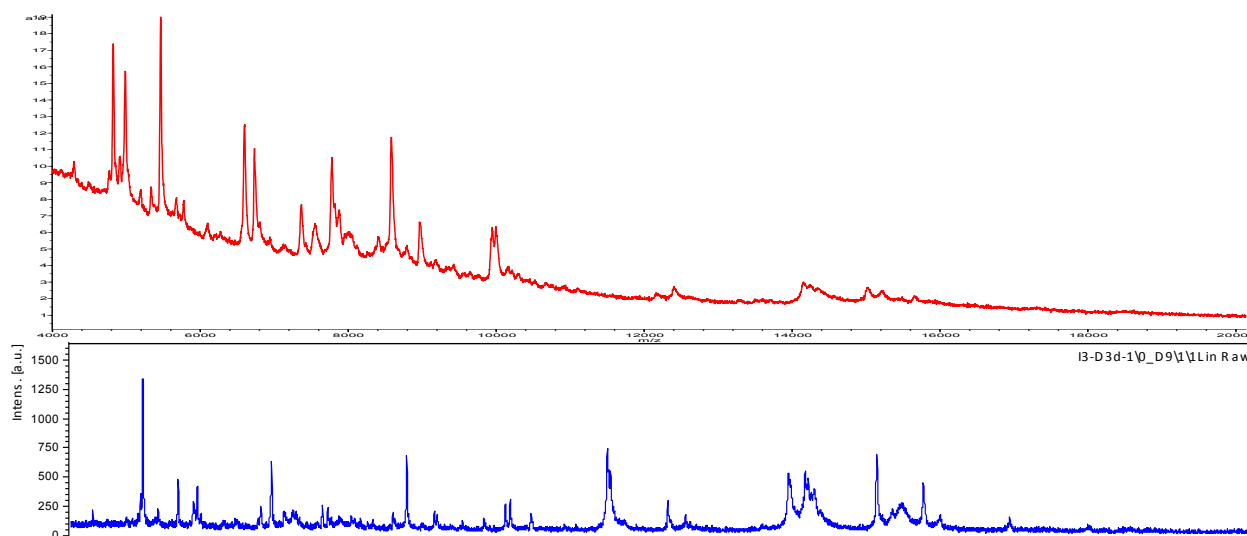
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Figure 7: Comparison of spectra obtained with META (top) and 50/50 ACN/1% TFA (bottom) as matrix solvent at 3 depositions of 20 mg/mL matrix solution.

50/50 ACN/1% TFA Optimization

Optimization studies similar to previous META optimization studies were performed with the 50/50 mixture at varying concentrations of matrix solution and the number of depositions. A fundamental difference between the application of META and 50/50 mixture to the tissue was spatial resolution. META was applied across an entire tissue section, and the resolution of the MALDI-MS image was only limited by instrument capabilities. However, 50/50 mixture is known to move proteins, so it must be applied to the tissue in discreet droplets by manually laying down an array of spots applied by micropipette. This limits the spatial resolution of the image greatly, with a spot diameter of approximately 300 μm. However, we were willing to sacrifice spatial resolution to initially search for differences present within the motor cortex of control and ALS-affected tissue. Also, at this point we wrote a computer script within flexAnalysis to analyze the data more efficiently. Rather than looking at the spectra by eye and assessing the quality visually, the method of analysis used for all META spectra obtained, a method was written to smooth, baseline subtract, and find peaks within a given set of parameters.

This data was processed in MATLAB to match the number of peaks to the data file name, and statistical analysis was performed in Microsoft Excel.

The first experiment with the 50/50 mixture tested a range of matrix solution concentrations from 5 mg/mL to saturated solution at 1, 3 and 6 depositions on slides that were and were not washed with META. This experiment was performed to determine whether a
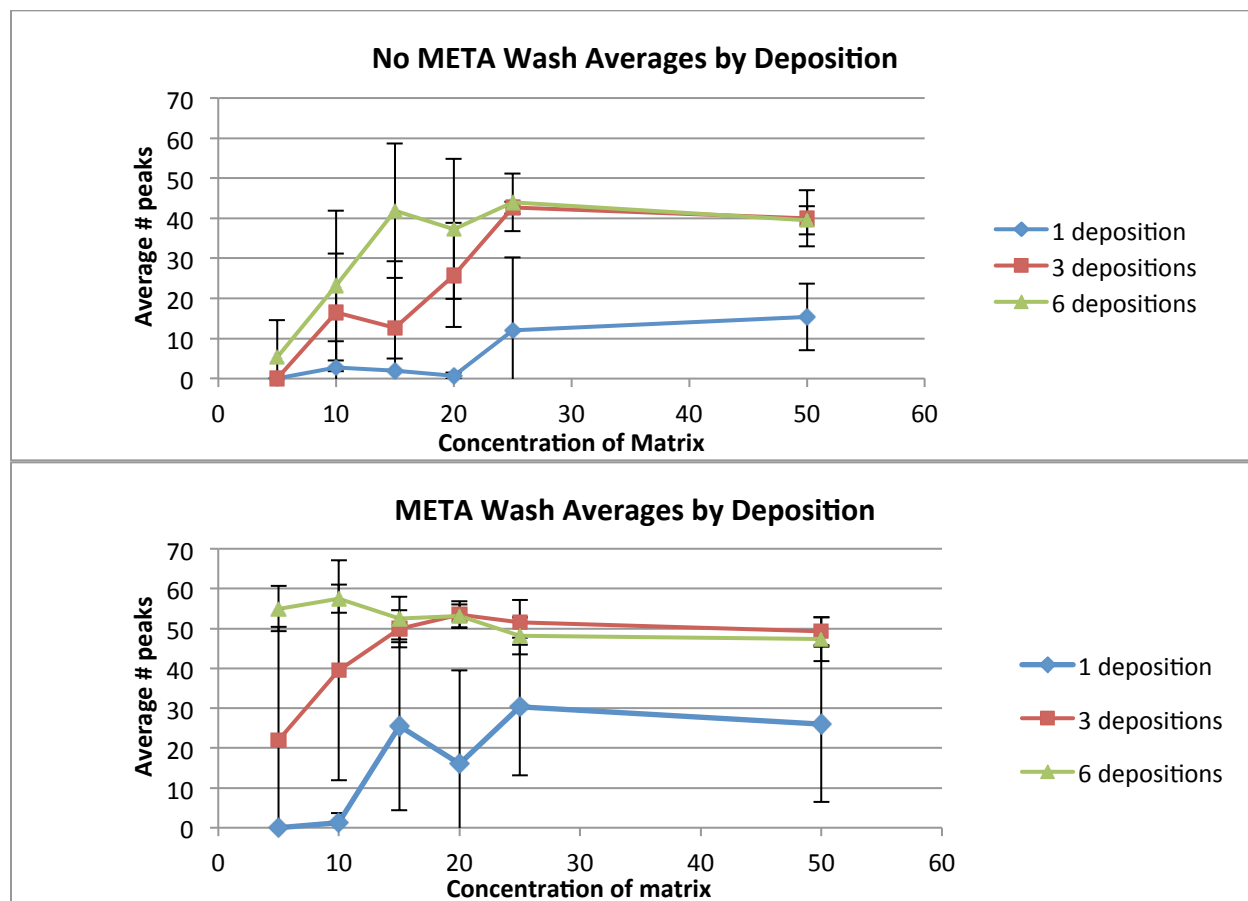

Figure 8: Graphs of the average number of peaks found in a given spectrum acquired through a particular set of conditions using the spectral peak analysis script by deposition. Each color represents a different number of depositions. No META wash before application of matrix (top) shows that the quality of spectra generally follow this trend: 6 depositions > 3 depositions > 1 deposition and the number of peaks range from 0-40 peaks/spectrum. Implementation of a META wash before application of matrix (bottom) shows a significant difference in the 5-10mg/mL region and the number of peaks range from 0-55 peaks/spectrum, with decreased variability at higher number of depositions.
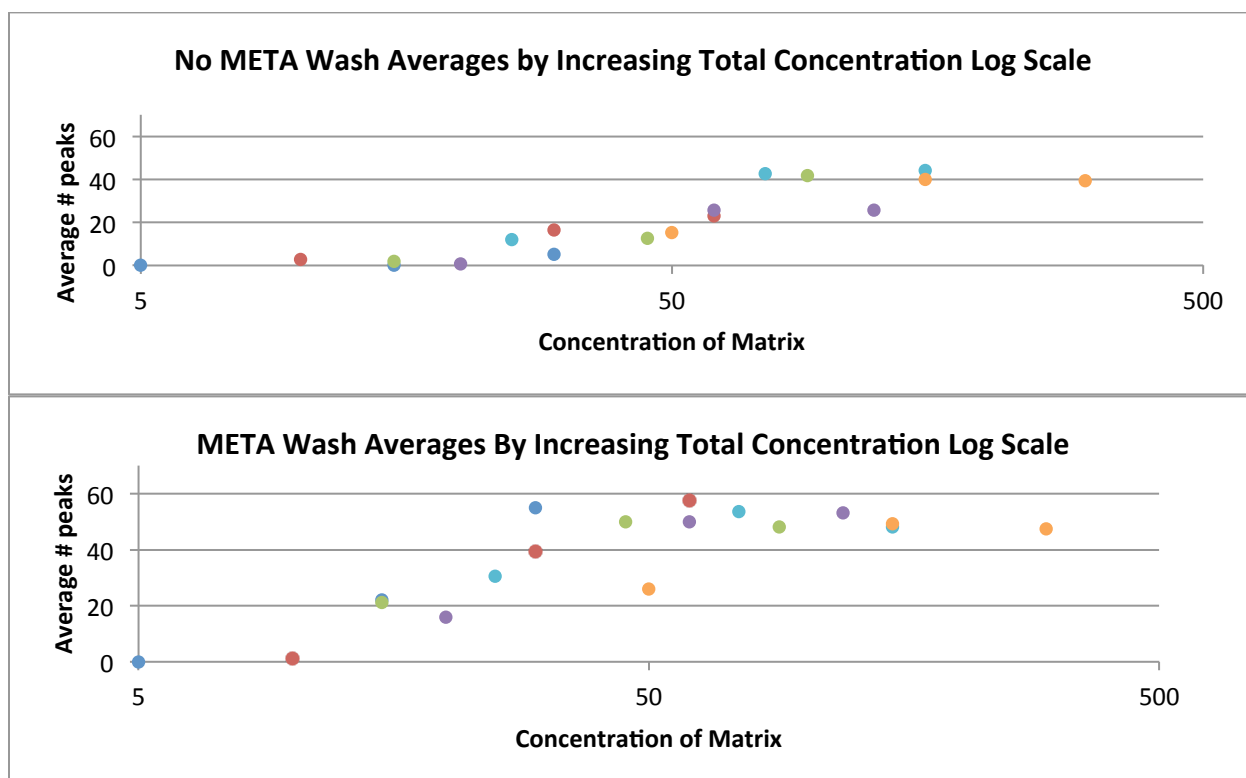
Figure 9: Graphs of the average number of peaks found in a given spectrum acquired through a particular set of conditions using the spectral peak analysis script by total matrix concentration on a log scale. The different colors represent different initial concentrations of matrix. Blue is 5 mg/mL, red is 10 mg/mL, green is 15 mg/mL, purple is 20 mg/mL, teal is 25 mg/mL and orange is saturated solution. There is a general upward trend, with an observable maximum with a META wash. The average number of peaks increases with the use of a META wash.

META wash had a significant effect on the quality of spectra obtained using the 50/50 mixture as a matrix solvent compared to using META as a matrix solvent. Through this experiment, it was found that a META wash greatly affects the spectra acquired. For the slide without a META wash (Figure 8), 1 deposition was always worse than 3 depositions, which was always worse than 6 depositions at any given concentration of matrix. Below 20 mg/mL, the difference between 3 and 6 depositions is large, but above 20 mg/mL, the average number of peaks in a spectrum is comparable.

It is also interesting to note that except for the case with saturated matrix, a higher concentration of matrix with more depositions will yield more peaks, even if the total concentration of matrix is the same (Figure 9). With a META wash step, looking at the graph the
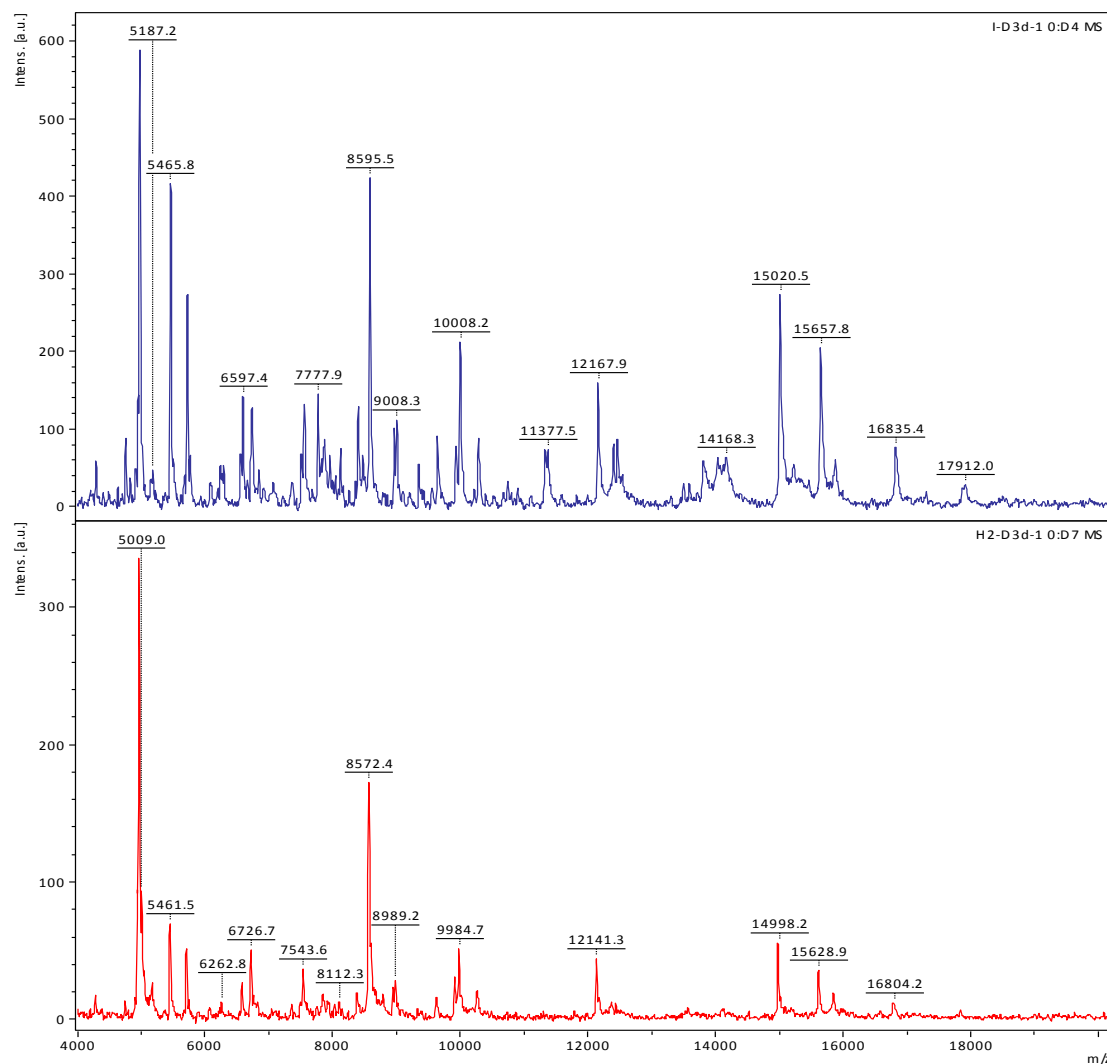
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Figure 10: Comparison of spectra obtained with 50/50 ACN/1% TFA in water at 20 mg/mL and 3 depositions with (top) and without a META wash (bottom). The number of peaks and intensity greatly increase with the use of a META wash prior to matrix application.

average number of peaks increases with total concentration of matrix, and reaches a definite

maximum before decreasing after about 60 mg/mL (Figure 9). However, the initial concentration

of matrix still has a large impact on the quality of spectra. For example, at the maximum there is

a red dot signifying 6 depositions of 10 mg/mL matrix solution. This has the same total

concentration as the purple dot below it, which signifies 3 depositions of 20 mg/mL matrix

solution. So even though the two conditions have the same total concentration, an initial

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concentration of 10 mg/mL yielded a greater number of average peaks, and thus higher quality spectra.

In contrast, for the slide that was washed with META, the spectra with the highest number of peaks were obtained with low initial concentrations of matrix solution at a high number of depositions. The use of 6 depositions of matrix at concentrations of 5mg/mL and 10 mg/mL yielded higher number of peaks in spectra than obtained with 3 depositions. The highest number of peaks in a spectrum was obtained using 10 mg/mL matrix at 6 depositions. In all cases, 1 deposition yielded the lowest average number of peaks in a spectrum, and matrix at a concentration of 15 mg/mL or greater yielded comparable results using 3 and 6 depositions. It is also interesting to note that when looking at the total concentration of matrix applied to the tissue, the results for the META washed tissue are exactly the opposite as for the non-washed tissue. Except for the case with saturated matrix, a lower concentration of matrix with more depositions will yield more peaks, even if the total concentration of matrix is the same. Also, in general spectra from META washed tissue had a greater average number of peaks (~50-60 peaks) than spectra from non-washed tissue (~35-45 peaks) (Figure 10).
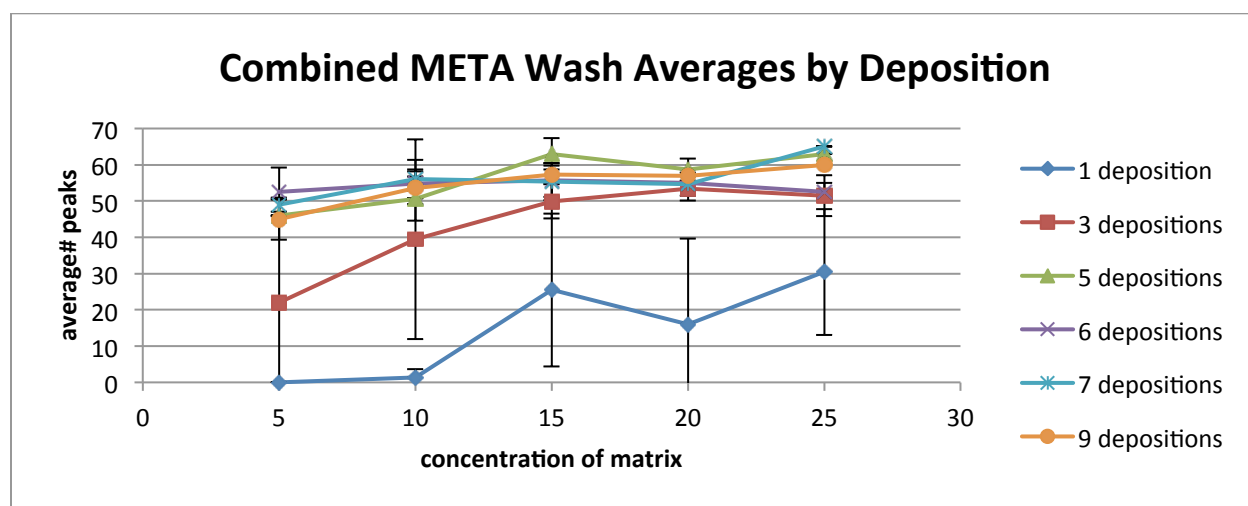

Figure 11: Graph of the data acquired from both 50/50 ACN/1% TFA in water optimizations combined into a single graphic to better understand the results obtained. Here it is evident that there is not a large difference is the average number of peaks at depositions greater than 5.

A further optimization study was conducted with a META wash at a more contained range of depositions and concentrations of matrix. Since a lower concentration of matrix with more depositions yielded a greater number of peaks in the previous experiment, concentrations of matrix ranging from 5 mg/mL to 25 mg/mL were tested at 5, 6, 7 and 9 depositions. The data was combined with the previous data to gain a better understanding of the whole picture (Figure 11). From this data, it was found that 5 depositions generally yielded spectra with a high average number of peaks, though results for 5, 6, 7, and 9 depositions were generally similar at all concentrations of matrix. A final optimization experiment determined that 20 mg/mL at 5 depositions would be optimal because of the average number of peaks, the overall spectral quality, and small number of depositions. Through preparing the tissue for these optimization experiments, it was quickly realized that it is difficult to place a spot of matrix on the exact same spot on the tissue multiple times, and the greater the number of depositions, the greater the probability for error.

A spatial test was conducted after these results were obtained, because each different condition was tested on different parts of the tissue section, and different regions of the brain inherently contain more or less peaks due to protein distribution within the brain. To make sure that the results obtained were not a result of where each spot was located on the tissue section, an entire tissue section was covered in spots of the same condition. Comparison of the data obtained to previous data revealed there were no major differences, and the number of peaks only varied by an average of about +/- 4 peaks (Figure 12). Thus, through this series of experiments, it was found that the optimal conditions for the use of 50/50 ACN/1% aqueous TFA as a matrix solvent are applying 5 depositions of 20 mg/mL matrix on META washed tissue.

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Experiments using different matrix applications were also conducted. Slides were

prepared with 5 mg/mL, 10 mg/mL and 15 mg/mL matrix solution in 50/50 ACN/1% aqueous

TFA via ImagePrep automatic sprayer, which applies matrix to the slide in a fine mist, coating

the slide until an optimal thickness of matrix is achieved. This technique allows for much greater

spatial resolution with this matrix solvent, which is known to move proteins since the matrix is

still being applied to the tissue in small discreet spots, but the spots are just much smaller.

Experiments with the ImagePrep yielded disappointing spectra, with few peaks at low intensities

and very low signal to noise, leading to poorly resolved spectra. Unfortunately, there was not

enough time to optimize conditions with the ImagePrep to obtain high quality spectra.

Total Concentration (mg/mL)	Concentration (mg/mL)	Depositions	Average Peaks 1	Average Peaks 2	Difference
90	15	6	55.79	58.17	-2.38
100	20	5	58.67	57.94	0.72
125	25	5	63.00	58.00	5.00
135	15	9	57.33	57.53	-0.20
180	20	9	57.00	49.00	8.00
225	25	9	60.00	50.36	9.64

Figure 12: Table of data from the 50/50 ACN/1% TFA in water spatial tests. The first column of average peaks represents the data acquired in the initial optimization study. The second column of average peaks represents the data acquired in the spatial study. The average difference in the number of peaks at any given concentration and number of depositions is +/- 4 peaks.

Further Wash Experiments

After determining optimal conditions for the use of the 50/50 mixture as a matrix solvent,

we went back to look at the META wash. Conventionally, tissue is washed with ethanol before

application of matrix, and the Agar group at Harvard Medical School reported good quality

spectra using a delipidation protocol. Thus the META wash was compared to these two wash

protocols, along with repeated META washes (x8). Spectral peak analysis revealed that the

delipidation protocol yielded the highest quality spectra (Figure 13), but fluorescent imaging

showed YFP fluorescence was lost (Figure 14). All other washes preserved fluorescence, and of

these wash protocols, the standard META wash used for all previous experiments yielded the highest number of peaks behind the delipidation protocol with the smallest standard deviation, or lowest variability.

Thus, these wash experiments confirmed that the META wash we were using was indeed the best method to follow because the low variability will allow us to effectively compare ALS-affected tissue to healthy tissue without the variability of the technique to skew results. The comparison of the two tissues can then eventually lead to the discovery of a biomarker for ALS. It also exemplifies META's versatility not only as a matrix solvent, but also as an effective wash solvent. Use of a META wash increases spectral sensitivity and reproducibility by both increasing the average number of peaks obtained within a given spectrum and decreases the variability in the results, regardless of matrix solvent applied. This can been seen in Figure 5 for use of META as a matrix solvent, and Figure 8 for use of 50/50 ACN/1% TFA in water as a matrix solvent.
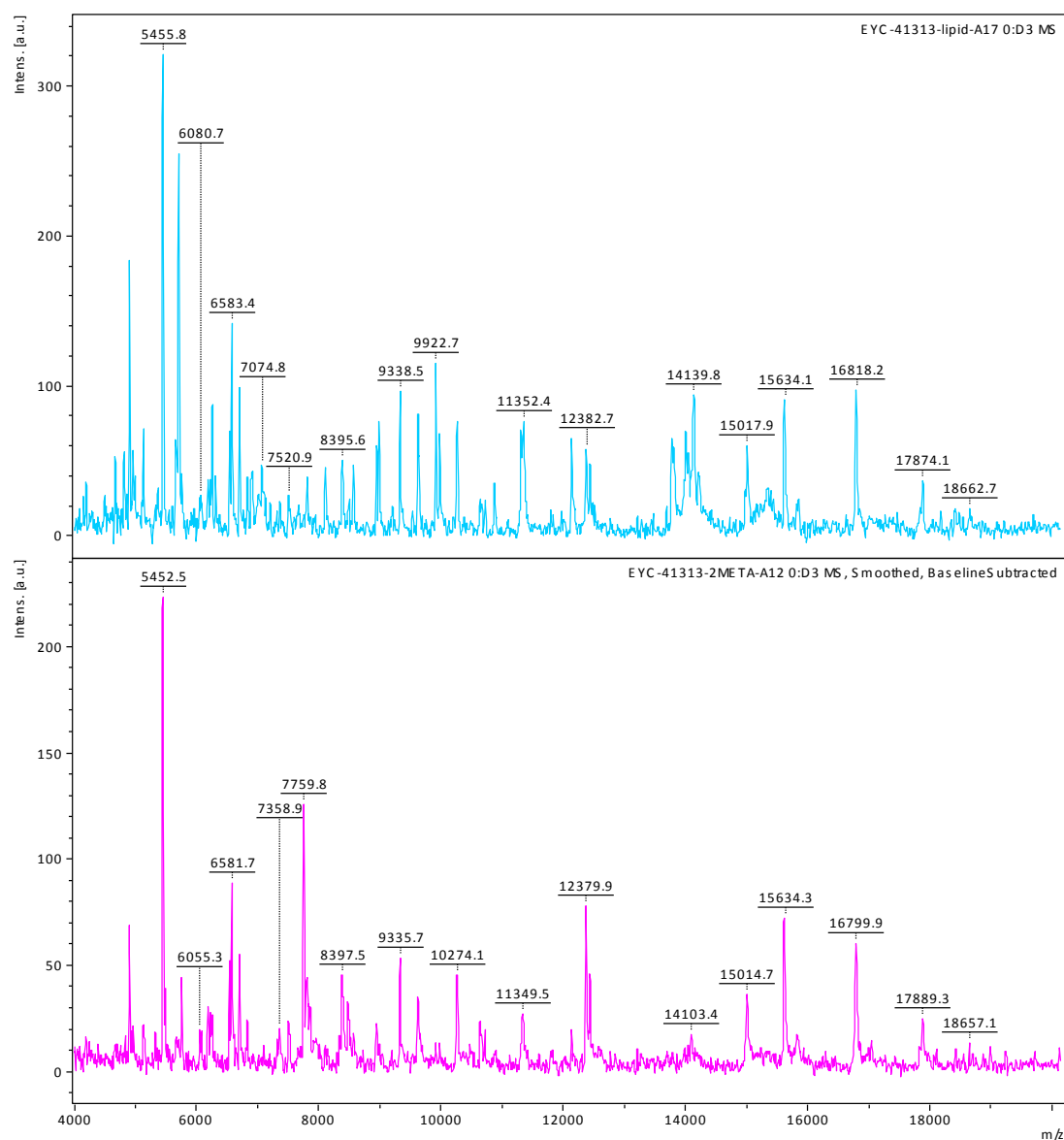
Figure 13: Comparison of spectra obtained from the use of a delipidation wash (top) or META wash (bottom) before the application of 5 depositions of 20 mg/mL matrix made in 50/50 ACN/1% TFA. The delipidation wash resulted in higher quality spectra – with a higher number of peaks at higher intensity, but the META wash resulted in the lowest variance in the quality of spectra obtained.
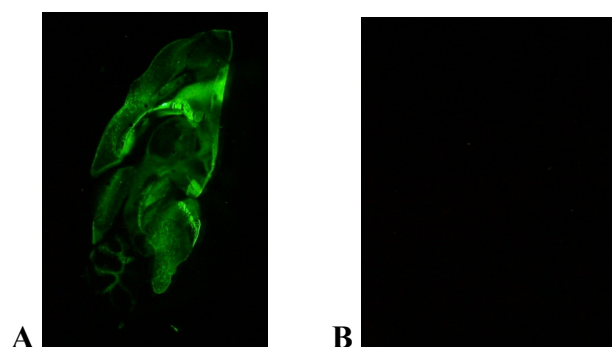

Figure 14: Fluorescent images of YFP tissue treated with a META wash (A) and a delipidation wash (B). Fluorescence was preserved with the META wash, but lost with the delipidation wash

G93A/Control Comparison Studies

Now that optimal conditions for preparation of tissue for biomarker discovery have been determined, a study was conducted to compare G93A positive mice to their control littermates. G93A positive mice are genetically affected with ALS and express the protein SOD1. Tissue was sliced coronally, from the anterior to the posterior of the brain every 300 microns. The slices were matched to a mouse brain atlas to determine where the motor cortex was located in each section. The sections were washed with META via vertical dip in solution and then 5 depositions of 20 mg/mL matrix solution in 50/50 ACN/1% TFA were manually spotted with micropipettes on the motor cortex, and analyzed via MALDI-MS. The data was compared both by visually looking at the spectra (Figure 15), and also using the program ClinProTools by Bruker Daltonics (Figure 16). It was found that there are peaks that differ between the G93A tissue and control tissue.
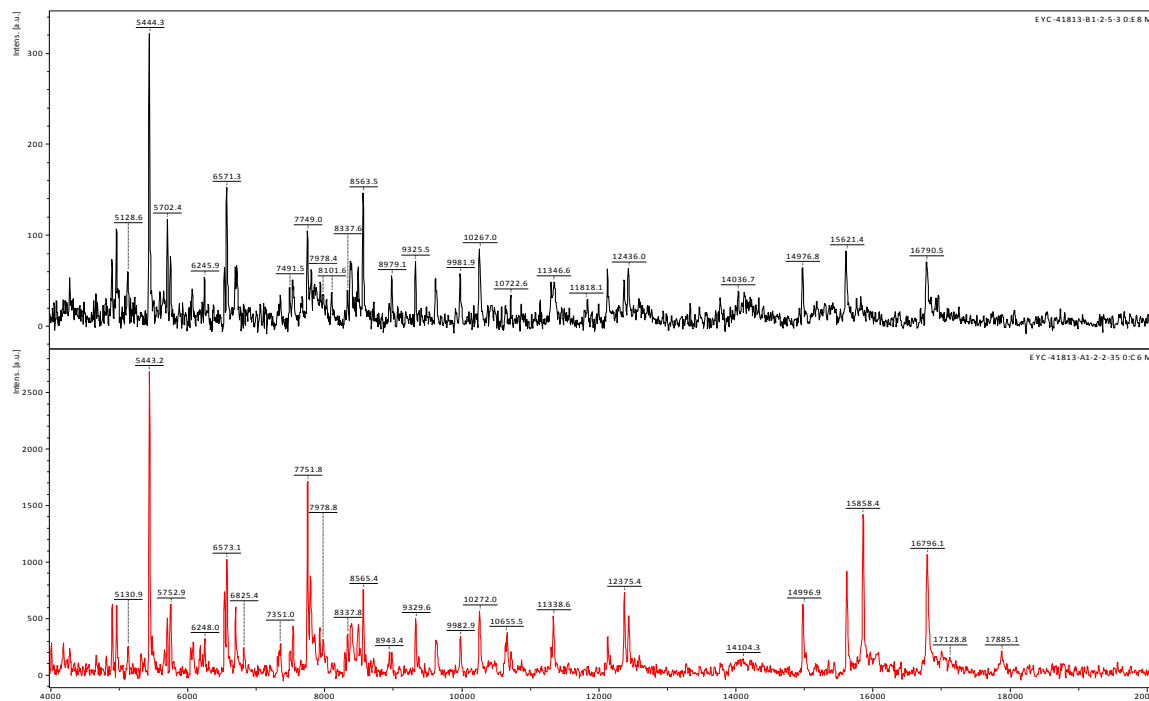

Figure 15: Comparison of spectra obtained from the motor cortex of G93A positive (bottom) and control littermate (tissue) tissue. There are observable peak differences between the two spectra, most notably the peak at 15858.1 m/z, which corresponds to SOD1, a protein implicated in familial ALS, which should only be present in the G93A spectra and not in the control.

The largest difference between the two was the peak at 15848.1 m/z, which corresponds to SOD1 and only exists in the G93A data. This finding validates the experiment design, in that a clear difference in spectra was observed. Other differences were also observed at 17872.1 and 6180.3 m/z, which were present more in the control and not the G93A tissue, and 16057.9, and 8559.5 m/z, which were present more in the G93A and not the control tissue. These differences are a great start towards the discovery of an ALS biomarker, though they are not definitive. This study was only performed once, so the validity of the results is questionable, and repeated experiments must be performed to substantiate the differences found in this initial study.
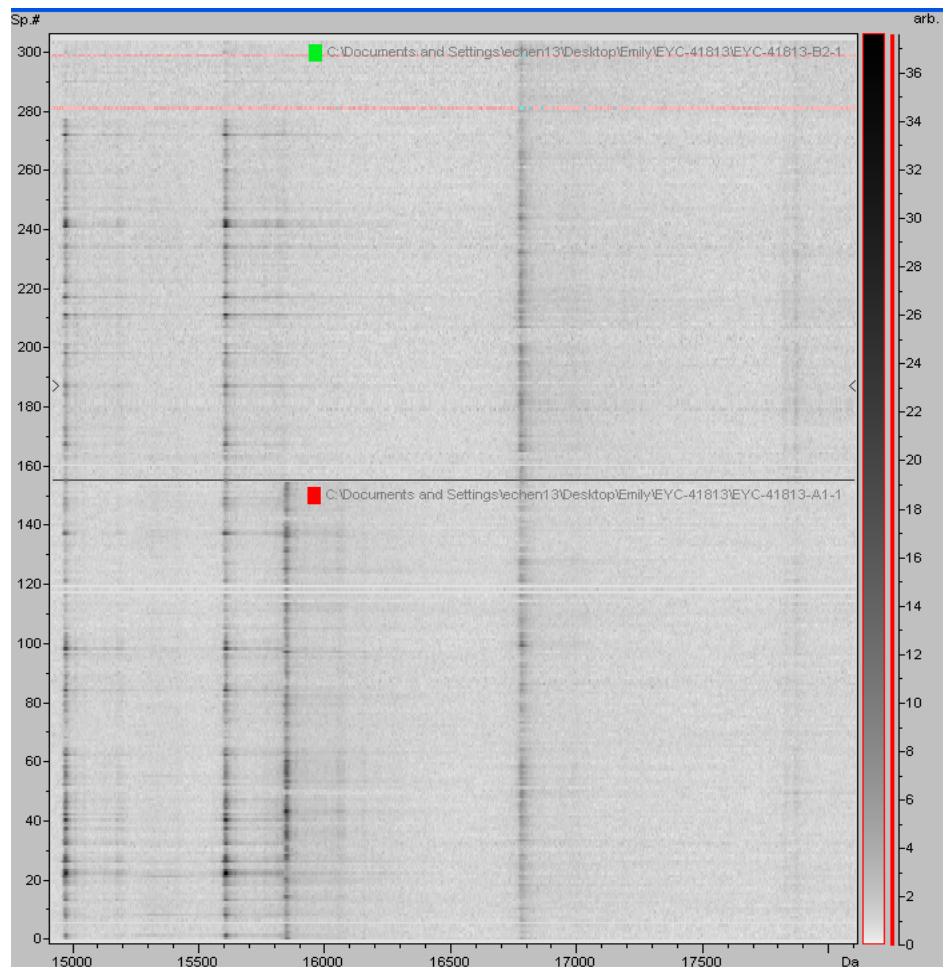

Figure 16: ClinProTools generated diagram of the occurrence of the peak of 15848.1 m/z in G93A spectra (bottom), and its absence in the control spectra (top). The diagram is a representation of all spectra acquired across the tissue, and the band corresponding to the protein of this molecular weight is lost in the control spectra.

Conclusions

The focus of this study was the optimization of sample preparation for the study of protein distribution and relative abundance in the motor cortex of ALS-affected mice and its application towards the discovery of a biomarker for ALS. Through a large series of experiments, tissue preparation with the use of META as a matrix solvent was optimized, and it was found that 3 depositions of matrix at 20 mg/mL yielded the highest quality spectra. This finding is a remarkable discovery in the field of MALDI-MS imaging, as there were no optimal conditions or universal method used in MALDI-MS imaging previously. This method maintains the spatial distribution of proteins across a tissue section, while acquiring sensitive and reproducible spectra.

The lessons learned from the META solvent optimization – that the initial concentration of matrix applied to the tissue and the number of depositions determine the overall quality of spectra more than the total concentration of matrix on the tissue – were then applied to a more general MALDI solvent which is widely used, the 50/50 mixture of acetonitrile and 1% aqueous TFA. Subsequent optimization experiments conducted with 50/50 matrix solvent concluded 5 depositions of 20 mg/mL matrix solution yielded the highest quality spectra. In addition, multiple META wash experiments were performed, and found that META is also an optimal wash solvent, which removes salts and increases the quality of spectra acquired as well as decreasing the variability, thus allowing one to obtain the best spectra for the discovery of a biomarker.

Preliminary results from my comparison study show differences in protein distribution within the motor cortex, which is extremely promising. Further experiments must be conducted to validate the results found and eventually identify the proteins which differ in the ALS-affected

and control tissue. Correspondingly, a new mouse colony is currently being bred with both the G93A positive and YFP labeled neurons to eventually correlate differences seen in the spectra to specific motor neurons via MALDI-MS imaging at a cellular level. Though this study focused solely on the upper motor neurons located in the motor cortex, spinal cord studies will also be performed in the future to look at the effect of ALS on the lower motor neurons in a similar fashion. Our results suggest the methods and preparation have been developed to the point where protein differences in the motor cortex can be detected, which can lead to the discovery of a biomarker for ALS or enhance the understanding of the underlying mechanism behind the disease.

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