

SANDIA REPORT

SAND2008-0077

Unlimited Release

Printed December 2007

Final LDRD Report: Development of Sample Preparation Methods for CHIPMA-Based Imaging Mass Spectrometry of Tissue Samples

Richard Behrens, Deneille Wiese-Smith, Sean Maharrey, Aaron Highley

Prepared by
Sandia National Laboratories
Albuquerque, New Mexico 87185 and Livermore, California 94550

Sandia is a multiprogram laboratory operated by Sandia Corporation,
a Lockheed Martin Company, for the United States Department of Energy's
National Nuclear Security Administration under Contract DE-AC04-94-AL85000.

Approved for public release; further dissemination unlimited.



Sandia National Laboratories

Issued by Sandia National Laboratories, operated for the United States Department of Energy by Sandia Corporation.

NOTICE: This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government, nor any agency thereof, nor any of their employees, nor any of their contractors, subcontractors, or their employees, make any warranty, express or implied, or assume any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represent that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government, any agency thereof, or any of their contractors or subcontractors. The views and opinions expressed herein do not necessarily state or reflect those of the United States Government, any agency thereof, or any of their contractors.

Printed in the United States of America. This report has been reproduced directly from the best available copy.

Available to DOE and DOE contractors from
U.S. Department of Energy
Office of Scientific and Technical Information
P.O. Box 62
Oak Ridge, TN 37831

Telephone: (865) 576-8401
Facsimile: (865) 576-5728
E-Mail: reports@adonis.osti.gov
Online ordering: <http://www.doe.gov/bridge>

Available to the public from
U.S. Department of Commerce
National Technical Information Service
5285 Port Royal Rd
Springfield, VA 22161

Telephone: (800) 553-6847
Facsimile: (703) 605-6900
E-Mail: orders@ntis.fedworld.gov
Online order: <http://www.ntis.gov/help/ordermethods.asp?loc=7-4-0#online>



Final LDRD Report: Development of Sample Preparation Methods for ChIPMA-based Imaging Mass Spectrometry of Tissue Samples

Richard Behrens, Deneille Wiese-Smith, Sean Maharrery, Aaron Highley
Remote Sensing and Energetic Materials Department
Sandia National Laboratories
P.O. Box 969
Livermore, California, 94551-0969

ABSTRACT

The objective of this short-term LDRD project was to acquire the tools needed to use our chemical imaging precision mass analyzer (ChIPMA) instrument to analyze tissue samples. This effort was an outgrowth of discussions with oncologists on the need to find the cellular origin of signals in mass spectra of serum samples, which provide biomarkers for ovarian cancer. The ultimate goal would be to collect chemical images of biopsy samples allowing the chemical images of diseased and non-diseased sections of a sample to be compared. The equipment needed to prepare tissue samples have been acquired and built. This equipment includes a cyro-ultramicrotome for preparing thin sections of samples and a coating unit. The coating unit uses an electrospray system to deposit small droplets of a UV-photo absorbing compound on the surface of the tissue samples. Both units are operational. The tissue sample must be coated with the organic compound to enable matrix assisted laser desorption/ionization (MALDI) and matrix enhanced secondary ion mass spectrometry (ME-SIMS) measurements with the ChIPMA instrument

Initial plans to test the sample preparation using human tissue samples required development of administrative procedures beyond the scope of this LDRD. Hence, it was decided to make two types of measurements: (1) Testing the spatial resolution of ME-SIMS by preparing a substrate coated with a mixture of an organic matrix and a bio standard and etching a defined pattern in the coating using a liquid metal ion beam, and (2) preparing and imaging *C. elegans* worms. Difficulties arose in sectioning the *C. elegans* for analysis and funds and time to overcome these difficulties were not available in this project.

The facilities are now available for preparing biological samples for analysis with the ChIPMA instrument. Some further investment of time and resources in sample preparation should make this a useful tool for chemical imaging applications.

Intentionally Left Blank

Contents

Abstract.....	3
Introduction	6
Background.....	6
ChIPMA Instrument.....	9
Research Objectives and Technical Issues.	10
Technical Challenges.	11
Project Organization and Results.....	13
Project Organization	13
Original scope of work.	13
Modifications to original scope of work	13
Executed project plan	14
Results	14
Microtome acquisition	14
Sample coating system.....	15
Preparation and analysis of thin film bio test samples	17
Preparation of <i>C. elegans</i> samples.....	17
Working with <i>C. elegans</i>	17
Sectioning of <i>C. elegans</i>	18
Current status of Chemical Imaging of Bio Samples.....	19
Conclusion	20
References	21
Appendix I. Mathematica code for spray parameters.....	22
Distribution	27

Figures

Figure 1. MALDI protein profile obtained from 12 μm thick human grade IV glioma tissue section (From Caprioli[1]).....	8
Figure 2. IMS analysis of a 12- μm thick coronal mouse brain section containing a tumor.....	8
Figure 3. Direct molecular imaging of <i>Lymnaea stagnalis</i> nervous tissue by ME-SIMS.....	9
Figure 4. ChIPMA-SIMS imaging of AP-based rocket motor propellant sample...	10
Figure 5. Schematic illustration of MALDI and ME-SIMS sample preparation issues	13
Figure 6. Picture of the Leica UC-6/FC-6 cyro-ultramicrotome	14
Figure 7. Pictures of electrospray coating device.....	15

Development of Sample Preparation Methods for ChIPMA-Based Imaging Mass Spectrometry of Tissue Samples

Introduction

The purpose of this short-term LDRD was to develop the methods required to prepare biological tissue samples for examination with imaging mass spectrometry (IMS) implemented on Sandia's Chemical Imaging Precision Mass Analyzer (ChIPMA) instrument. Results from these measurements would enable us to develop new programs in proteomic-based development of new diagnostic tests, patient-based treatment therapies, and new methods to combat and treat ovarian cancer. In discussions with GYN surgical oncologists (James Lilja, MD – Bay Area Gynecology Oncology Group) and a clinical laboratory focused on developing new tests for diagnosing, treating and improving the lives of cancer patients (Oncotech, Tustin, CA), it was apparent that our unique ChIPMA instrument had the potential to make measurements on tissue samples that would enable the more reliable development of biomarkers for cancer detection. If successful this could potentially open new opportunities to collaborate on biomedical research.

To develop a new potential collaboration in this area required obtaining equipment to prepare tissue samples for analysis with the ChIPMA instrument. Two tools were required: (1) a cryo-microtome for sectioning samples for analysis, and (2) a coating system to spray small drops of organic compounds onto the tissue samples. The focus of this LDRD was to develop these capabilities.

The new capabilities have general utility for preparation of samples beyond the scope of our initial vision. The tools can be used to prepare any type of biological samples. For example, they have been used to prepare biomass samples, such as switchgrass, for analysis. They also have utility for examining other composite materials found in our NW systems. For this application, the tools have been used to prepare EPDM rubber samples for analysis with the ChIPMA instrument.

Background

Certain types of cancer, such as pancreatic and ovarian cancer, are typically detected during the later stages of the disease (stages III and IV), resulting in very high mortality rates. To provide earlier detection, the development of biomarkers for detection of disease is an active area of research. For example, recent mass spectrometry (MS) based methods have been developed for the early detection of ovarian cancer.[3] The MS methods were used to develop a set of “training” spectra based on the analysis of serum from 50 unaffected women and 50 women with ovarian cancer. When the discovered pattern was applied to 116 masked serum samples (50 cancer, 66 unaffected or non-malignant), all cases of ovarian cancer were correctly identified, 63 out of 66 cases of non-malignant patients were correctly identified. This was considered by many to be a huge success at the time the study was released. However, subsequent testing, in a clinical setting, has had difficulty in reproducing the initial results. To date the development of reliable biomarkers for cancer screening remains an elusive goal.

The problem with methods such as Petricoin's is that they are based on observing peaks in the mass spectrum that are associated with the group of patients having the disease in the clinical study. They do not measure the compounds that are associated with the tissue affected by the disease. Thus, both patient-to-patient variations and variation in instrument configuration and calibration can adversely affect the diagnostic test, rendering it useless in clinical applications.

An improved approach. The progression of many diseases, such as cancer, exhibits a high degree of spatiotemporal dependence of the biochemical markers associated with a disease. For example, cancer originates at the cellular level, progresses to invade and alter cells in the surrounding tissue, and eventually metastasizes, invading other organs, and eventually leading to death. Early intervention to remove cancerous cells, through surgery, chemotherapy or radiation treatment, can eliminate the growth or the cancerous cells and increase longevity. Thus, both the progression of the disease and its treatment illustrate the spatiotemporal dependence.

The ability to identify and track the chemical, physical and cellular processes that underlie the onset and development of these diseases in cells and tissues will provide a new means to understand their underlying causes and develop new diagnostic tests and therapies for their treatment.

Tissue analysis by IMS. Utilizing imaging mass spectrometry (IMS) to examine tissues and cells from different types of organisms is a recent development. Recent work by Caprioli employed matrix laser desorption and ionization (MALDI) as an IMS method to image tissue

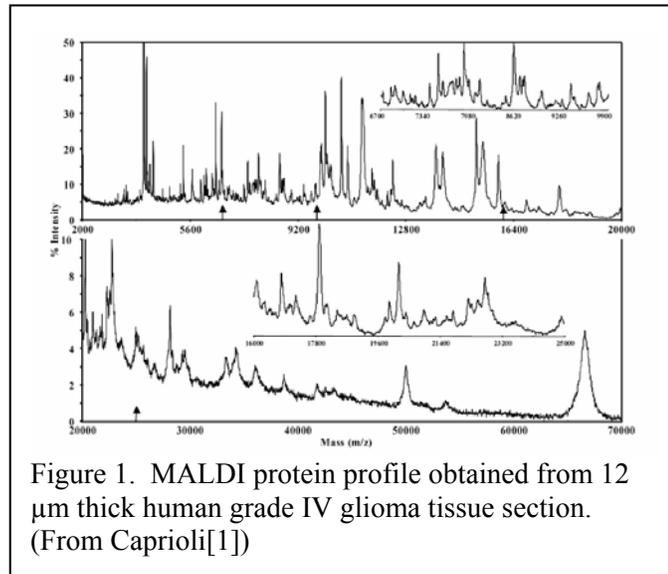


Figure 1. MALDI protein profile obtained from 12 μm thick human grade IV glioma tissue section. (From Caprioli[1])

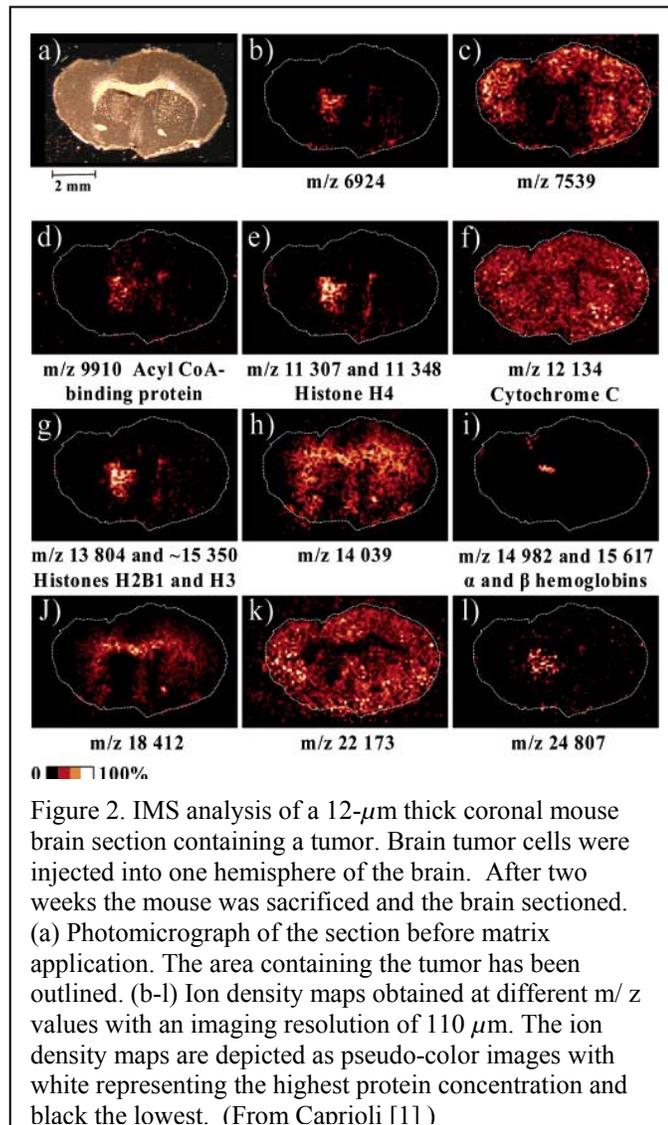


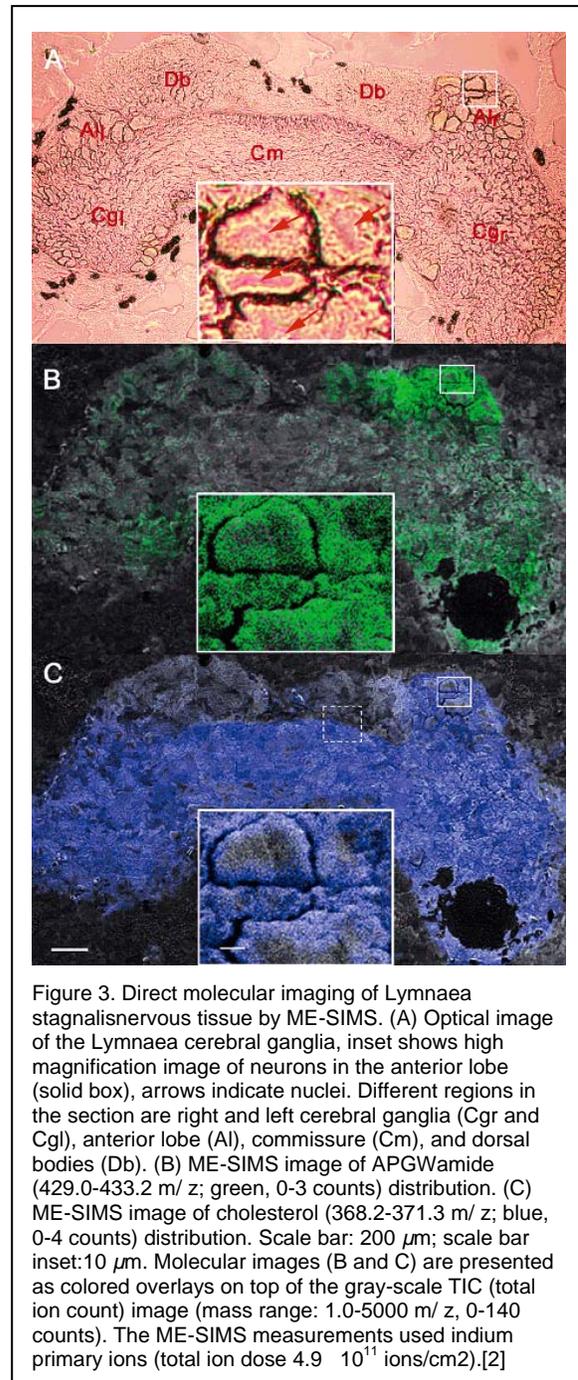
Figure 2. IMS analysis of a 12-μm thick coronal mouse brain section containing a tumor. Brain tumor cells were injected into one hemisphere of the brain. After two weeks the mouse was sacrificed and the brain sectioned. (a) Photomicrograph of the section before matrix application. The area containing the tumor has been outlined. (b-l) Ion density maps obtained at different m/z values with an imaging resolution of 110 μm. The ion density maps are depicted as pseudo-color images with white representing the highest protein concentration and black the lowest. (From Caprioli [1])

samples and characterize the progression of disease[1, 4, 5]. For example, Figure 1 illustrates the protein profile obtained with a MALDI MS analysis of a 12 μm thick human grade IV glioma tissue section. The peaks in this spectrum represent neuroproteins and neuropeptides from a brain tissue.

By carefully depositing the MALDI matrix on the surface of a tissue section, mass spectra may be collected as a function of location. For example, Figure 2 presents several ion intensity maps from a mouse brain in which a tumor was grown. Figure 2a shows the photomicrograph of the brain section prior to applying the MALDI matrix of sinapinic acid. Images shown in Figure 2b-l show different protein expression maps across the tissue section. Some of the individual proteins have been identified, for example those showing histones (H4, H2B1, and H3) are consistent with the presence of fast developing tumor cells[6]. Proteins associated with normal cell activity, such as Cytochrome C, are found uniformly distributed throughout the tissue section.

In 2005 Ron Heeren's group demonstrated higher spatial resolution IMS using matrix enhanced secondary ion mass spectrometry (ME-SIMS) methods.[2] Figure 3 illustrates direct molecular imaging of *Lymnaea stagnalis* nervous tissue by ME-SIMS. Note the higher spatial resolution compared to the MALDI based IMS images shown in Figure 1. The ME-SIMS method allows imaging with sub-cellular resolution.

These results from SIMS and MALDI IMS measurements demonstrate the wealth of new information that can now be obtained to investigate the pathways that underlie the development of disease.



ChIPMA Instrument

ChIPMA Instrument. The ChIPMA instrument was designed and built at Sandia to address aging issues of materials in the NW stockpile. An example of using the instrument to characterize and ammonium perchlorate propellant sample is shown in Figure 4. The ChIPMA instrument has capabilities that allow it to surpass the current instruments being used for SIMS and MALDI IMS measurements.

The ChIPMA instrument incorporates a high resolution and high mass accuracy Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer in addition to a reflectron TOF mass

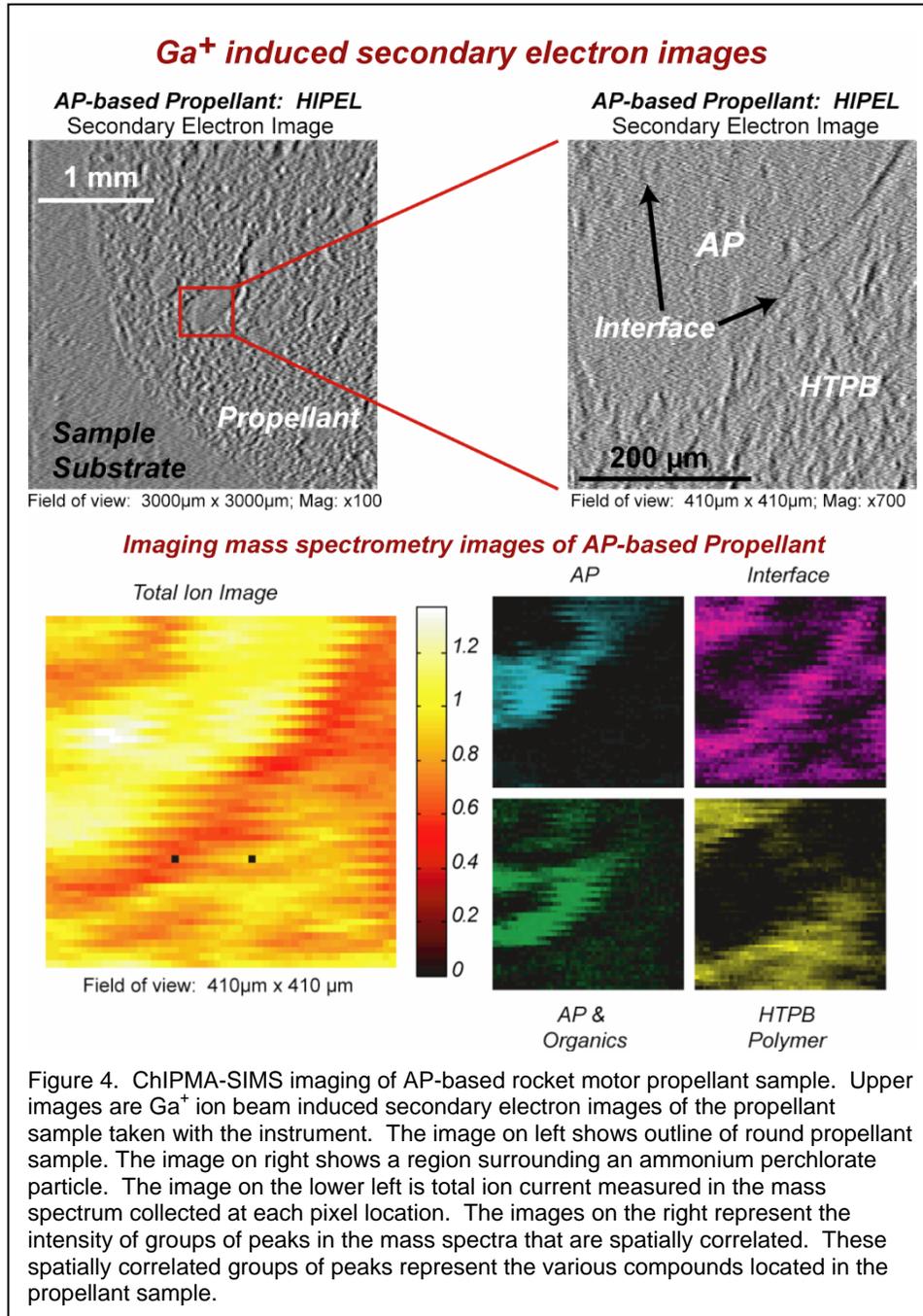


Figure 4. ChIPMA-SIMS imaging of AP-based rocket motor propellant sample. Upper images are Ga⁺ ion beam induced secondary electron images of the propellant sample taken with the instrument. The image on left shows outline of round propellant sample. The image on right shows a region surrounding an ammonium perchlorate particle. The image on the lower left is total ion current measured in the mass spectrum collected at each pixel location. The images on the right represent the intensity of groups of peaks in the mass spectra that are spatially correlated. These spatially correlated groups of peaks represent the various compounds located in the propellant sample.

spectrometer, which is typically used in IMS instruments. The FTICR mass spectrometer allows more accurate and definitive assessments of the types of peptides and proteins present in a tissue section due to its inherently high precision in identifying large mass molecules and fragments. This analysis capability also allows reactions involving various proteins, signaling compounds, or drugs to be probed as a function of location within the tissue. New automated spectral image analysis (AXSIA) algorithms[7] are used to examine the data collected with the ChIPMA instrument, which provide correlations of chemical compounds based on spatial location. For example, using AXSIA to analyze the data shown in Figure 2 would provide lists of m/z values in the IMS spectra that are associated with individual compounds in specific spatial regions. Thus, peptide, proteins or other compounds associated with specific regions of the developing tumor could be identified. The instrument is equipped with UV lasers for MALDI-based IMS measurements and a liquid metal Ga⁺ ion source (50nm beam diameter) for ME-SIMS measurements.

Research Objectives and Technical Issues.

Current research in the biological sciences focus on understanding the progression of disease by identifying and measuring biochemical compounds that are associated with the initiation, development, progression and reoccurrence of disease. Typical clinical diagnostic methods rely on either observing morphological changes in tissues caused by disease with various microscopic methods, or biochemical assays on bulk samples such as serum. In contrast, a new approach to achieve these research objectives is to determine what biochemical compounds are formed at various stages in diseased tissue by directly identifying and locating biochemical compounds within diseased tissues. This requires the ability to locate and identify compounds that are associated with disease at spatial resolution down to the cellular level. The technical issues to accomplish this are quite challenging. The work covered in this proposal will enable us to examine biological samples with the ChIPMA instrument, thus enabling us to address the primary research objectives.

Research Objectives. The primary research objectives focus on understanding the basis for the progression of disease. IMS with ChIPMA, if successful, could be applied to a range of different diseases. When this project was originally proposed, we had had discussions with a group that is interested in developing a CRADA with Sandia in the area of ovarian cancer research. Their objective was to develop better methods for early detection of ovarian cancer and improved assays to evaluate the efficacy of cancer treatment methods. The partners in this CRADA would bring three things to the table that are required to meet the biological research objectives and their health care objectives: (1) A source of diseased and non-diseased tissues samples for analysis (J. Lilja, Bay Area Gynecology Oncology Group), (2) a clinical laboratory with the facilities to handle and prepare tissue samples, cell cultures and current clinical diagnostics, plus a characterized bank of cancer samples (Oncotech), and (3) the ability to examine the spatial dependence of biochemical compounds in cell cultures and tissue samples (Sandia). (*Note: By the time funding was approved for this project and the equipment was acquired, Oncotech had decided to move in other research directions. They are currently being acquired by a Norwegian company.*)

This fundamental research approach could be applied to other types of cooperative efforts in the future. Please note that this project does not directly support these research efforts. It provides a critical link that is required to address the technical challenges.

Technical Challenges.

The technical challenges to perform chemical imaging of biological samples that will provide insight into the development of disease is substantial. The main challenges that must be met to enable this scientific approach are:

1. Identifying the wide range of different compounds that are present in a cell culture or tissue sample.
2. Measuring the location of the various compounds at sufficient spatial resolution that will enable their origin within the tissue sample (or within the cell) to be identified. This allows matching various chemicals to biological function.

To meet these challenges imaging mass spectrometry based approaches are used. To implement IMS for biological sample analysis requires:

1. Removing microscopic samples from the surface of the sample and creating ions of the molecules that are indicative of the compounds in the tissue sample or cell culture.
2. Analyzing the mass spectra of the resulting ions so that identities of the chemical compounds can be established.
3. Collecting the data with sufficiently high spatial resolution.
4. Rapidly analyzing large data sets.

The IMS work currently being undertaken throughout the world in this area is limited and quite technically challenging. World wide, there are five to ten groups exploring research in this area. To meet the challenges we build on the previous work of two of the more successful groups (Caprioli's work using MALDI and Ron Heeren's work using ME-SIMS).

The critical feature that we bring to the table, which is unavailable in any other group, is the ability to identify the ions formed in the IMS measurements. This unique ability is based on two aspects of the ChIPMA instrument. First, in addition to utilizing a reflectron TOF mass spec, as is the case for the other groups, we also can analyze ions removed from the sample surface with the high resolution ($m/\Delta m \sim 200,000$ compared to $m/\Delta m \sim 5000$ for TOF instruments) and high mass accuracy ($< 1\text{ppm}$) using an FTICR mass spectrometer. This enables us to identify chemical compounds that cannot be identified by TOFMS measurements.

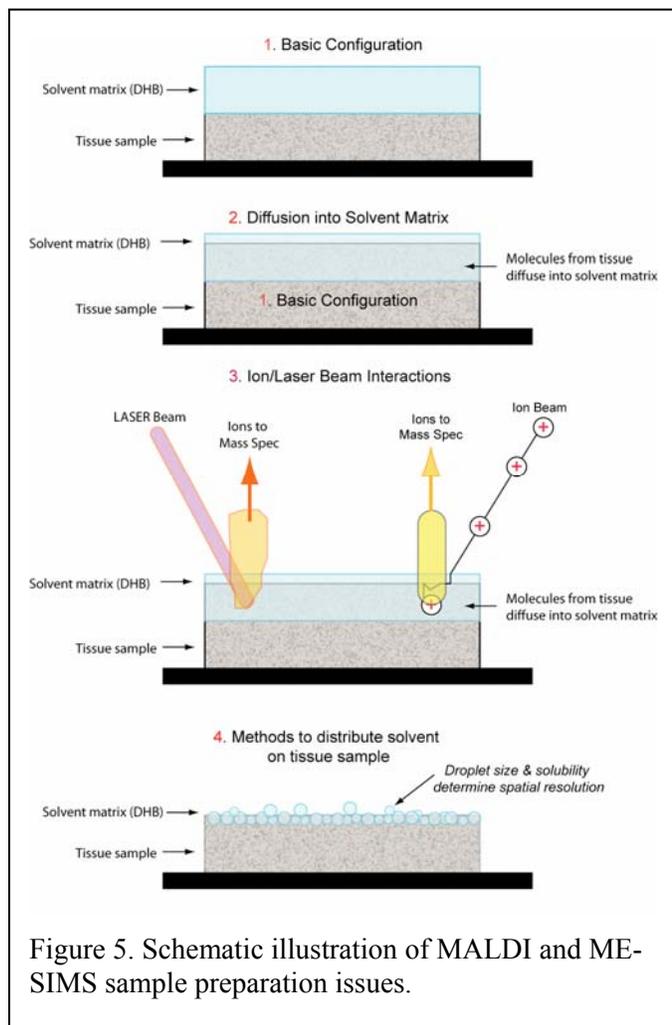


Figure 5. Schematic illustration of MALDI and ME-SIMS sample preparation issues.

Second, we utilize Sandia's AXSIA program to analyze the mass spectra for principal components as a function of spatial location. In contrast to Caprioli's data shown in Fig. 2, in which signals at one m/z value are shown, the AXSIA analysis provides all of the ions in a mass spectrum that are associated with specific spatial locations

The main issues that we need to address before we can start using the ChiPMA instrument for investigating biological samples deal with sample preparation. This involves developing the methods to section the samples and coat the samples with the required analysis matrices (e.g. dihydroxybenzoic acid). While this may sound simple, sample preparation is critical to the IMS analysis method. The main issues related to sample preparation are illustrated in Figure. 5. The critical step is preparing the sample so that the molecules of interest move from the tissue sample (or cell culture) into the solvent matrix without losing spatial resolution. This requires very careful and controlled deposition of microdroplets of solvent matrix on the sample surface and controlled interaction of the droplet with the sample at the tissue interface. These are the issues that we address in this project.

Project Organization and Results

Project Organization

Original scope of work.

This project will develop the methods to prepare and coat tissue samples with matrix solvents and collect images of these samples using MALDI-based IMS and ME-SIMS. FTICR mass spectra from selected regions of the tissue samples will be collected to demonstrate ability of the ChIPMA instrument to identify the compounds that evolve from the tissue sample.

The following tasks were planned in FY06 (\$88k):

1. Acquire non-hazardous tissue samples for testing.
2. Cryo-microtome tissues samples and prepare for mounting.
3. Design and construct electrospray matrix deposition station and sample holders.
4. Prepare samples for MALDI-IMS, ME-SIMS analysis.
5. Collect MALDI-IMS, ME-SIMS and FTICR/MS data on tissue samples.
6. Prepare report and illustrations of results for program development.

The following tasks were planned for FY07 (\$40k):

1. Obtain specific samples related to ovarian cancer proteomics.
2. Prepare samples for ChIPMA analysis.
3. Collect IMS data on new samples
4. Relate data to results from conventional biochemical analysis of samples.

Modifications to original scope of work

Shortly after the start of this project, it was recognized that two modifications to our original plans was necessary. First, acquisition of human tissue samples could not be implemented with the limited funding in this project. The administrative procedures to implement the required safety and controls for handling these types of tissues would be extensive and the interests of our initial contacts had waned. Second, collecting data with the ChIPMA instrument was non-routine.

To circumvent these issues we modified our objectives. To address the issue of tissue acquisition for method development and evaluation, we decided to focus on the imaging of *C. elegans* worms. These organisms have been studied extensively. They are comprised of approximately 1000 cells and have a well defined and extensively studied nervous system. Many different genetic modifications of *C. elegans* are readily available. Genetic modifications may be obtained in which specific proteins will be over expressed. Thus, comparison of chemical images of normal *C. elegans* with chemical images of the genetically modified organism provides a good system to test the development of our chemical imaging methods.

To address issues of making measurements with the ChIPMA instrument more routine and to evaluate the spatial resolution of the instrument when examining biological samples, a pattern with different spatial dimensions was made from a set of thin film samples. The films were made from a mixture of several different peptides in DHB.

Executed project plan

The following tasks were undertaken during this project:

1. Evaluate and secure a cryo-microtome for the preparation of samples.
2. Design, fabricate and test system to control the coating and preparation of tissue samples
3. Evaluate MALDI and ME-SIMS measurement of patterned thin film samples to develop the chemical imaging methods.
4. Develop capabilities to acquire, grow and section *C. elegans* for analysis.

Results

Microtome acquisition

Our original plans were to purchase a histological cryo-microtome similar to those used in other laboratories doing this type of work. In examining various types of cryo-microtomes, we came to consider the acquisition of a cyro-ultramicrotome. These instruments are normally used to prepare samples for transmission electron microscopy (TEM) measurements. In evaluating the various instruments we used the following criteria for selection:

1. The ability to prepare very thin sections of material without the need to fix the sample with a cross-linking agent prior to sectioning. Typical histological cryo-microtomes can prepare sections down to several microns thick. In contrast, a cyro-ultramicrotome typically creates sections that are 30 to 80 nm thick. Some manufacturers claim to be able to create sections

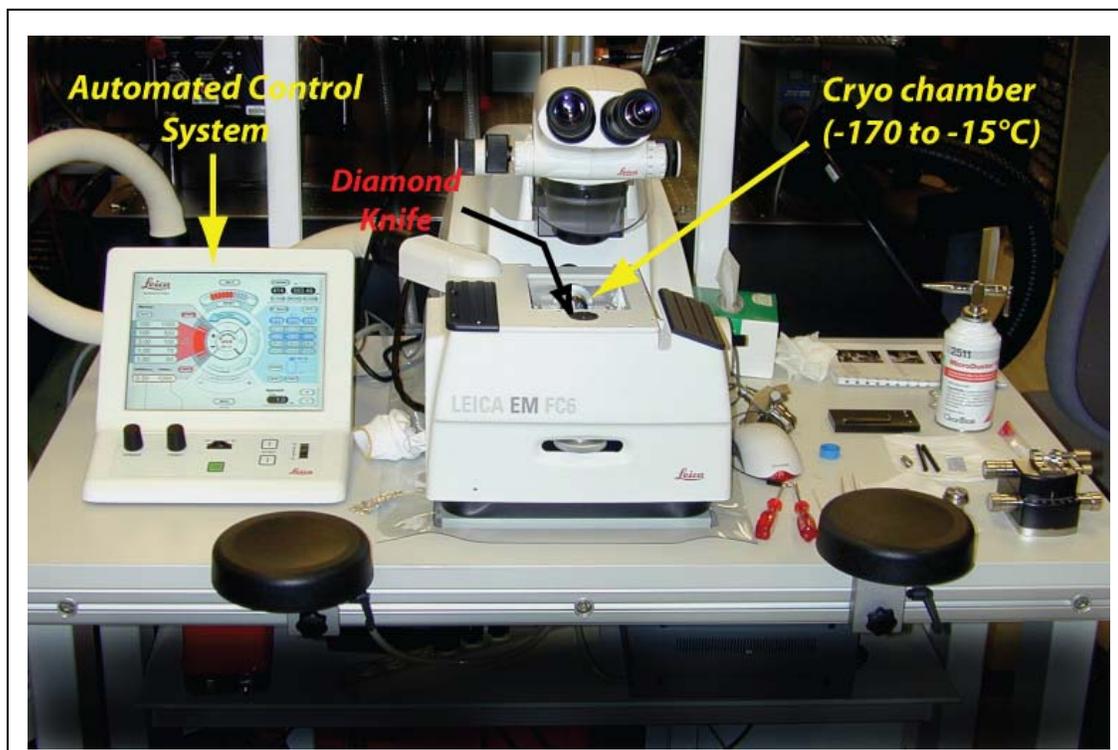


Figure 6. Picture of the Leica UC-6/FC-6 cyro-ultramicrotome.

down to a thickness of 5 nm.

2. The ability to prepare sections of sample in the range of 5 to 50 nm opens new possibilities for imaging diagnostics. The thin samples are similar to thin films of polymers that have been analyzed by placing them on different types of substrates. For example, we have imaged samples of dimethylsiloxane and polystyrene polymers on silver films. Thin samples also opens the possibility of using techniques such as direct ionization on silicon (DIOS)[8] to image the samples.
3. The instrument had to provide good control of the working temperature of the knives and the sample. It also had to have a long working time (hours) before ice accumulates in the working area.

With some funds available from another program with interest in preparing samples for imaging we decided to purchase a cyro-ultramicrotome. Two instruments were evaluated: one from RMC and another from Leica instruments. In discussions with other users, it was apparent the Leica instrument was the better of the two and more versatile.

The Leica UC6-FC6 cyro-ultramicrotome was purchased for preparing samples. A picture of the instrument is shown in Figure 6. The instrument uses diamond knives to cut the samples. An automatic feed system advance the sample to cut slices of a desired thickness.

We have prepared samples in the 30 to 70 nm thickness range with some practice. One of the critical issues is determining the working temperature to use for sectioning.

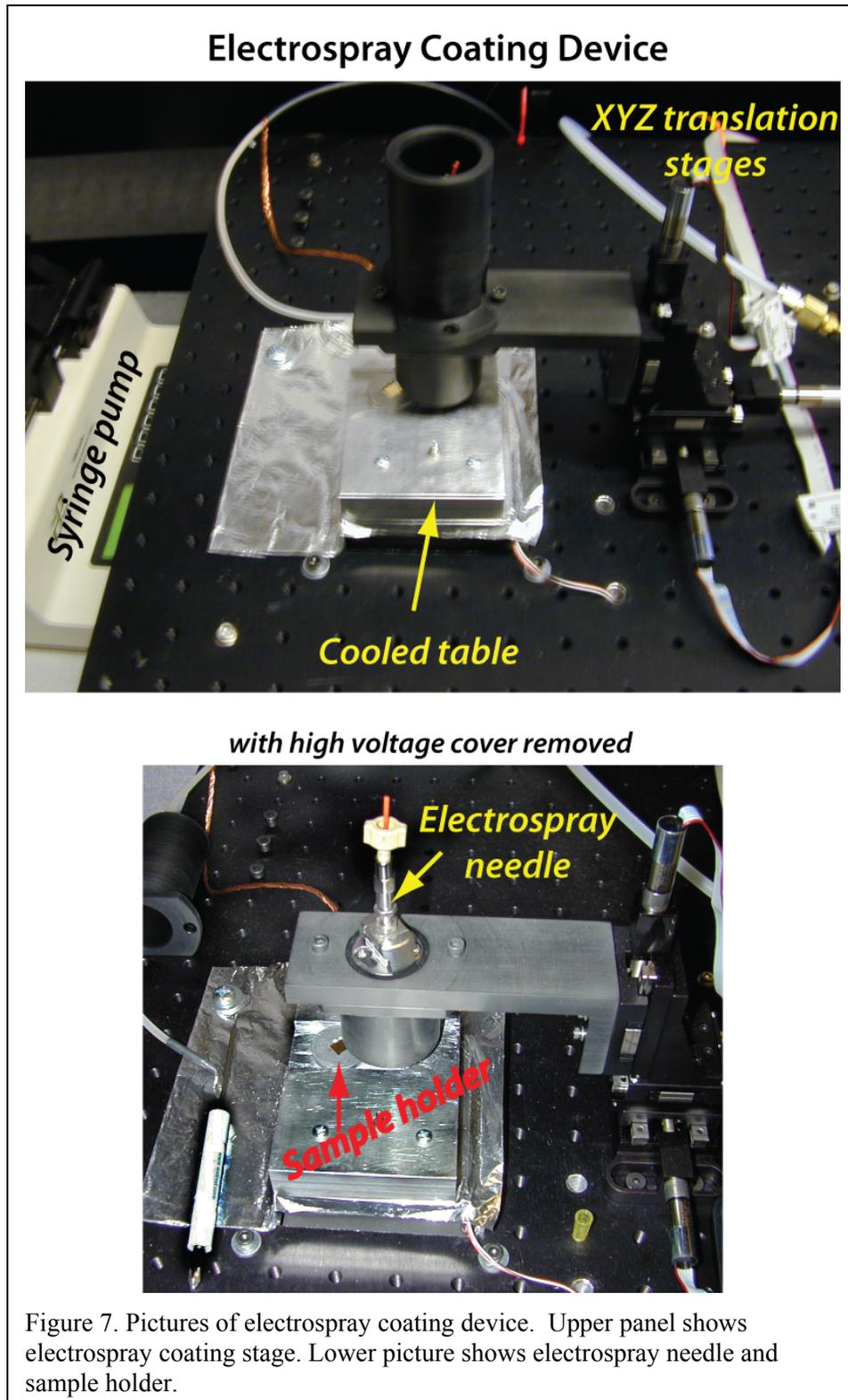
Sample coating system

A sample coating system used for coating samples for ME-SIMS measurements has been described by Heeren et.al.[2] Based on this work, we established the following criteria for the design of our coating device:

1. Temperature controlled specimen holder.
2. X-Y control of translation table. (Automated – LV)
3. Control of Z position (electrospray needle to sample surface) with translation stage (computer control).
4. Control of spray rate. Computer control of syringe pump.
5. Control of localized heat where spray is deposited.
 - a. Small CO₂ laser – computer control of energy deposition.
 - b. Optic to focus beam on region of sample where spray hits sample.
 - c. Beam enclosure system.
6. Computer control system – National Instruments Labview

The design of the device focuses on the need to control the interaction of the solvent with the tissue sample. The organic UV-photo absorbing compound (e.g., DHB) is dissolved in solvent and then sprayed on the tissue sample. Based on our knowledge of the chemical physics underlying the MALDI process, it is necessary to get the biological molecules of interest to mix with the UV-photo absorbing compound.

With this in mind, successful coating of the surface entails: (1) having a droplet deposit on the surface of the tissue; (2) dissolving of the biological compound into the solvent/UV-photo absorber; and (3) the evaporation of the solvent. To maintain good spatial resolution of the location of various compounds in the tissue sample, the diffusion of the biological molecules from their original location



in the sample to their final resting position after the solvent evaporates from the droplet will determine the ultimate resolution. The use of small droplets under conditions where droplets do not coalesce to any significant extent would appear to be the optimum conditions.

The electrospray coating device is located in a plastic enclosure to allow control of the atmosphere and to shield users from the CO₂ laser. A picture of the electrospray unit with the outer housing removed is shown in Figure 7. The coater has been used to prepare the thin film samples to be used for testing of the MALDI and ME-SIMS measurements with ChIPMA.

A short Mathematica code has been written to calculate the spray deposition parameters. This code is listed in Appendix I.

Preparation and analysis of thin film bio test samples

A sample of peptides (Bradykinin, Angiotensin II, Angiotensin I, Substance P, Bombesin, Renin Substrate, ACTH clip 1-17, ACTH clip 18-39, Somatostatin 28) and the MALDI matrix, cyano-4-hydroxycinnamic acid (CCA), were dissolved in ??? and used for preparing the films. The samples were first spotted on a substrate, loaded into the instrument, and then analyzed using MALDI and ME-SIMS methods. Spectra mass spectra of the peptides in the standard were detected with MALDI. The peptides were not observed with the ME-SIMS measurements.

The solutions were then sprayed onto the surface of silicon wafers using the new matrix-coating device. Samples were prepared using coating parameters specified in the literature. The samples were examined using ToF-SIMS. The samples were also examined using a line of SIMS measurements through the sample to determine if heterogeneity of the sample led to the inability to detect the peptides. No peptide signals were observed with any of the sprayed samples.

After preparing a set of sample using different electrospray conditions and not being able to observe any peptides in SIMS measurements, the samples were then analyzed with MALDI. MALDI also showed no peptide signals for any of the spray-coated samples. It was apparent from the lack of any MALDI signal and the fact that the samples coated onto the Si wafer were a dark color, while drop spotted samples that work in MALDI were clear, that the MALDI solution was not actually spraying from the needle.

Problems were found with the liquid delivery setup and the fact that the needle was actually grounded within the electrospray assembly and not floating at the applied voltage. By the time these problems were fixed, the end of the project had been reached and no new attempts were made to test the system. The electrical grounding of the needle tip has been fixed, and it does now float at the applied voltage, as well the liquid delivery system problems were fixed.

Preparation of *C. elegans* samples.

Preparation of the facilities to handle and work with *C. elegans* was done in conjunction with Cathy Branda. This work entailed defining, ordering and setting up the equipment to handle *C. elegans*. It was also necessary to prepare a summary of this work for review by the Institutional Biosafety Committee (IBC). The preparative work was completed and Sandia now has a facility to handle and work with *C. elegans*. The following working procedure was provided by Cathy Branda.

Working with C. elegans

Nematodes are the most numerous multicellular animals on earth; a handful of soil will contain thousands of the microscopic worms. The non-parasitic nematode species *Caenorhabditis elegans* (*C. elegans*) is an excellent simple eukaryotic research model due to the worm's transparency

(permitting the observation of cell division and cell migration throughout development), ease of maintenance (for example, *C. elegans* are hermaphrodites and thus self-fertile), and extensive genetic characterization (the genome is fully sequenced and annotated, the complete cell lineage and neural circuitry has been determined and a vast collection of behavioral and anatomical mutants are available). *C. elegans* are maintained in the lab in petri dishes containing nematode growth medium (NGM) agar streaked with their food source, OP50 *E. coli* (non-toxic, non-pathogenic). Both *C. elegans* and OP50 *E. coli* can be obtained from the Caenorhabditis Genetics Center (CGC) located in St. Paul, MN.

Reagents used to generate NGM agar plates

- Agar
- Peptone
- Cholesterol (dissolved in ethanol)
- Magnesium sulfate
- Potassium phosphate
- Water

The NGM agar mix is autoclaved (to sterilize and dissolve the agar), allowed to cool briefly, and then poured into petri dishes, similar to the production of LB agar plates. Once the plates are cooled, a few drops of OP50 culture is added to the top of each plate and allowed to dry. *C. elegans* can be transferred from one NGM agar plate to another, or onto a microscope slide, or into an embedding media for cryosectioning using a sterile spatula. For the purpose of cryosectioning, a sucrose-based embedding media containing the worms would be placed onto a bath of dry ice and ethanol for rapid freezing. The procedures described above will take place in building 968. For long-term storage, *C. elegans* can be frozen indefinitely in liquid nitrogen (in glycerol in a 2 milliliter cryovial). This vial can be stored in a liquid nitrogen container we currently use in building 968 for the storage of mammalian cell lines.

Sectioning of the *C. elegans* and subsequent Mass Spectrometry will take place in building 905. The frozen block will be transferred across the site on dry ice in a closed styrofoam container. To ensure that the styrofoam container is decontaminated prior to leaving building 968, we will thoroughly wipe the outside of the container with a 70% ethanol solution.

Sectioning of *C. elegans*

The *C. elegans* were prepared for sectioning by immobilizing them in a OCT solution. The first attempt to prepare sections of *C. elegans* began by placing a few worms in an OCT solution spotted to a series of small cryo-molds and freezing the molds in liquid N₂. In trying to section this sample with the cyro-ultramicrotome, it was not possible to see the worms in the matrix with the microscope. Next a sample was prepared with many more worms. Again, it was not possible to see the worms with the microscope on the cyro-ultramicrotome. The transparency of the worms outer membrane made them difficult to observe.

On closer examination it was found that the OCT solution may in fact be dissolving the worms. Next use of a sucrose solution was investigated. The worms were observable. However, a useful cutting temperature was not found for this solution before it was decided to try a rapid freezing approach. The few samples from a sucrose solution that were tried simply gummed-up the knife as the sucrose was not hard enough. Colder samples to harden the sucrose simply shattered upon knife contact. Actual cutting temperature probably between these two limits, but we chose the freezing technique as a better alternative instead of continuing with the sucrose.

We are currently investigating to possibility of using a high-pressure rapid freezing system, located at UC, Berkeley, to prepare the worms.

Current status of Chemical Imaging of Bio Samples

Chemical imaging of the bio samples has required more than standard operation of the ChIPMA instrument. In our work to date on imaging bio samples, we have placed the bio samples in the ChIPMA instrument along with other types of samples that we use for ChIPMA chemical imaging. These other samples include metal and ceramic substrates and CsCl salt, which we use to achieve initial operation of the instrument.

In our test on the bio samples to date, we have been able to successfully image the CsCl, metal and ceramic samples using SIMS. The signal strength and mass resolution is good for these samples. However, when we move the sample holder to analyze the bio thin film samples, the signal strengths are very low or not detectable. To check that these samples contained the peptides in the DHB matrix, we ran the sample on a Q-TOF mass spectrometer. The measurements showed that the sample did indeed contain the peptides. Next we ran similar sample using the MALDI capabilities of the ChIPMA instrument. Again we were able to see the peptides in these samples.

From these measurements we can conclude that our attempt to make ME-SIMS measurements with the Ga liquid metal ion beam were not successful. Further work must be done to understand and control the physics of the interactions between the ion beam and the bio sample in the DHB matrix.

Of the limited time that we have had to examine the bio samples, our results indicate that we should be able to do MALDI imaging of the samples using the N₂ laser. Results so far using SIMS indicate that it will take significant effort to develop this analysis tool (more than the \$20k we were able to spend on this project). Thus, at this point MALDI is the more promising method to develop in the near term.

We have also examined using the quadrupled (266nm) line from a Nd/YAG laser to ionize the MALDI samples. Our objective here was to use a laser that has a smaller beam diameter than which we can obtain with the N₂ laser (~ 5 to 10µm). However, we had little success in using the Nd/YAG laser. In discussions with Gary Kruppa from Bruker Daltonics, we learned that the high intensity of the Gaussian beam from the Nd/YAG laser provides too much power for the MALDI measurements. Bruker has had to downgrade the beam quality of the Nd/YAG lasers in their system to make them effective for MALDI measurements.

As of the end of this LDRD, our imaging of bio samples is limited to using the N₂ laser in MALDI measurements for chemical imaging. In light of our plan to develop chemical imaging using *C. elegans* this is somewhat problematic. The dimensions of the *C. elegans* are approximately 1000µm long and 100µm wide. If we are limited to using the N₂ laser for MALDI measurements the spatial resolution for trying to image the worms will be low. However, it still may be useful to test our ability to discern the variation of different proteins in normal and genetically modified worms.

Clearly, other research groups have been able to use MALDI and ME-SIMS to image biological samples. Starting with these published methods we were not able to reproduce similar results in a brief period of time. Using the new equipment to parametrically investigate and find the optimum sample preparation techniques will require further investment of time. It may also be useful to enlist the help of others who may have more experience in SIMS and MALDI imaging.

Conclusion

This project has achieved its main objective of designing and obtaining the equipment necessary to prepare samples for chemical imaging with the ChiPMA instrument. We have obtained a cryo-ultramicrotome that can be used to prepare samples for analysis in a manner beyond what we had originally envisioned for this project. This instrument will enable us to prepare samples that can be imbedded in a solvent matrix or used with DIOS analysis methods.

A sample coating device was designed and built that allows many parameters to be varied in a controlled manner in the coating process. The use of this device will be critical for learning how to prepare samples for analysis properly.

The focus for biological samples was shifted from tissue analysis to analysis of thin film test samples and imaging of *C. elegans*. Given our limited success to date in obtaining images of these samples, this shift in focus was prudent. Trying to image the more complex tissue samples, especially using ME SIMS, would be quite difficult.

Our limited chemical imaging effort with bio samples has shown that MALDI measurements are easier to do. This is consistent with what has been found in several other research groups working in this area. It is clear that MALDI based chemical imaging is a useful method for investigating tissue samples. Caprioli has shown that this is a viable technique based on work on a number of different samples. Bruker Daltonics has designed a MALDI imaging system that they now sell with their mass spectrometers.

The more difficult technique is chemical imaging of biological samples using ME-SIMS. While several groups have been successful in this area, close examination of their published work reveals that the measurements that have been made, while impressive, are difficult to make and still provide limited information on the biological processes in the system.

The path forward at Sandia in this area is uncertain. We have clearly developed the equipment needed to investigate how to prepare biological samples for chemical imaging. The use of MALDI based chemical imaging would be a practical tool to develop further. The creation of new experimental protocols to prepare biological samples for chemical imaging using MALDI, especially when coupled to the FTICR mass spectrometer, would be especially useful for examining reaction processes in biological systems. This could be quite useful for the development of biofuels.

The path forward for the development of ME-SIMS is less obvious. For investigating biological systems this is not a well-developed analysis tool. It has the potential to develop into a useful tool in the future. However, this will require a significant effort investigating the physics of beam interactions with these complex substrates. If high spatial resolution is required, it may be necessary to use a dual probe system, consisting of an ion beam to ablate a small portion of the substrate and laser ionization to create ions from the evolving gas for analysis. The ability to analyze sample using an ion beam for both ablation and ionization may be too constrained to form a basis for the development of a useful analytical instrument.

Finally, it is important to point out that the lack of obtaining images of tissue samples in the work covered under this LDRD should not leave the impression that this approach will not succeed. Most of the effort in this project was devoted to designing, fabricating and procuring the equipment required to prepare sample for analysis in a well-controlled manner. The amount of time spent developing sample preparation methods and integrating this with the ChiPMA imaging methods was very limited. The funds allocated to this effort was about \$40k of which we were only able to spend ~\$15k due to a higher priority NW project that had to be addressed in FY07.

References

1. Chaurand, P., S.A. Schwartz, and R.M. Caprioli, *Assessing protein patterns in disease using imaging mass spectrometry*. Journal of Proteome Research, 2004. **3**(2): p. 245-252, 235.
2. Altelaar, A.F.M., et al., *Direct Molecular Imaging of Lymnaea stagnalis Nervous tissue at Subcellular Spatial Resolution by Mass Spectrometry*. Analytical Chemistry, 2005. **77**: p. 735-741.
3. Petricoin III, E.F., et al., *Use of proteomic patterns in serum to identify ovarian cancer*. Lancet, 2002. **359**: p. 572-77.
4. Schwartz, S.A., et al., *Protein profiling in brain tumors using mass spectrometry: Feasibility of a new technique for the analysis of protein expression*. Clinical Cancer Research, 2004. **10**(3): p. 981-987.
5. Yanagisawa, K., et al., *Tumor proteomic patterns predict classification and tumor behavior in human non-small cell lung cancer*. Proceedings of the American Association for Cancer Research Annual Meeting, 2003. **44**: p. 963.
6. Masumori, N., et al., *Cancer Res.*, 2001. **61**: p. 2239-2249.
7. Kotula, P.G., M.R. Keenan, and J.R. Michael, *Automated analysis of SEM X-ray spectral images: A powerful new microanalysis tool*. Microscopy And Microanalysis, 2003. **9**: p. 1-17.
8. Thomas, J.J., et al., *Desorption/ionization on silicon (DIOS): A diverse mass spectrometry platform for protein characterization*. Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(9): p. 4932-4937.

Appendix I. Mathematica code for spray parameters

Coater Calculations

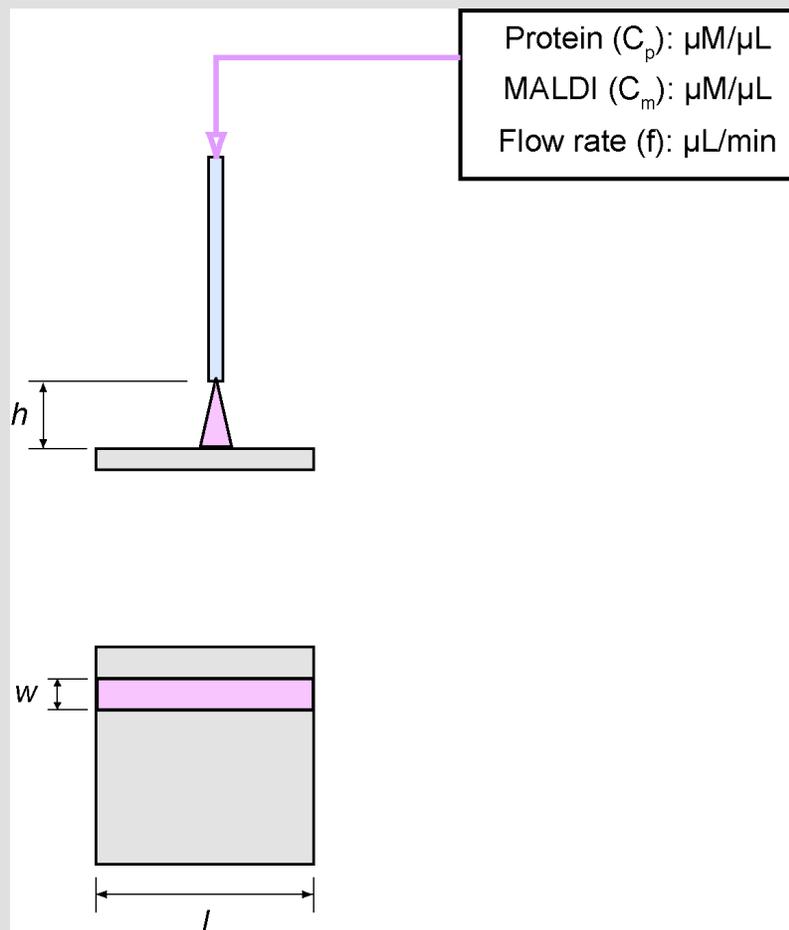
Introduction

This program calculates the parameters to deposit films of MALDI coatings or protein/peptide dissolved in a solution. The geometric aspect of the problem are defined by the following illustration.

```
image1 = Import["/Users/rbehen/Documents/DocumentsTransferred/  
Projects/LDRD - Bioimaging/Bioimage coating/Flow  
calculations/Biocoater Calculation illustration.tif"];
```

- Graphics -

```
Show[image1, ImageSize -> 300];
```



The following variable are used in the calculation:

rhoMALDI = density of MALDI solvent (g/cm3)
 rhoProtein = density of protein (g/cm3)
 rhoSolvent = density of solvent (g/cm3)
 mwMALDI = molecular weight of MALDI solvent
 mwProtein = molecular weight of protein
 mwSolvent = molecular weight of solvent
 concMALDI = concentration of MALDI solvent in solution (mol/L)

```
nAvogadro = 6.02*^23
```

$$6.02 \times 10^{23}$$

```
molecularDiameterCm[molWt_, density_] :=  
2. * (3. ((molWt / density) / nAvogadro) / (4. * Pi)) ^ (1. / 3.)
```

```
molecularDiameterCm[80000., 2.]
```

$$5.02522 \times 10^{-7}$$

```
amountRequired[molWt_, density_,  
widthCm_, lengthCm_, numberOfLayers_] :=  
Block[{molecularDiameterCm, moleculesPerLayer,  
nAvogadro, numberOfMolecules},  
nAvogadro = 6.02*^23;  
molecularDiameterCm =  
2. * (3. ((molWt / density) / nAvogadro) / (4. * Pi)) ^ (1. / 3.);  
moleculesPerLayer = widthCm * lengthCm / molecularDiameterCm ^ 2;  
numberOfMolecules = numberOfLayers * moleculesPerLayer;  
Return[numberOfMolecules]  
]
```

```
amountRequired[100., 1.5, 0.3, 0.7, 10]
```

$$5.91576 \times 10^{14}$$

```

layerThicknessMicrons[molWt_, density_, numberOfLayers_] :=
Block[{molecularDiameterCm,
moleculesPerLayer, nAvogadro, numberOfMolecules},
nAvogadro = 6.02*^23;
molecularDiameterCm =
2. * (3. ((molWt / density) / nAvogadro) / (4. * Pi)) ^ (1. / 3.);

thicknessMicrons = numberOfLayers * molecularDiameterCm * 10^4;
Return[thicknessMicrons]
]

```

```
layerThicknessMicrons[100., 1.5, 2000]
```

```
1.19161
```

```

depositionTimeMinutes[molWt_,
density_, widthCm_, lengthCm_, numberOfLayers_,
concentratorMolPerLiter_, flowRateMicroLiterPerHour_] :=
Block[{amountToDeposit, nAvogadro, numberOfMinutes},
nAvogadro = 6.02*^23;
amountToDeposit = amountRequired[molWt,
density, widthCm, lengthCm, numberOfLayers] / nAvogadro;
numberOfMinutes = amountToDeposit / concentratorMolPerLiter /
(flowRateMicroLiterPerHour * 10*^-6) * 60;
Print["Amount to deposit = ", amountToDeposit, " moles",
"\nVolume to deposit = ",
amountToDeposit / concentratorMolPerLiter, " Liters",
"\nConcentration = ", concentratorMolPerLiter,
" moles/liter",
"\nConcentration = ",
concentratorMolPerLiter * molWt, " gm/liter",
"\nNumber of monolayers = ", numberOfLayers,
"\nLayer thickness = ",
layerThicknessMicrons[molWt, density, numberOfLayers], " microns",
"\nDeposition time = ", numberOfMinutes, " minutes"];
Return[numberOfMinutes];
]

```

```
depositionTimeMinutes [154., 1.5, 0.3, 0.7, 4000, 1.*^-1, 10]
```

```
Amount to deposit =  $3.93073 \times 10^{-7}$  moles  
Volume to deposit =  $3.93073 \times 10^{-6}$  Liters  
Concentration = 0.1 moles/liter  
Concentration = 10. gm/liter  
Number of monolayers = 4000  
Layer thickness = 2.38322 microns  
Deposition time = 2.35844 minutes
```

```
2.35844
```

Distribution

- 2 MS0123 D.L. Chavez, LDRD office
- 1 MS9052 Richard Behrens, 08368
- 1 MS9052 Sean Maharrey, 08368
- 1 MS9052 Deneille Wiese-Smith, 08368
- 1 MS9052 Aaron Highley, 08368
- 1 MS9056 Wen Hsu, 08368
- 1 MS9292 Joe Schoeniger, 08321
- 1 MS9291 Cathy Branda, 08321

- 3 MS9018 Central Technical File, 8945-1

- 1 MS0899 Technical Library, 9616

- 1 MS9021 Classification Office, 8511 for Technical Library, MS 0899, 9616 DOE/OSTI via URL