The Application of Ultrafast Laser Pulses to Laser Desorption Mass Spectrometry

BY

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THESIS
Submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry (Analytical Chemistry) in the Graduate College of the University of Illinois at Chicago, 2015

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The thesis is dedicated to my family, colleagues and friends, without them it would never have been accomplished.
ACKNOWLEDGEMENTS

I would like to thank my thesis committee – (Luke Hanley, Jerry Moore, Igor V. Veryovkin, Tim A. Keiderling and Scott A. Shippy) – for their unwavering support and assistance. I think my advisor Dr. Hanley for his support of my PhD research. If it was not him, I would not had a chance to learn how to build a scientific instrument. I would also like to thank Dr. Moore for his guidance in my early PhD studies. He showed me how to design and build instrument. I also thank Dr. Veryovkin for pointing out the design issues in the instrument and eventually lead to much better instrument performance.

I thank the all the group members in Hanley Lab, especially Dr. Chhavi Bhardwaj, Dr. Slobodan Milasinovic and Dr. Artem Akhmetov for the happy collaboration between us. During all five years, I received a tremendous help from Don Rippon at electronics shop of Chemistry department, also Frank Tobias, Joseph Dublin, and Francisco Alvarez in Chemistry machine shop, also, Kevin Lynch, Richard Frueh, and Richard Dojutrek in LAS machine shop later on. I would not achieve so much in instrumentation without them.

Finally, I thank all the friends I met at UIC chemistry department, particularly, my girlfriend Ms. Xuejing Chen for the company during the years.
CONTRIBUTION OF AUTHORS

Chapter 1 is an introduction of my dissertation, highlighting the significance of my research.

Chapter 2 represents two published paper (Review of Scientific Instrument 83, (2012) 093702, ACS Applied Materials & Interfaces, 19, (2013) 9269) for which I was the primary author and major driver of the research. For the first paper, Dr. Jerry Moore contributed designing and building the instrument. Dr. Slobodan Milasinovic and Dr. Yaoming Liu involved in the building instrument processes. Dr. Slobodan Milasinovic was also responsible for preparing sample in Figure 38. Robert Gordon and Luke Hanley were advisers and contributed to the writing of the manuscript. For the second paper, Ross P. Carlson provided the biofilm bacteria sample. Dr. Chhavi Bhardwaj grown the testing samples as needed. The samples were used in Figure 41-44. Dr. Slobodan Milasinovic involved in upgrading of the instrument. Robert Gordon and Luke Hanley were advisers and contributed to the writing of the manuscript.

Chapter 3 represents manuscript which will be submitted later, in which I was the first author. I played a large role in writing the software. Dr. Luke Hanley was my adviser and contributed to the writing of the manuscript.

Chapter 4 represents a published paper (Review of Scientific Instrument 83, (2012) 093702) for which I was the primary author and major driver of the research. For the first paper, Dr. Jerry Moore partially designed and built the instrument. Dr. Slobodan Milasinovic and Dr. Yaoming Liu involved in the building instrument processes. Dr. Slobodan Milasinovic was also responsible for preparing sample in Figure 38. Dr. Robert Gordon and Dr. Luke Hanley were advisers and contributed to the writing of the manuscript.

Chapter 5 represents a published paper (ACS Applied Materials & Interfaces, 19, (2013) 9269) for which I was the primary author and major driver of the research. Dr. Ross P. Carlson provided the biofilm bacteria sample. Dr. Chhavi Bhardwaj grown the testing samples. The samples were used in Figure 41-44. Dr. Slobodan Milasinovic involved in
upgrading of the instrument. Dr. Robert Gordon and Dr. Luke Hanley were advisers and contributed to the writing of the manuscript.

Chapter 6 represents a published paper (Analytical Chemistry, 1, (2015), 367) for which I was the primary author and major driver of the research. Dr. Igor Veryovkin involved with improvement of instrument transmission and part of data processing. Michael Majeski prepared sample used in Figure 45. Daniel Cavazos prepared samples in Figure 50 and 51. Dr. Luke Hanley were advisers and contributed to the writing of the manuscript.

Chapter 7 represents a series of my own unpublished experiments directed at faster imaging mass spectrometry. I anticipate that this line of research will be continued in the laboratory after I leave.

In Chapter 8 represents my conclusion of the research presented in this thesis/dissertation.
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LIST OF ABBREVIATIONS

Br$_2$-Tyr  Dibromotyrosine  
FHG     Fourth harmonic generation  
f.l.    focal length  
fs     femtosecond  
IE     Ionization energy  
IR     Infrared  
ITO    Indium tin oxide  
LD     Laser desorption  
LDI    Laser desorption/ionization  
LDPI   Laser desorption positionization  
MACA   2-methoxy-4-amino-5-chlorobenzoic acid  
MALDI  Matrix-assisted laser desorption/ionization  
MPI    Multiphoton ionization  
MS     Mass spectrometry  
Nd:YAG Neodymium-doped yttrium aluminum garnet, Nd:Y$_3$Al$_5$O$_{12}$  
NIR    Near infrared  
ns     nanosecond  
NPM    N-(1-pyrene)maleimide  
PEM    Polyelectrolyte multilayer  
ps     picosecond  
SHG    Second harmonic generation  
SIMS   Secondary ion mass spectrometry  
S/N    signal-to-noise ratio  
SPI    Single photon ionization  
THG    Third harmonic generation
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<td>TOF</td>
<td>Time of flight</td>
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<td>UV</td>
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<td>VUV</td>
<td>Vacuum ultraviolet</td>
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SUMMARY

Ultrashort femtosecond laser pulses display exceptional performance for the selective ablation of materials, includes metals, semiconductors, and biological tissues. They do not damage the remaining unabluted portion of a sample, which permits the possibility of depth profiling by repeat sampling at the same location. Imaging mass spectrometry can also benefit from the lack of damage to the remaining sample. With sufficiently micro-focused fs laser pulse length beam, high lateral resolution imaging is possible, while sample damage may degrade ultimate lateral resolution in some other methods. Combining imaging and depth profiling could ultimately lead to tomographic mass spectrometry or 3D imaging MS.

Laser postionization, a “soft” ionization method, was combined with ultrafast laser desorption for enhancing molecular analysis. Laser postionization improves sensitivity for laser desorption ionization, which is essential when the shrinking of sampling area leads to significantly less signal.

In this dissertation, a customized femtosecond laser desorption/ablation postionization time-of-flight mass spectrometer was designed and built. The instrument’s construction went through two phases. The first phase included a linear time-of-flight and micro-focused laser ablation ion source. The second phase included a reflectron TOF and 118 nm vacuum ultraviolet ionization laser source. The construction and performance of both phases including the VUV source are detailed. Some auxiliary tools and components were also designed and built to diagnose and optimize the instrument. Beyond hardware
construction, instrument control software was created to operate this instrument. Many automated experiments were successfully carried out by this software.

Elemental analysis was carried out on the first phase of the instrument and demonstrated exceptional performance for fs laser pulse sampling of small areas. Molecular studies were done after the postionization source and reflectron TOF were added. Molecular studies demonstrated the imaging and depth profiling capability of fs-LDPI on intact biofilm tissues. Attempts were made to reach the limit of lateral resolution of imaging by fs-LDPI-MS. The results showed similar lateral resolution of <2 μm for both fs 800 nm and 400 nm desorption beams.

To improve the repetition rate for high speed imaging application, an alternative LDPI scheme was designed and constructed. The fs 800 beam was tripled to 267 nm and delivered into the ion source as an ionization laser, while a ns 349 nm pulse laser was used for desorption. Preliminary data showed certain intact molecular ions can be detected. Fragmentation tendency was measured against various ionization laser pulse energies and photoionization time delays.

In short, a fs-LDPI mass spectrometer was designed and constructed. Elemental and molecular analysis were carried out to demonstrate the ultrafast laser’s capability for imaging and depth profiling mass spectrometry, including molecular perservation, depth profiling and ultimate lateral resolution.
I. INTRODUCTION

A. General introduction to mass spectrometry

Mass spectrometry is one of the most important analytical chemistry techniques for organic species and is complementary to ultraviolet/visible spectroscopy, fluorescence, and nuclear magnetic resonance.\(^1\) Two critical components of mass spectrometers are the ion source and mass analyzer. The ion source generates ions so that molecules or atoms can be manipulated by an electromagnetic field such that the mass analyzer will separate them by their mass to charge ratio (m/z). The m/z value of a peak can lead to its chemical composition by variety of methods. Most mass spectrometers are capable of resolving isotope peaks, which enables isotopic analysis. The mass resolution of a mass spectrometer can be as high as \(10^6\) \(m/\Delta m\) (\(\Delta m\) is defined as peak full width half maximum).\(^2\)\(^3\) Selective chemical analysis can be achieved by single ion monitoring, where selectivity is linked to mass resolution and isobaric composition. A particular chemical can be monitored by even a regular unit mass resolution spectrometer with help from chromatography, often achieving better selectivity than is available in other techniques. Ultrahigh mass resolution spectrometers also enable atomic composition determination by mass defect.

The development of ion sources is critical to MS analysis.\(^4\) The ionization efficiency and ion transmission in the source are the most critical aspect: low transmission or ionization efficiency leads to poor limit of detection or linearity. An ideal ion source should ionize as many molecules as possible and transport all the ions into the mass analyzer. The failure of either part leads to a drop in sensitivity, linearity, or both. Common ion sources include electron impact (EI) and chemical ionization (CI) for gas phase
samples; electrospray (ESI) and inductively coupled plasma (ICP) for liquid samples; and secondary ionization mass spectrometry (SIMS) and matrix assisted laser desorption/ionization (MALDI) for solid samples. \(^1\)

Mass spectrometric (MS) imaging is increasingly being used for the spatially resolved molecular analysis of human tissue, as well as the interfaces of biomaterials with invasive microbial biofilms and other biological materials. \(^5\) The methods used for MS imaging include SIMS, \(^6\) as well as those based on ESI and MALDI using nanosecond (ns) pulse length lasers. \(^5, 7-11\)

**B. Imaging and depth profiling technique**

1. Matrix-assisted laser desorption/ionization

MALDI is the most widely used biological tissue imaging method. \(^12-15\) In MALDI, a pulsed ns UV laser, typically a nitrogen laser or the third harmonic of a Nd:YAG laser, shoots samples that are coated or mixed with matrix compounds. Protonated molecules are generated during the process and extracted into the mass analyzer. Matrix plays an important role in the desorption and ionization process. Ions formed in MALDI are usually singly charged, includes those of protonated proteins, peptides, DNA, and polymers. The most common mass analyzer is time-of-flight, because its theoretically unlimited mass range. Ions of over 10\(^6\) Dalton has been demonstrated. Other mass analyzers are also successfully coupled with MALDI, including FT-ICR, Orbitrap, and ion trap. \(^16-18\)

MALDI-MS imaging of species within organic, polymeric, or biological samples preserves information about their spatial distribution while correlating the ion signal to macroscopic structures. \(^7, 11, 19\) Although MALDI-MS is most commonly performed with ns
laser pulses, the use of ultrafast (i.e., <2 ps) pulses for intact desorption of biological material promises to greatly extend the sensitivity and lateral resolution of MS imaging.\(^{20-23}\)

Despite its popularity and numerous advantages, MALDI has several shortcomings when used for MS imaging of molecular species from biomaterials interfaces. Sample preparation often requires multiple steps including tissue washing and multiple matrix applications to enhance ion yields.\(^{7,10,24}\) MALDI displays overall low ion yields, with typically \(10^3\) to \(10^7\) neutrals desorbed along with every ion.\(^{18,25,26}\) Salts, sample-specific ion suppression, and interferences between multiple analytes lead to strong dependence of ionization efficiency on both analyte and local chemical environment.\(^7\) These effects collectively limit the classes of species readily detected by MALDI and its capability for quantification.

2. Secondary ion mass spectrometry

In SIMS, a primary ion beam with typical energy on the order of magnitude of tens of keV is used to bombard materials. Secondary ions will be formed after this process, which are then detected by a mass analyzer. Typical primary ion beams include \(\text{Ar}^+\), \(\text{Au}_3^+\), \(\text{O}_2^+\), \(\text{Ga}^+\), and \(\text{C}_{60}^+\). Developed in the 1960s, SIMS is a standard surface analysis method, which has been widely used in the semiconductor industry to diagnostic semiconductor devices.\(^{27-29}\) SIMS is also used for high lateral resolution elemental analysis as low as tens of nm.\(^{30}\) High lateral resolution biological molecular imaging can be achieved via elemental analysis by isotope labeling.\(^{31}\) High depth resolution depth profiling can be achieved by low energy ion milling on SIMS.\(^{32-35}\) Disadvantages of SIMS includes low ion yield, excessive sensitivity to sample surface chemistry, damaging of sample, mixing of
inorganic layers, and surface charging. Surface charging on semiconductor or insulating samples can lead to a mass calibration shift on the final spectrum. The high energy imparted by the primary ion beam will also lead to molecular fragmentation, which hinders organic analysis on traditional SIMS instruments. Nevertheless, cluster primary ion beams show less fragmentation of small molecular species, leading to emerging molecular analysis by SIMS in the past decade.\textsuperscript{29,36-46}

\section*{C. Introduction of ultrafast laser technology}

Ultrafast lasers are a type of laser defined by having a time span of less than one picosecond (ps). The most typical ultrafast laser is the Ti:sapphire laser. Due to the time-energy uncertainty relationship in quantum mechanics, the short pulse duration of an ultrafast laser leads to a broadband emission on the frequency spectrum. The most common 100 fs pulse length Ti:sapphire lasers have a bandwidth about 20 nm, centered at 800 nm, although their emission wavelength spans from as low as 690 nm and as high as 1040 nm in commercial systems. With addition of nonlinear optical crystals, the emission wavelength can be shifted to as short as 200 nm or as long as 3 μm.\textsuperscript{47}

\subsection*{1. Oscillator}

To create ultrafast laser pulses, a continuous wave green laser is used typically as pumping source. The near infrared (NIR) output of a Nd:YVO\textsubscript{4} laser is doubled to green and injected into a Ti:sapphire crystal. The Ti:sapphire crystal is positioned inside a cavity and able to generate a very broad band useful fluorescence emission, which is crucial to achieve fs output. An active component, such as acousto-optic modulator (AOM), is also inserted into the cavity to control light passing/blocking periodically inside the cavity. The
AOM period is synchronized with the laser pulse propagation inside the laser cavity to achieve mode-lock. Due to the wide spectrum span of the output and dispersion from the air, a group velocity compensator has to be integrated to compensate for air-induced pulse duration broadening.\(^{48}\)

2. Amplifier

The laser pulse energy from a fs oscillator alone is insufficient for a laser ablation experiment, hence, a Ti:sapphire amplifier is needed. Due to the very short pulse duration of an ultrafast laser pulse, traditional amplification will lead to a significant nonlinear effect in the gain media. In the worst case, the laser beam will be self-focused inside the gain media and thus damage the crystal due to Kerr effect.\(^{48,49}\) To work around the self-focusing problem, the concept of regenerative amplification was introduced. When the 82 MHz repetition rate pulses are injected into the amplifier through a Faraday optical isolator to prevent back reflection that can interfere with the upstream oscillator, a Pockels cell selectively passes pulses through at a 1 kHz rate, which is synced to the Nd:YLF pump laser of the amplifier. The seed laser will pass through a grating pair, lens and several mirrors to be stretched by introducing group velocity dispersion (GVD). The assembly of these gratings and mirrors are called a pulse stretcher. The fs pulse will be stretched to about \(~300\) ps in the particular system used here. The stretched pulse will pass through the Ti:sapphire gain media several times back and forth, controlled by the Pockels cells and a high precision delay generator, then the pulse will be ejected out of the gain cavity. A compressor assembly, which is very similar to a stretcher, will compress the \(~300\) ps back to \(~100\) fs time scale by introducing reversed GVD. The typical amplification of an amplifier is \(~10^6\). In this way, the peak power of the pulse during amplification is
significantly reduced, and detrimental nonlinear effects, such as optical damage, are avoided.49

3. Application

Laser ablation with ultrashort pulses has the remarkable ability to remove material from the surface layer of a solid while doing minimal damage to the remaining sample.50 This effect has been utilized in laser surgery, where femtosecond laser pulses impart negligible damage to surrounding tissues as compared with nanosecond laser pulses.51-56 This property extends beyond biological tissues to a wide variety of materials, including polymers, metals, semiconductors, and insulators.23, 50 It was previously shown that ablation with ~75 fs, 800 nm laser pulses can remove material from bacterial biofilms and bovine eye tissue with only minor chemical disruption to the underlayer, in principle allowing depth profiling by subsequent mass spectrometric (MS) analysis in separate instruments.24, 57

Moreover, the increasing availability of reliable and easily operated ultrafast lasers50, 51 is opening up new possibilities for laser desorption ionization that have not been fully exploited by the MS imaging community. For example, several researchers have begun to use ultrafast laser ablation of biological material for MS applications by either directly forming gaseous ions58-60 or by desorbing neutral species that are subsequently ionized by electrospray.61, 62 Those studies took advantage of the ability of sub-100 fs laser pulses to induce nonresonant desorption events. Although some of those studies using ultrafast laser pulses for sampling were performed under vacuum, most were done at atmospheric pressure. In parallel, the past decade has experienced a rapid increase in the use of atmospheric pressure desorption ionization methods, including applications for MS
These atmospheric pressure-based methods allow the direct analysis of samples while avoiding the dehydrating effects (and resultant structural modifications) of high vacuum.

D. Introduction of laboratory VUV sources

Photoionization of gas phase organic species requires a photon energy higher than the ionization energy of the analyte molecule, which lies in the vacuum ultraviolet (VUV) region. Common laboratory VUV light sources include the excimer laser, excimer lamp, and laser wavelength conversion techniques. Each technique has its own advantage and drawbacks.

1. Excimer laser

Excimer lasers were developed in the 1970s, where excimer stands for “excited dimer”. Most of excimers are of the rare gas halide type. A notable exception is the fluorine laser, in which the active media is an excited state fluorine molecule. Common excimer laser types includes ArF\textsuperscript{+}, KrF\textsuperscript{+}, XeCl\textsuperscript{+}, XeF\textsuperscript{+} and F\textsubscript{2}\textsuperscript{1}. General operation of common excimers involve use of a gas mixture consisting of fluorine and balance gas, typically helium. Excimers are generated by electrical discharge by multiple electrodes induced within the laser cavity. Output beam quality is usually not as high as in solid state lasers.\textsuperscript{68}

Thanks to the large demand in the semiconductor industry, LASIK laser eye surgery, and academia, decades of ongoing design and refinement has led to very robust excimer laser solutions for customers.\textsuperscript{68} The cost of an excimer laser could range from $30,000 to

\textsuperscript{1} F\textsubscript{2} is not an excimer technically, but F\textsubscript{2} lasers share same design as other excimer lasers.
millions, depending on application. The repetition rate of benchtop units can reach 200 Hz continuous operation for a low cost unit or 2 kHz for an industrial quality unit.\textsuperscript{69}

For research applications, a fluorine laser can generate sufficient photon flux to saturate single photonionization within the desorption plume, at 157 nm with 7.87 eV photon energy. The output stability is high, provided the premix gas quality is under control. The high energy of emitted photons from the fluorine laser tends to ionize residual gas molecules in the vacuum chamber, which tend to deposit on the laser output window and lead to a significant drop in laser output power. However, periodically cleaning of the window will recover the full laser power.\textsuperscript{70}

2. Excimer lamp

An excimer lamp relies on similar excited molecules as in an excimer laser.\textsuperscript{71, 72} However, the excimer molecule undergoes spontaneous emission in the lamp, unlike in the excimer laser. The lamp output is continuous, with very low peak power density compared to lasers. Available wavelengths include 126 and 146 nm from Ar\textsubscript{2}\textsuperscript{*} and Kr\textsubscript{2}\textsuperscript{*}, respectively. Gas refill is generally not required, unlike lasers.\textsuperscript{73, 74}

3. Harmonic generation

Besides excimer lasers, the other common method to generate VUV laser radiation in the laboratory is wavelength conversion from a high energy pulsed laser. The ninth harmonic of the Nd:YAG laser, 118 nm or 10.5 eV, is one of the simplest ways to reach VUV photon energy. It requires only one laser and one gas cell. No complicated beam alignment is involved, such as matching polarization direction, or co-aligning two lasers. The difficulty mostly lies in gas mixture purity and cleanness of the optics and gas cell.\textsuperscript{70, 75-77}
The tripling of 355 nm, the third harmonic output of Nd:YAG laser, relies on a 5p-5d transition of Xe around 117.2 – 119.2 nm\textsuperscript{75, 78}. In this region, Xe undergoes negative dispersion, hence phase matching should also be achieved by balancing the refractive index through adding an inert buffer gas. In most cases, Ar is used to for this purpose.

Due to the nature of VUV photons, a vacuum compatible laser energy meter is required to measure generated VUV pulse energy. The existence of the high energy pump beam also limits how VUV can be measured. Thus, the actual VUV generation flux is rarely reported in the literature. Nevertheless, some groups reported \(\sim 10 \mu J\) is achievable with 30-50 mJ, 355 nm pump energy, with a conversion efficiency of \(\sim 10^{-4}\).\textsuperscript{76, 77} The high photon energy of 118 nm output can also generate color centers in window and lens materials, which lead to decreasing material transmission.

4. Others sources of VUV radiation

There are also other techniques to generate VUV photons.\textsuperscript{70} Successfully generating tunable VUV is very delicate work which requires considerable knowledge of laser physics. A more efficient but also more complicated method is four-wave mixing in a rare gas or metal vapor.\textsuperscript{79} Four-wave mixing generally involves two pump beams. One is tuned to a resonant absorption frequency of the rare gas in the UV region and the other is in the near-IR region. Because the pump beam wavelength has to be tuned, a dye laser most likely must be used to provide the UV beam. The near-IR beam can also be tuned by an optical parametric oscillator (OPO) and thus provides the capability to achieve tunable VUV emission.\textsuperscript{80} Besides tunability, resonant four-wave mixing in Hg vapor can generate VUV output with much higher flux. VUV pulse energy of 200 \(\mu J\), more than 20 times brighter than 118 nm by tripling in Xe/Ar cell, has been demonstrated by using dye lasers.\textsuperscript{81}
Other techniques such as up conversion by anti-Stokes Raman shifting can also be used to generate VUV photons.\textsuperscript{82}

\section*{E. Introduction to laser desorption postionization (LDPI)}

MALDI has been extensively used in biological research, but the addition of matrix reduces spectral quality in the low mass region, where matrix fragments and cluster ions appear. This effect limits the capability of MALDI to detect the small molecules, known to play a very important role in biology as metabolites. Low ion yields from the laser desorption event also limit the sensitivity of MALDI and the surface chemistry can additionally interfere with ion formation.\textsuperscript{83}

Laser desorption postionization isolates the event of material desorption and ionization by introducing a secondary VUV ionization laser into the ion source. With the separation of desorption and ionization events, the majority of species in the desorption plume, which are neutral, may be ionized and detected. Thus, LDPI improves sensitivity of molecular detection. For successful ionization of molecular species, the photon energy has to be above the ionization threshold of the analyte species. Thus, a 7.87 eV fluorine laser, although permitting very high irradiance, can only ionize very limited classes of chemical compounds, about \(\sim 10\%\) of all organic molecules. Higher photon energy light sources, such as the 118 nm, ninth harmonic of the Nd:YAG laser, can be used to detect more molecules.\textsuperscript{73}

LDPI is generally a soft ionization method, in which the molecular ion is the most abundant peak observed in most small molecular cases. Large molecules can also be observed by the LDPI method. It has been shown that N-(1-pyrene)maleimide (NPM) and
iTRAQ tagged peptides can be observed by the LDPI method, with molecular weights up to 4 kDa and the Markus group also showed the possibility of acquiring photoions beyond 10 kDa.  

VUV single photon ionization of isolated molecules in vacuum occurs only when their ionization energies are below that of incident photons, rendering 10.5 eV VUV radiation sufficient for a wide range of analytes. Sensitivity in VUV single photon ionization in vacuum depends on the ionization yield to produce the radical cation $M^{++}$. The ion yield $Y = \sigma_{\text{spi}} I N_{\text{gas}}$, where $\sigma_{\text{spi}}$ is the photoionization cross section at a given photon energy $h\nu$, $I$ is the intensity of VUV radiation, and $N_{\text{gas}}$ is the density of gaseous neutrals. This linear relationship of ion yield to gaseous neutral density has allowed ns laser desorption combined with 7.87 eV single photon ionization (ns-LDPI-MS) to be used for quantification of an antibiotic infused into a drug delivery multilayer. It was also used to observe that the formation of a molecular cluster can lower ionization energy, which broadens the application of the 7.87 eV fluorine laser.

UV multiphoton ionization (MPI) can occur when photon energy is below a chemical species’ ionization energy. In such case, multiple photons are absorbed by the molecule and the total energy surpass the ionization energy required to form a photoion. In case of resonant two photon ionization (R2PI), one of the various types of MPI methods and the most efficient one, the ion yield $Y = \sigma_a \sigma_b F^2 N_{\text{gas}}$, where $\sigma_a$ is the cross section for absorption to the intermediate state at a given photon energy $h\nu$, $\sigma_b$ is the cross section from intermediate state to ion state, and $I$ is the intensity of UV radiation. The relationship of ion yield to UV radiation intensity is nonlinear, unlike the case in SPI. R2PI and other MPI methods are more selective than SPI, but suffer from more fragmentation. The
technical benefit of MPI is the availability of optics and lasers: the selection of UV optics is much broader than VUV optics and vacuum is not required, which eases the engineering of instrumentation.

The various combinations of desorption and ionization lasers leads to a wide variety of LDPI methods. In the Hanley lab, the LDPI operating mode could be any practical combination of desorption laser and ionization laser, limited only by whether both lasers can be fired within sufficient time precision and also whether the laser can be delivered to the ion source as needed. Up until now, four operational modes of LDPI have been thoroughly studied as listed below.

1. **Ns-LD-ns-VUV(157 nm)-SPI**

   This was the first operational mode of LDPI achieved in the Hanley lab.\textsuperscript{73, 96, 97} In this mode, a ns 349 nm laser was used to desorb organic samples and the plume was ionized by a 157 nm, 7.87 eV, fluorine laser. This mode provides the highest sensitivity for molecules whose ionization energies are below 7.87 eV, because of the high irradiance of the fluorine laser. The laser pulse energy can exceed 1 mJ and is able to saturate photoionization in certain cases.\textsuperscript{73, 85, 97-99}

2. **Ns-LD-ns-VUV(118 nm)-SPI**

   The 118 nm, 10.5 eV, VUV source can substitute for the fluorine laser in the previous mode, to provide ionization for a broader range of molecules. Over 90\% of all organic molecules have ionization energies below 10.5 eV, and thus can be ionized by the 118 nm source.\textsuperscript{73} Due to the lower conversion efficiency of 118 nm, typically 10\textsuperscript{-4}, this
method has lower sensitivity compared to 157 nm photoionization. The ability to detect a much broader range of organics offsets the drop in sensitivity.88-90, 100

3. Fs-LD-ns-VUV(118 nm)-SPI

Ultrafast fs 800 nm laser pulses generate very little to no thermal damage to the underlying sample when used for sampling materials. Hence, a second LDPI instrument was built utilizing an ultrafast laser for desorption and this is the instrument discussed in this thesis. The combination of ultrafast laser and 118 nm VUV ionization source provides the capability to sample and detect a majority of organic samples, if not as complicated as biological tissue. The lack of thermal damage to the remaining samples, also opens the opportunity to achieve depth profiling at the same sampling location by continuously firing the laser. Such depth profiling is arguably problematic on a ns laser, because of ns laser damage to the remaining sample, leads to changes in chemical composition. Alternatively, the 800 nm desorption laser beam can be doubled to 400 nm and used for desorption, which may improve lateral resolution due to smaller diffraction limited spot size.

4. Ns-LD-fs-UV(267 nm)-MPI

The repetition rate of the 118 nm VUV source is limited by the pump laser, typically 30 Hz for ~300 mJ 1064 nm fundamental pulses, which is needed to generate 30-50 mJ 355 UV pulses. Although 200 Hz, 20 mJ 355 nm ns lasers are available on the market, the VUV generation efficiency is uncertain in those cases, because of the different output mode. The need for a high repetition rate laser for imaging applications led to the development of the third harmonic output of the Ti:sapphire laser as ionization source. In a ns multiphoton ionization scheme, a ns 266 nm source, from the fourth harmonic of a Nd:YAG laser often causes ions to dissociate into fragments.101 Such fragmentation makes it difficult to identify
unknown peaks, especially if the spectrum is acquired on complicated samples. In the case of fs lasers, the pulse duration is shorter than the lifetimes of the excitation state, and ionized molecules are more likely to be preserved as intact molecular ions in the final spectrum instead of as fragments. With molecular preservation and high repetition rate, the combination of ns UV desorption and fs UV multiphoton ionization may finally provide the capability for high speed (1 kHz) molecular ion sample imaging by laser desorption postionization.
Figure 1. Demonstration of some achieved LDPI operation modes. (top) Overview of all lasers available and geometry on the instrument presented in this dissertation. The red beams are fs laser beam, delivered by either a doublet lens or objective lens. The slate blue beam is ns 349 nm laser, used for ns laser desorption. The purple beam is ns VUV ionization laser for SPI. The blue beam is fs 267 nm laser used for MPI. Both optical imaging objective and laser focusing objective are showed. (bottom left) ns-LD-fs-UV(267nm)-MPI mode. The ns desorption laser and fs ionization laser are showed. (bottom right) fs-LD-ns-VUV(118nm)-SPI mode. Two fs laser beam path (red) are showed, only one of them is chosen for each experiment depending on the required focus condition. The VUV beam (purple) intersects with desorption plum (green).
5. Applications

Ns-LDPI-MS with 7.87 eV radiation has been used to detect derivatized peptides and select antibiotics in microbial biofilms. While 7.87 eV energy photons only ionize low ionization energy species such as fused ring systems and tertiary amines, the high intensity of the molecular fluorine laser saturates photoionization to achieve maximum detection of neutrals with low background signal. Higher photon energy sources such as those at 10.5 eV can detect a wide range of laser-desorbed species such as endogenous species in biofilms, although the relatively low intensity of such VUV sources does not allow saturation of photoionization. VUV single photon ionization for postionization has also been coupled to UV ns laser desorption as discussed above.

Various aspects of fs-LDPI-MS are described in this thesis. Chapter II covers the design and construction of the laser desorption postionization mass spectrometer, including ion optics, ns VUV generation, and fs UV generation. Chapter III and the appendix cover the instrument control software, created for driving the in-house built instrument. Elemental analysis, imaging and depth profiling are covered in Chapter IV. Chapter V covers molecular analysis on intact biofilm samples. Chapter VI covers the ultimate imaging lateral resolution by fs-LD-VUV-SPI method. Finally, Chapter VII discusses a novel fs UV 267 nm beam used for alternative photoionization scheme followed by some conclusions in Chapter VIII.
II. INSTRUMENTATION

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A. Ablation time-of-flight mass spectrometer (ATOF-MS)

1. Overview of instrument

   The early stage experimental setup consisted of the following components: a femtosecond laser and associated optics for delivering the laser beam, a high-precision translation stage mounted in a differentially-pumped vacuum chamber, customized ion optics, an ion detector, a high-voltage pulse generator and associated power supplies, and a high-voltage radio frequency (RF) generator. Those components located in or adjacent to the ion source are depicted in Figure 2. Customized controlling software was written using a commercial data acquisition and analysis package.
Figure 2. Ion source section view of ATOF-MS. The fs laser was focused by objective (NA 0.28) on the sample plate (orange). The sample plate is mounted on a vacuum compatible motion stage (yellow). Ions went through ion funnel (grey) and were focused by Einzel lenses (green). Teflon spacers were used to isolate low pressure ion source from high vacuum mass analyzer. The vacuum chamber wall was depicted in dark grey.

2. Femtosecond laser

Ultrashort laser pulses (800 nm, 45 fs) were generated at a 1 kHz pulse repetition rate by a Ti:sapphire oscillator (Tsunami, Newport Spectra-Physics, Irvine, CA) pumped by a diode pumped solid-state laser (532 nm, 4.3 Watts, Millenia, Newport Spectra-Physics, Irvine, CA). The pulses were amplified by a Ti:sapphire regenerative amplifier (Spitfire 50FS, Spectra-Physics) pumped by an Nd:YLF laser (527 nm, Evolution-30, Coherent, Santa Clara, CA). The output of this laser had a near transform-limited bandwidth of 40 nm and a pulse energy of up to 2 mJ. The pulse energy was attenuated to typically 0.1 - 10 µJ for use in the present experiments by a half-wave plate followed by a Rochon polarizer. The laser pulse passed through a beamsplitter and delay line for two-pulse experiments.
The beam was steered by several mirrors, then focused onto the sample by an infinity-corrected long working distance objective lens (10×/NA0.28, NT59-877, Edmund Optics, Barrington, NJ) mounted inside the ion-source vacuum chamber. The laser pulse energy was measured by a pyroelectric detector (J-10MB-LE, EPM2000, Coherent, Santa Clara, CA), located near the optical window of the ion source chamber.

The spatial profile was characterized by scanning a knife-edge across the beam, while measuring the transmitted light with a thin photodiode (FDS1010, Thorlabs, Newton, NJ). The knife edge was mounted on a sample plate in front of the photodiode. Multiple z-positions were scanned to find the focal point position. The 1/e² radius of the laser beam was 8 µm at the focal point, and the Rayleigh range was 75 µm. The beam was incident at an angle of 60° from the normal, producing an elliptical, 32×16 µm² spot on the target surface. The laser pulse length, measured with an autocorrelator, was 65 fs before entering the optical window of the ion source chamber. The objective lens was estimated to stretch the pulse length to ~75 fs (as measured by inserting a 5-mm thick quartz flat into the beam to simulate the dispersion of the objective).

Later studies showed the non-optimized beam focus size was due to poor flatness of one dichroic mirror in the beam path. The poor flatness could be due to under specification of the beamsplitter and/or incorrect mounting of the dichroic.

3. Sample motion stage

Samples were placed on a high-vacuum-compatible 3D motion stage (consisting of three separate PLS-85 stages, miCos USA, Irvine, CA) which allowed movement over a 34×34 mm² area with typical positional resolution of ±0.05 µm. The conductive sample plate holder (44 mm × 44 mm, AB SCIEX, Framingham, MA) was mounted on the motion
stage by an insulating polytetrafluoroethylene (PTFE) bracket. A PCI bus motor control board (Corvus-PCI, PI miCos, Irvine, CA) was used to drive all three motors directly, with all power and cabling coming from the host computer.

4. Ion optics and detection

The electrically isolated sample plate holder was connected to a custom high voltage pulse generator (described below) and biased by one channel of a four channel power supply (HV-RACK-4-250, UltraVolt, Ronkonkoma, NY). An aluminum cone electrode was used to extract ions from the source region. An ion funnel constructed from 12 aluminum plates insulated by PTFE spacers followed the cone. The plates of the ion funnel were connected via 11 vacuum-compatible resistors (ITT Power Solutions, West Springfield, MA). The first 8 resistors were 2 MΩ, while the three closest to the TOF tube were 3 MΩ. RF was coupled to the ion funnel plates by 10 nF capacitors. The DC gradient on the ion funnel was provided by a floating regulated power supply (Bertan 214, Spellman, Hauppauge, NY). RF was generated by a custom-built, high voltage RF generator. A gas-limiting aperture plate followed the ion funnel for differential pumping, with a diameter of 1.5 mm. Two high-transmission grids that follow the gas-limiting aperture were used for trapping, selecting, and/or extracting ions when the source chamber was at elevated pressure. The two grids were glued to copper ring plates by vacuum-compatible silver paint and insulated from each other by a machined ceramic piece. The grids and insulating ceramic piece were held by a custom-made PTFE adapter, separating them from the elevated pressure region to prevent high voltage discharge.

Vacuum-compatible Kapton-coated wire passed into the PTFE adapter, connecting the grids to the high voltage pulse generator. Extra electrical insulation on the high voltage
feedthroughs in the source region was achieved by vacuum-compatible heat shrink tubing. This extra insulation was required to prevent high voltage discharge during keV pulsing at ~0.3 Torr pressure, near the Paschen minimum. The custom high voltage pulse generator used to drive the grids was built from two solid-state high voltage switches (HTS 151-03-GSM, Behlke USA, Billerica, MA), having measured rising and falling times of ~10 ns. Under the high vacuum conditions used in the present study, the grids were grounded, and the ion kinetic energy was defined by the 5 keV DC-biased sample plate rather than by the two pulsed grids.

An Einzel lens following the two grids was used to focus ions onto a second, 2.5 mm diameter aperture which provided another stage of differential pumping. A split-lens deflection assembly was used to steer the converging beam through this aperture and towards the detector. DC voltages for the deflectors, ion funnel, cone, and gas-limiting aperture were provided by a six-channel, bipolar power supply (LE-300 vacuum lens controller, Analytica of Branford, Branford, CT). PTFE was used as insulation and supporting materials in between the aluminum Einzel lenses and also as a gas-tight seal around the outside of the lens stack. The outer diameter of the PTFE spacer was the same as the inner diameter of the vacuum chamber flange, thereby serving as a differential pressure barrier between the source and the TOF region. Polyetheretherketone (PEEK) threaded rods were used to fix the PTFE spacers and Einzel lenses in place under tension, while stainless steel 304 rods were used to connect the high voltage.

Ions that passed through the linear flight tube were detected by a microchannel plate (MCP) detector in an anti-chevron configuration (C-0701, Jordan TOF Products, Grass
Valley, CA), normally operated in analog mode because of the high dynamic range of the ablation signals.

5. Differential pumping

For intermediate pressure (10^{-3} - 0.3 Torr) experiments, differential pumping was achieved using the two apertures described above: one immediately after the ion funnel and the other close to the detector (not shown). Three ion gauges were installed for vacuum measurement, placed near the detector, inside the flight tube region, and inside the ion source chamber. Two manometers (1 Torr maximum, MKS 622B, MKS Instruments, Andover, MA, and 1000 Torr maximum, Datametrics 6000A, Edwards US, Sanborn, NY) were also installed in the source chamber for measuring absolute pressures in the intermediate regime. In the current configuration, six orders of magnitude vacuum isolation was achieved between the source and the detector.

An oil-free scroll pump (SC15D, Oerlikon Leybold, Cologne, Germany) was used to evacuate both the flight tube and detector turbomolecular pumps, so that the base vacuum in this foreline routinely remained <0.01 Torr. The dry pump allowed the vacuum to recover to <10^{-6} Torr in just a few hours after venting or inadvertent shutdown, significantly decreasing instrument down time. A standard oil-sealed rotary vane pump was used to back a 300L/s turbomolecular pump for the ion source chamber.

6. Data acquisition hardware and software

Timing was controlled by a digital pulse delay unit (BNC-565, Berkeley Nucleonics Corporation, San Rafael, CA). This unit was triggered by a TTL pulse from the laser Pockels cell driver. The delay unit then triggered the high voltage pulse generator and
the computer data acquisition card. The ion signal was recorded by a PCI digitizer card (8 bit, 1 GS/s, Cobra CS11G8, Gage Applied, Lockport, IL).

Instrument control software was developed entirely in a standard commercial data acquisition and analysis programming environment (LabVIEW, National Instruments, Austin, TX). Customized code was written to ensure the highest possible data transfer rate to the main memory of the computer. Although the data transfer rate was ultimately limited by the PCI bus (effectively 100 MB/s), the onboard hardware averaging of the digitizer could be used in many situations to compress significantly the data to be transferred. Data were saved as either ASCII text files, when speed was not a concern, or in custom binary format for high speed streaming.

Separate routines were written to process the raw data, including processing depth profile data and converting customized binary format to BioMAP format (based on Analyze v7.5, www.maldi-msi.org), which was used as the primary MS imaging data analysis software. A special program was written for laser beam profile measurements using the knife-edge approach described above. The control software here is an early version of the ChiMS package, which will be discussed in detail in the following chapter.

7. Optical microscopy

Two digital single lens reflex cameras (T1i and T2i, Canon USA) were employed for optical imaging of the sample. The first camera was equipped with a macro lens (EF 50mm f/2.5 Compact Macro, Canon USA) and was used for imaging the entire sample plate. The second camera was mounted behind the objective lens viewport, using a dichroic mirror to deliver the 800 nm laser beam into the objective lens while passing visible light to the camera. This camera was equipped with a 250 mm focal length achromatic doublet.
lens that formed an image on the camera CCD with a magnification of 12.5×. A kinematic tube mount was used to tilt the camera and lens assembly, so that the laser focus was maintained in the field-of-view. The optical resolution of this arrangement was ~2 µm, as shown in Figure 3.

Figure 3. Demonstration of optical imaging on ATOF. (left) 20 µm size fluorescent beads were observed by optical imaging camera. (right) USAF resolution test target shows ~2 µm lateral resolution on optical image.

B. Fs-laser desorption postionization mass spectrometer (fs-LDPI-MS)

1. Reflectron installation

The second phase instrument was constructed from a two-stage reflectron TOF was scavenged from a donated instrument (Pegasus III, LECO) and coupled to the fs-LDPI-MS after the detector (31374, Photonis, Lancaster, PA) was refurbished. High voltage power supplies (PMT series, Bertan/Spellman, Hauppauge, NY) removed from this instrument were reused for the reflectron with customized controlling circuits. A conical nipple vacuum adapter was used to couple the TOF drift tube to the instrument main chamber. The reTOF had a screen inside the vacuum tube chamber that could be biased negatively
but was grounded for these experiments. The reflector consists of 16 rings and 2 grids in the front. There was a 1 MΩ resistor between every two rings, except the first grid. The control of the reflectron was performed by adjusting the second grid and back plate of reflectron, named reflectron back and reflectron front, respectively, on the final instrument control panel. Mass resolution optimization was usually done by adjusting the reflectron front potential. Because the ion source was located outside of the reflectron’s focal point, a second-order energy correction could not be achieved. Nevertheless, mass resolution up to ~1100 was demonstrated in both LDI and LDPI modes.
Figure 4. Fs-LDPI-MS overview and ion trajectory.
(top) Overview diagram of femtosecond laser desorption postionization mass spectrometer (fs-LDPI-MS) showing the 10.5 eV (118 nm) VUV generation cell, optical layout, UV laser beam blocker, VUV diagnostic detector, and reflectron time-of-flight MS assembly. (bottom) Photoion trajectory acquired by SIMION simulation. The green trajectory represents the ion flight path.
Figure 5. Ion source section view of fs-LDPI-MS. The ion source and the front end of ion optics are showed in 3D. The critical components of the ion source are labeled in the figure. Red beams represent two options of delivering fs laser onto the sample.

Figure 6. Section view of reflectron assembly. The reflector assembly and deflector are showed in the figure. The screen (green) is used for isolating the potential of the free flight region from grounded vacuum chamber.

2. Ion optics

Ion optics were changed significantly from the previously described ion funnel design. The cone in front of the sample plate was split into two symmetrical pieces. A short
metal insert right after the cone shielded ions away from the plastic insulator that was used for mechanically supporting the two half cones. Without the shielding tube, ablated ions charged the plastic piece, and induced unwanted focusing effects, leading to severe loss of signal at higher laser fluence. Both of the half cones were biased to \( \sim 2 \) keV when the ionization laser fires to extract photoions, while one of them was grounded to deflect direct ions away from the TOF when the desorption laser fires. Three sets of static electrical lens assemblies with deflectors were used to focus ions and correct ion trajectories to have them pass through the gas conductance limiting tube in between the reflectron assembly and main chamber. There were two sets of deflectors before the reflectron to successfully transfer as many ions as possible into the reflectron. A delay generator (DG535, Stanford Research Systems, Sunnyvale, CA) was used to control the timing of the pulsed half cone and starting time of the TOF spectrum.

Ions formed directly from laser desorption usually move towards the detector along with the photoions. When large molecular ions are involved, two separate processes can have their TOF spectra merge together. In this case, the high mass peaks from laser desorption/ionization (LDI) appear in the low mass region of the photoion spectrum in the composite spectrum. Beyond these confusing peaks in the low mass region, LDI ions also contribute to “grass”-like baseline noise, which increases the limit of detection for LDPI. To solve this problem, the ion optics were pulsed to remove direct ions before photoions entered the mass analyzer. Two methods for actively suppressing direct ions were implemented.

In the first method, direct ions were mostly removed from LDPI mass spectra by pulsing a deflector to +400 V when firing the desorption laser. Several µs after firing the
VUV-generating Nd:YAG laser, the deflector voltage was pulled down to ground to shunt all photoions into the reTOF. There was also a balance between the upper m/z limit of direct ions suppressed and lower m/z limit of photoions passed to the detector, which was determined by the delay between Nd:YAG firing and the falling edge of the deflector bias. The typical delay used was chosen such that direct ion rejection was up to m/z ~300 and photoions transmitted down to m/z ~50. The deflector could also be placed closer to the ion source so that the direct ion suppression would extend above m/z ~1000 while allowing photoions above m/z ~50 to pass into the reTOF.

Figure 7 shows the benefit from pulsed direct ion suppression. The black spectrum with no pulse showed significant amount of grass-like baseline noise and some direct ion peaks merged into the photoion spectrum. However, with suppression in effect, the red curve shows mostly only the molecular peak from N-(1-pyrenyl)maleimide (NPM), together with gas phase calibration peaks. Figure 8 and Figure 9 demonstrate of how pulsed direct ion suppression works for both direct ions and photoions.
Figure 7. MS spectra comparison with and without LDI ion suppression. Baseline noise between m/z 50-150 is removed in red curve.

The second method of direct ion suppression solved the problem of the pulsed deflector design by splitting and pulsing the cone in the source. In this case, one of the half cones was biased to the collection voltage, typically 2-3 kV and the other one was grounded when the desorption laser fires. All direct ions were pulled out of the ionization region due to the high potential difference. In the meanwhile, the high deflection field between the two half cones guided direct ions to impact the grounded cone. The potential difference was much higher than the pulsed deflector scheme, and direct ions from all masses were suppressed in this case. As shown by Figure 8, direct ions did not even see the focusing lens. The grounded cone was biased to the same potential as the other one, ~2 μs before ionization laser firing, so that the potential on both half cones could come to equilibrium when the photoionization laser fires. As shown by Figure 9, a majority of the photoions
moved towards the mass analyzer. The typical rise time of the cone was about 100 ns, and the overshoot / ringing can span as long as ~1 μs. Based on the Figure 10 oscilloscope plot, 2 μs is mostly sufficient to have both half cones at exactly the same potential, so no beam deflection was introduced by the half cones.

Figure 8. Ion trajectory when desorption laser fires shows that most of the ions are hitting half cones (generated by SIMION 8.1)

Figure 9. Ion trajectory when ionization laser fires shows that most of the ion are going towards TOF (generated by SIMION 8.1)
Figure 10. High voltage pulse generator test waveform. Showing rise time of pulsed HV of ~100 µs and ringing time scale of 1 µs.

3. 118 nm and 267 nm beam coupling

A 118 nm (10.5 eV) photon energy source was built and installed on the instrument for VUV postionization, employing a design that was improved from the third harmonic generation gas cell described previously. More details will be presented in the following chapters.

A ~4.6 eV (267 nm) fs beam was generated by passing fs 800 nm fundamental beam through two BBO nonlinear crystals. The 267 nm beam was delivered to the chamber via a viewport on the top of the instrument. A periscope, two translation stages, and several mirrors were used to deliver the beam. Two of the mirrors are mounted on translational stages instead of conventional kinematic mounts, so that a precision beam position can be
read out by micrometer. The geometry of beam delivering is showed in Figure 11. This method gives the instrument the capability to quantify the influence of ion source position on the final spectra. More details will be discussed in a subsequent chapter.

![Figure 11. Fs 267 nm beam laser entrance optical layout.](image)

**4. Precision motion stage for imaging**

A high precision sample plate holder had to be designed to achieve the ultimate lateral resolution on the instrument. The initial commercial sample holder was purchased
to mount the sample. The initial sample plate holder was designed for an old commercial MALDI instrument, thus the mounting fixture had to be designed around it. There was no specification provided by the manufacturer, and the sample mounting fixture suffered from poor positioning reproducibility and non-parallel motion. The worst issue was non-parallelism, which caused the sample to move out of focus when an objective lens was used for sampling. The initial sample holder is shown in Figure 12. As shown by Figure 13, even for a relatively small 200×200 μm² area scan, the sample moved out of focus and lost signal on the top-left corner. This issue significantly limited how well the instrument could perform at the ultimate lateral resolution, especially when nonlinear effects play a crucial role, because it led to a defocused laser and rapid drop in laser fluence. Mechanically, the issue originated from two sources. The most important source was insufficient number of plungers on the back of the sample plate. The initial design had only two plungers, which left room for the sample plate to angle differently. The second source was due to a non-polished mounting bracket, which was not made to sufficient flatness, exaggerating the previously mentioned issue.

Figure 12. First version of sample mounting assembly. Showing non-polished mounting brackets and PTFE insulator with low precision.
Figure 13. Artifact due to non-parallel sample holder. Non-parallel sample plate holder causes out of focus issue when running imaging experiment, lower signal appears at the end of imaging scan.

To fix these issues, a second version sample mounting holder was designed and constructed, shown in Figure 14. The mounting brackets were made out of stainless steel for strength then polished to sufficient flatness. Four plungers were inserted into the fixture on the back of the sample plate holder and adjusted so that all of them pushed the holder against brackets. Tested with common test indicator (0.0005” per grid, generic brand), sample plate z position (the normal direction of sample plate) measurement did not change more than 0.0001” (2.5 μm), less than 1/4 of the Rayleigh range of the objective focused beam when moving across the whole scan area, 34 × 34 mm.

Beyond the non-parallelism issue, the second version holder was made much larger, to mitigate ion optical alignment issues due to sample plate position. The second version also had mounting space for a thin photodiode for a beam profiling experiment. The center was drilled through, so that the laser can transmit through the holder and be measured by a
photodiode. This allows beam profiling at the optimal condition with optimal signal to noise ratio, and leads to the minimum measured focus size in later experiments. This reduced some beam focus diameter values of the previous measurements, which showed larger beam diameter than actual ones.

![Second version of sample mounting assembly. Showing large sample plate, polished stainless steel brackets and PEEK insulator.](image)

Figure 14. Second version of sample mounting assembly. Showing large sample plate, polished stainless steel brackets and PEEK insulator.

5. Laser beam delivery

Two optical beam paths were used. One consisted of a long working distance objective (10× NA 0.28, Mitutoyo, Japan) with a 60° incidence angle with respect to the sample surface normal. The second beam path used an achromatic doublet lens (200 mm focal length, Thorlabs, Newton, NJ) with a 30° incidence angle. Switching between the two beam paths was achieved by demountable magnetic kinematic mount (Thorlabs,
Newton, NJ. A 2× Galilean beam expander was inserted into the beam upstream to reduce the laser focus size on the sample. The 30° fs laser focusing optics provided a focused beam diameter of ~25 μm and a Rayleigh range of ~100 μm, which is better suited for rough surfaces such as dried biofilms. The 60° objective focused the fs laser beam to a ~4 μm diameter and was used for high lateral resolution. To further improve the beam quality, the previously described double-pulse setup and dichroic beamsplitter were bypassed. Optical imaging was done using a separate objective, as illustrated in Figure 4.

C. VUV generation gas cell

1. 118 nm VUV generation

The 355 nm output of a ns Nd:YAG laser was used to pump a Xe/Ar frequency tripling cell. Three lasers with similar specifications (Tempest-10, New Wave Research, Portland, OR; Surelite I-10 and I-30, Continuum, San Jose, CA) were used from time to time due to occasional instrumental failures.

A beam sampler was also inserted in the beam path, extracting 6% of the beam for monitoring the pulse energy. Two mirrors were used to deliver the fundamental beam into the gas cell. One of the mirrors was mounted on a micrometer kinematic mount (SN100C-F2M, Newport, Irvine, CA) to have numerical control of beam direction. The micrometer mount was mounted on a translational stage to position the beam at the correct entrance location on the cell. The translational stage helped the optical alignment when an off-axis LiF lens was also used as the dispersion element.

The picture and geometry of the cell is showed in Figure 15 and Figure 16, respectively. On the gas cell side, a UV coated 250 mm focal length fused silica lens
(PLCX-38.1-128.8-UV-355, CVI, Albuquerque, NM) was mounted on a detachable viewport flange (112667, Accuglass, Valencia, CA) for isolating the gas mixture from the atmosphere and focusing the beam to generate VUV radiation. An off-axis LiF lens (Beijing Scitilon, China) was used as a VUV collimation lens. It was also used as a dispersion element to separate and block the 355 nm primary beam by an aluminum beam blocker. The LiF lens material quality was crucial to achieve good 10.5 eV yield as the transmission at 118 nm varied significantly from vendor to vendor.

Figure 15. Picture of VUV generation gas cell with 125 mm quartz lens. Metering valve (Xe) and bellows valve (Ar) are showed in the picture. A capacitance diaphragm gauge was used to measure pressure. A quartz lens is mounted on a 2.75” conflate flange viewport to isolate the cell from atmosphere. A 2.75” gate valve was used to isolate the cell from vacuum pump, which is used for emptying the cell during gas refilling.
Figure 16. Top section view of VUV generation cell.
The pump laser entered the gas cell from quartz lens on the right. The
generated VUV radiation passed off-axis LiF lens and be focused into the
ion source. The pump beam will be separated from VUV beam by dispersion
of the LiF lens. A gate valve isolated the cell from turbomolecular pump,
which is used to pump the cell during gas refilling. Metering valve was used
to inject Xe into the gas cell.

A metering valve (SS-4BMRG-VCR, Swagelok, Solon, OH) with shutoff feature
was used to control the introduction of Xe, and a regular bellows valve was used for
introduction of Ar. Venting relied on a 2 ¾” conflat flange gate valve (01032-UE01, VAT,
Switzerland). The large opening of the gate valve allowed high conductance when pumping
by turbomolecular pump (TURBOVAC 50, Oerlikon Leybold, Cologne, Germany). A
capacitance diaphragm gauge (1000 Torr, MKS, Andover, MA), whose response is
independent of gas species, was used for monitoring total pressure in the cell. An offset
flange was machined to position the output VUV beam ~1 mm in front of the sample plate.
Dimensions were calculated by CAD software (SolidWorks, Dassault Systèmes
SOLIDWORKS, Waltham, MA) with assistance from a simple optical ray tracing software
(OpticalRayTracer, http://arachnoid.com/OpticalRayTracer), based on LiF refractive index
values. The custom machined offset flange was essential to ease cell positioning and laser alignment.

Using an antireflection coated quartz lens as a window transmitted more 355 nm radiation into the system, as compared to the previous design which lost ~20% of the pump laser power by reflection from the quartz windows. Fixing the lens to the cell also decreased the possibility of laser damage, commonly caused by moving the focusing lens too far and focusing the beam onto the window.

An off-axis LiF lens was used to pick up generated VUV output and focus it to near the desorption plume. Figure 17 shows the optical layout of the scheme. Meanwhile the fundamental UV beam diverged after the LiF lens and fellowed a different path due to relative large refractive index difference. A beam blocker was positioned so only the VUV beam entered the ion source, although the separation of UV and VUV in space was sufficient to avoid molecular ion fragmentation due to an intense UV beam.

![Figure 17. Illustration of the optical layout and 355 nm UV beam removal. Red line represents the 355 nm beam on the left side of the LiF lens while the purple lines represent the 355 beam on the right side.](image)

Despite the VUV generation cell being a very robust source that required no beam re-alignment for a single whole year, it still can be improved. The off-axis UV beam entrance on the quartz lens led to spherical aberration, coma, and astigmatic focus. All three aberrations were mostly due to the use of a plano-convex singlet lenses. The use of a
single lens was also a compromise due to the high cost of aberration corrected custom UV optics. However, the singlet lens can be positioned at a certain offset location and angle, so that UV beam passes through the optical axis of the UV lens, whereby coma and astigmatism would be mostly avoided. Spherical aberration would not be not too significant in such case, since the numerical aperture would only be ~0.010. However, this strategy would involve careful calculation of laser entrance angle and a custom made viewport and might only result in marginal improvement.

2. Determination of dimensions

To determine several critical dimensions of the VUV cell, the focal length and cell length as well as several other parameters of the two lenses had to be taken account. First, the laser damage threshold for quartz and LiF lenses was considered. Second, the viable quartz lens focal length was adjusted to achieve phase matching in the Xe/Ar gas mixture. Third, the distance from LiF lens to ion source was optimized. To prevent slow laser damage to the LiF lens due to high UV beam irradiance, the distance between UV beam focus in cell to either the LiF lens or quartz entrance lens were made roughly equal. This way, if the laser did not damage the quartz lens, it was assumed it would not damage the LiF lens either. Fortunately, for up to 30 mJ/pulse this assumption has held up for over a year. To achieve the highest possible conversion yield, Xe/Ar gas mixture was used. Short focal length quartz lens, or more precisely higher numerical aperture focusing, did not benefit VUV generation from phase matching by mixture of gases.\textsuperscript{75} Two quartz lenses were tested: 250 mm f.l. lens benefited from phase matching and produced more VUV flux than did the 125 mm f.l. lens. Fundamentally, the determining factor was the numerical
aperture of the focus. In practice, most Nd:YAG lasers, at this power level, have similar output beam diameters of ~5 mm, which led to the choice of the 250 mm focal length lens.

3. More details of VUV generation cell

The initial pump down had to be done by turbomolecular pump and heating was necessary, requiring over one week to reach sufficient cleanliness in the cell.\textsuperscript{110} An example of the residual gas pressure in a cleaned cell is shown in Figure 18. However, after initial pump down, the refill gas could be pumped down by clean dry pump, or even an oil pump equipped with a trap. LiF lens material has a tendency to form color centers, which was found to absorb VUV up to 50%. Based upon experience with this cell, the transmission at 118 nm could drop by more than 50% after the first three experiments, and then stabilized at about half of the initial transmission. No experiment was attempted to determine whether or not this was a vendor specific issue.

![Figure 18. Residual gases partial pressure in gas cell. Spectrum taken after cell evacuated by turbomolecular pump, showing major residue gases.](image)
4. VUV photon detector

A simple photoelectron current detector was built based on a published design and mounted opposite the gas cell for the purpose of monitoring VUV generation and assisting beam alignment. Before the introduction of this detector, researchers commonly reported using acetone gas phase mass spectra to quantify the VUV generation. The problem with this method was that it is very difficult to diagnose issues when two under-development instrumental setups are involved, the VUV generation cell and the mass spectrometer. Specifically, it was very hard to know whether the lack of VUV signal was due to poor VUV generation or incorrect ion optical potentials or alignment. An acetone ionization cell was previously described that measured ionization current, which was a more robust method to quantify VUV generation. A VUV spectrometer under vacuum can also be used to test VUV generation, although at a significant cost.

As shown in Figure 19 and Figure 20, the VUV detector consisted of a Ni plate having an ~5 eV work function that allowed generated photoelectrons to be extracted from the cathode by a +250 V biased Ni mesh anode and monitored by an oscilloscope (TDS5104S, Tektronix, Beaverton, OR). The acquired signal is shown in Figure 21. The VUV detector had an ~10^4 discrimination of VUV vs. UV photoelectron current. An attempt was made to calibrate the detector to absolute VUV flux. Based upon literature reported quantum yield, 92% transmission of Ni grids, and measured photo current signal on an oscilloscope with 50 Ω termination, the lower limit estimate of VUV irradiance was 8 μJ, which is roughly consistent with literature reported efficiency. However, a slightly saturation was observed on the detector, although still inconclusive, which would only lead to higher actual irradiation.
The cathode was made of Ni and connected to the pin of vacuum feedthrough directly. A Ni grid (not drawn) with 90% transmission was attached to the anode ring. The anode was connected to the other vacuum feedthrough pin. A PTFE ring and a PEEK seat were used to electronically isolate anode and cathode and also used as mechanical support.

The picture showed an assembled VUV photon detector.
Figure 21. VUV detector response signal. Photoelectron current measured by 50 Ω terminated oscilloscope. FWHM of 8 ns roughly matches Nd:YAG laser pulse duration.

5. VUV generation results

A gas mixture consisting of acetone, hexane, toluene, octane, and α-pinene was leaked into the vacuum chamber and detected by photoionization as a complementary check of VUV generation and additionally for mass calibration. An example gas calibration mass spectrum is shown in Figure 22. In most cases, an accurate calibration with the aforementioned molecules could be extrapolated to m/z 500 with less than m/z 1 mass shift. Higher molecular weight molecules such as C$_{60}$ and sexithiophene were used to calibrate the higher mass range.
The VUV yield dependence on pump pulse energy, Xe pressure and gas mixture composition was also studied. Shown in Figure 23, the yield tops at ~ 6 Torr, with pure Xe under tight focus by 125 mm f.l. lens. Shown in Figure 24, the optimum gas composition to achieve highest yield by 250 mm f.l. lens is 10.5:1 (Ar:Xe). A slightly low Ar composition did not decrease yield too much, but a slightly higher Ar composition quickly ruined the VUV generation. Hence, the routine gas ratio was kept at 10:1, with Xe partial pressure at 10 Torr.
Figure 23. VUV output vs. pure Xe pressure, with 125 mm f.l. lens. The figure showed optimum VUV generation condition is 6 Torr of Xe with 125 mm f.l. entrance lens.

Figure 24. VUV output vs. gas mixture composition, with 250 mm f.l. lens. The figure showed optimum condition of VUV generation is with 109 Torr Ar and 10.4 Torr Xe. The figure also showed that Ar gas does not help improve VUV yield at 6 Torr Xe pressure.
VUV generation yield at various pump powers were also investigated. Figure 25 shows the gas phase acetone ionization signal vs pump power, displaying an approximately linear dependence. Figure 26 also shows the pulse to pulse VUV generation stability and power dependency. In Figure 26, the VUV detector signal and UV beam pulse energy meter signal were fed into the customized software, which recorded pulse to pulse VUV intensity measurement. During the process, laser pump power was slowly increased to cover the whole pump power range. The span of red points in Figure 26 gives an idea of how much the VUV pulse energy fluctuated, assuming the VUV detector is ideal. In Figure 27, the gas phase ion signal is also plotted against VUV detector signal. The distribution of the data points show the mass spectrometer has a higher fluctuation than VUV detector in the case of monitoring VUV intensity.

![Figure 25. Acetone ion response vs. pump pulse energy.](image)
The acetone ion response showed near-linear relationship between VUV output and UV pump pulse energy.
Figure 26. Pulse to pulse correlation of VUV output vs. 355 nm beam pulse energy. The figure showed the relationship between VUV output, characterized by photo cathode detector, and UV pump laser pulse energy. Each dot on the plot represents a single measurement by high speed ADC and calibrated pulse energy meter.

Figure 27. Pulse to pulse correlation of acetone ion response vs. VUV pulse energy. Each dot represents a single measurement of VUV output by acetone ion signal and photo cathode detector signal. The graph shows the fluctuation by acetone ion signal is higher than it by photo cathode detector.
D. Fs 267 nm generation

1. Optical design

A fs 267 nm generation setup was built for implementing multiphoton ionization on the instrument. There are generally two approaches to generating fs 267 nm output. Both of them involve two nonlinear crystals for second harmonic generation (SHG) and third harmonic generation (THG), with their difference laying in how GVD compensation is done before the beam reaches the THG crystal.\(^{115,116}\) The nonlinear crystals usually have larger dispersion in the usable wavelength range. Because of this large dispersion, the fundamental beam and the doubled beam will be temporally separated after the SHG crystal. The time difference between these two wavelengths is about 200-700 fs, which is large enough to completely separate the 800 nm beam from the 400 nm in time, if the 50 fs fundamental beam is used. Traditional GVD compensation was done by splitting the beam after SHG into separate 800 nm and 400 nm beams by harmonic separator, inserting adjustable delay lines in both beamlines and merging them back together with another harmonic separator/combiner. The final merged beam was then passed through a THG crystal.\(^{116,117}\) The delay was be adjusted to minimum GVD (close to 0), so that the 800 nm and the 400 nm beams reach THG crystal at the same time. A \(\lambda/2\) waveplate was also inserted into either the 800 nm or 400 nm leg of the delay lines to match polarization of the two beams at the THG crystal. However, the delay line method added significant complexity into the system, and also could suffer from vibration, which can lead to delay line length shifts and THG efficiency losses.

A new approach was used to achieve 267 nm generation with simpler optics and less complexity, shown by Figure 28.\(^{116}\) Unlike the traditional method, two novel
components were used. The first was a time plate (225-2114, Eskma Optics, Vilnius, Lithuania), generally a calcite crystal, cut at a particular angle. The calcite crystal is birefringent, which means a 400 nm beam propagates through it faster than does a 800 nm beam when the crystal is positioned at a particular angle. The thickness of the calcite crystal determined the amount of introduced reversed GVD. Thus, by matching the thickness of SHG crystal ($\theta = 29.2^\circ$, $\varphi = 90^\circ$, thickness = 0.2 mm, BBO-602H, Eskma Optics) and calcite time plate, full GVD compensation could be achieved. The second novel component of this method was a dual wavelength waveplate (465-4211, Eskma Optics, Vilnius, Lithuania), which behaves as $\lambda/2$ waveplate at 800 nm and $\lambda$ waveplate at 400 nm. This results in polarization rotation of only the 800 nm beam, so that the polarization can be matched for Type I sum-frequency generation at the THG crystal ($\theta = 44.3^\circ$, $\varphi = 90^\circ$, thickness = 0.05 mm, BBO-608H, Eskma Optics).

As illustrated in Figure 28, the fundamental beam passes through the SHG crystal and is doubled to 400 nm. The type I SHG crystal generates the 400 nm at a polarization direction perpendicular to the fundamental beam. Due to the dispersion in the SHG crystal and air, the 800 nm pulse is in front of the 400 nm pulse. Both wavelength pulses go through the time plate, and the 800 nm beam is delayed to slightly behind the 400 nm beam, but later the dispersion of air corrects the two sets of pulses to co-align at the THG crystal. The third component rotates the 800 nm beam polarization to the same as the 400 nm beam for Type I sum-frequency generation. The fourth component, the THG crystal mixes the two beams into 267 nm. Several harmonic separators and a 267 nm mirror remove both 400 nm and 800 nm from the beam and deliver 267 nm light into the ion source.
Figure 28. Illustration of 267 nm generation scheme

2. Performance

The 267 nm conversion efficiency also relies on the fundamental beam properties. The amplifier needs to be adjusted to the highest beam quality and shortest pulse duration. A misaligned compressor will lead to pulse stretching and lower 267 nm yield. Poor spectral distribution in the fundamental beam will lead to non-Gaussian spectral distribution in the harmonic beams.

The fully tuned laser and nonlinear optics provides ~200 μJ of 400 nm and 42 μJ of 267 nm pulses by a 1.8 mJ pulse energy fundamental beam at 42 fs pulse duration. Routinely available 267 nm output was about ~30 μJ pulse energy with 80 fs from the 1.6 mJ fundamental beam. The measured efficiency was less than 50% of the value claimed by the manufacturer. The lower efficiency could be due to mismatched spectral bandwidth, although this was not fully tested. To prevent damage to the amplifier, the oscillator
bandwidth was kept at 45-50 nm, while the manufacturer specification quoted narrower bandwidth.

Figure 29 shows both 400 nm and 267 nm output beam spectral bandwidth. The bandwidth of second and third harmonic output were ~10 nm. The split peaks in the third harmonic spectrum were due to imperfect spectral bandwidth in the fundamental beam.

![Figure 29. Fs 267 nm and 400 nm output spectrum bandwidth. The red 400 nm peak shows a near-Gaussian profile with a minor imperfection. The black nominal 267 nm output shows two separated Gaussian profiles merged together, due to the imperfection of fundamental beam being exaggerated by harmonic conversion process.](image-url)
III. INSTRUMENT CONTROL SOFTWARE

A. Introduction

The data acquisition and control software that is provided with commercial mass spectrometers generally performs well for the modes of operation for which such instruments have been designed. Software also plays an important role in the customization of commercial mass spectrometers as well as in the implementation of entirely “homebuilt” instruments, both of which can be used to develop novel experimental strategies. However, commercial software is usually proprietary and often cannot be readily modified for alternate modes of operation without the express permission and cooperation of the instrument vendor. Cost, frequency of upgrades, and integration with evolving operating systems are among the additional issues that can arise with commercial software.

Free open-source software can lower the cost of owning, maintaining and operating custom-built scientific instruments. Furthermore, community support of open-source software can improve interoperability and flexibility. Open source software has been developed for the analysis of MS datasets, including those produced in imaging experiments. Publically hosted, open-source instrument control software can permit similar advantages, especially given that so many MS imaging configurations are actually ion source modifications on commercial MS analyzers.

LabVIEW software (National Instruments, Austin, TX) has been widely adopted for data acquisition and control of customized scientific instrumentation, including mass spectrometers, and many data acquisition programs written in LabVIEW are open source. While some users consider LabVIEW to be interpreted and therefore limited
by data transfer rates, it is actually a compiled programming language that uses the LLVM
compiler which does allow rapid execution high data transfer rates.\textsuperscript{130}

This chapter describes a versatile open-source instrument control software platform
called ChiMS that was written within LabVIEW for imaging and depth profiling mass
spectrometers. ChiMS was initially designed for a laser-based time-of-flight mass
spectrometer optimized for imaging and depth profiling. ChiMS was also designed for
transferring large datasets from a digitizer to computer memory at high repetition rate;
saving data to hard disk at high throughput; and automating data processing, imaging and
depth profiling experiments. However, the ChiMS software has now evolved so that it is
additionally well suited to non-laser based MS imaging and various other experiments in
laser physics, physical chemistry, and surface science. The data acquisition mode generally
simulates a digital oscilloscope, but with peripheral devices integrated for control as well
as advanced data sorting and processing capabilities. ChiMS also allows the repurposing
of useful hardware components by re-programming their manufacturer-provided software
drivers. Finally, customized user-designed experiments can be easily written based via
several templates included in the ChiMS software.

B. Software design and instrumentation

1. Software design.

The structure of the ChiMS software is shown in Figure 30: it is a modular multiple-
loop application framework with some parts involving a dynamic framework within a
subprogram. ChiMS is an extension of the compound design pattern concept as it is
constructed in a multiple single loop pattern as modules with communication by messaging
between loops. The hierarchy of modules includes: (1) a master subprogram for controlling all other modules in automatic mode, (2) high and low levels of user interface (UI) for flexible handling of peripheral devices, (3) module cores under a hardware abstraction layer (HAL) for communicating with hardware, and (4) processing and saving cores for simple signal processing and data storage.

Figure 30. Illustration of simplified software structure and hardware connections. Solid lines represent regular operating mode, and dotted lines show alternative mode. Dotted lines represent alternative operation mode. The cyan and green blocks represent actual hardware units and software communication cores, respectively. Colors for other blocks are chosen to group them by type. The general communication direction follows from dark to light yellow, then green and finally cyan. Pink colored blocks do not interfere with the instrument control sequence. See text for further description.
The unique features enabled by this design includes: (1) >1 kHz repetition rate data acquisition for MS imaging\(^2\) with the ability to collect generic, pulsed time-of-flight mass spectra without signal averaging or summing; (2) flexible peripheral device control; (3) real time data processing; and (4) ready implementation of dynamic or data dependent experiments.

Each block in Figure 30 represents a loop or module inside the software. There is a master subprogram on top of the high and low levels of the UI which runs the instrument in automatic acquisition mode to facilitate high volume data acquisition for applications such as MS imaging. The low level UI sends messages to the HAL modules that communicate bi- or unidirectionally with the hardware. Acquired data is fed into a processing core for simple digital filtering while the high level UI displays instrumental parameters. The processing core handles simple data filtering tasks such as pseudo pulse counting, baseline electronic noise removal and/or baseline correction, then passes the processed filtered data to either master subprogram for future data sorting, or the UI for display and streaming of raw data to storage media. The master subprogram is capable of processing raw data into images for display or saving to disk.

Module cores, labeled green in Figure 30, are designed to encompass or wrap around the function of traditional LabVIEW instrument drivers while additionally handling commands or other messages received from the upper level program. The module cores are usually single loop programs that respond to commands received from the queue structure, although some utilize two single loops (if the hardware device has both blocking and non-blocking operations). These module cores must be designed to handle all actions

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\(^2\) The typical sample rate is \(~1\) GS/s and the record length is 10-100 μs long, so the amount of data is huge.
gracefully while preventing unexpected module seizure. The module cores are also capable of running in standalone mode to facilitate testing and debugging. Running standalone also helps isolate potential issues from integration with the main program during the early prototyping stage of instrument development. A simple HAL is written on top of the LabVIEW instrument drivers to simplify switching between hardware with similar functionality from different vendors.

The user interface is separated into high and low levels. The high level UI accepts experimental parameters input by the user and converts these into hardware parameters that are then passed to the low level UI, where they are stored and passed to hardware without further modification. However, the hardware parameters in the low level UI are directly accessible to the user to permit trial experimental configurations without software modification.

Two types of master subprograms based on templates are included in ChiMS for different levels of customized experiments. The simpler, statically controlled master subprogram does not employ any online signal processing, but streams signal data to disk at the highest possible transfer rate. The more complicated, dynamically controlled master subprogram is used when online data processing is needed, such as in MS imaging. Thus, the dynamic master subprogram must control the operation, analyze the data, record results, and generate necessary instrumental parameters on the fly. Overall, general instrumental behavior is defined via the triggering scheme defined by hard-wired delay generator connections, timing values set in the hardware, and hardware action sequences defined in the static or dynamic subprograms of ChiMS.
2. Instrumental details.

The configuration of the laser desorption postionization mass spectrometer instrument has been detailed in Chapter II. For consistency, the instrument is in ns-LD-fs-MPI mode in this chapter. The computer used in the reference instrument was loaded with Windows 7 and LabVIEW 2013 and was equipped with a quad-core processor (Xeon E-1230, similar to Intel Core i5), 16 GB memory, a mechanical hard drive in a RAID 0 array, and a 256 GB solid state hard drive (SSD). ChiMS will also work (with minor changes) on computers with fewer hardware resources as well as those running Windows 8 or 8.1 and/or LabVIEW 2012 through 2014. A calibration test target, made by depositing pentacene through TEM grids as mesh, was used for demonstrating imaging.

C. Results and discussion

1. High throughput MS imaging.

Specialized hardware is required to achieve high throughput MS imaging: a high repetition rate laser, a digitizer with high data throughput and low latency such as a fast analog-to-digital converter (ADC) or time-to-digital converter (TDC), a motion stage with a rapid position update rate, and high slew rate pulse generators that can match the pulsed laser repetition rate. The instrument used here is a laser desorption postionization time-of-flight MS (LDPI-MS) configured to run at 1 kHz repetition rate with a ns pulse length, UV Nd:YLF laser for desorption and an ultrashort pulse, Ti:sapphire laser outputting UV for photoionization. The remainder of this section further describes the ChiMS software in the context of how it facilitates 1 kHz MS imaging with this particular instrumental configuration.
Figure 31 (a) shows the base user interface (UI) of ChiMS, which mimics the function of an oscilloscope. Most of the functions of the base UI involve high level control of the instrument, whereby mass spectra are displayed as long as the lasers are firing. The spectra are continually refreshed, which is particularly convenient for tuning instrumental operating parameters. The base UI interface provides control to all implemented hardware functionality. The application menu guides the user to a master subprogram configuration page depicted in Figure 31(b), which allows users to provide parameters for MS imaging such as imaged area, pixel size, and number of averaged spectra per pixel. ChiMS calculates all the required hardware parameters from input values such as motion stage velocity, then combines them with queried instrumental status for input to the master subprogram. Figure 31(c) shows the master subprogram interface for MS imaging that controls the instrument in automatic mode and performs data analysis online (see below).
Figure 31. Representative user interface screenshot of ChiMS (a) ChiMS base user interface during biofilm analysis experiment, (b) configuration master subprogram page for inputting experimental parameters and (c) dynamic control master subprogram interface during an MS imaging experiment.
2. Laser and timing cores.

Laser and timing cores are created to control laser and digital delay generator, so that all the devices fire or start at the expected time with an accuracy of nanoseconds. The amplifier of the Ti:sapphire laser employed here cannot be externally triggered and instead is used to drive the overall experimental timing sequence (see Appendix). Thus, the Ti:sapphire laser triggers the digital delay gate generator (delay generator), which in turn must wait slightly less than 1 ms to trigger the desorption laser (no photoion signal is produced from the first desorption laser pulse). The delay generator also triggers the HV pulse generator to ramp the voltage on the ion optical electrodes to reject direct ions while extracting photoions. Finally, the digitizer is triggered by the time zero output pulse on the delay generator, which serves as the starting time of the time-of-flight spectrum.

Switching between different laser desorption/ionization schemes is done via reconfiguring the delay generator by loading configuration files and swapping physical trigger lines (cables) between the lasers. The loaded configuration files (de)activate different delay generator channels and set both delay times and laser firing sequences. Drivers were written for two common delay generators (DG535, SRS and 500 series, BNC), allowing their rapid interchange despite hardware incompatibilities.

3. Main DAQ core.

The speed of the main DAQ core is responsible for acquiring and processing of the ion signal and is the central driving force of the ChiMS software data flow. The digitizer is typically a high speed ADC or TDC with an on-board histogrammer. The main DAQ core of ChiMS transfers binary integer data from the digitizer, then converts it from an
integer into a floating number in volts for module compatibility. After voltage conversion, the data is sent to the processing core for simple signal processing tasks that include fine adjusting zero baseline, baseline electronics noise removal, pseudo pulse counting, and/or spectral averaging/summing. Two types of digitizers are currently supported by ChiMS: various high speed ADCs (100 MS/s to 4GS/s, 8-bit to 16-bit PCI and PCIe, Gage Applied) and a low cost multipurpose device (100MS/s, 14-bit, 10 MHz, dual channel, Analog Discovery, Digilent). However, many other types of data acquisition hardware can also be supported with minor revisions to the ChiMS software.

ChiMS was specifically designed for rapid data collection and transfer rates. The typical sequence denoted in Figure 32(top) as the “common simple method” is simple to write and deploy, but it uses computer resources inefficiently. For example, this strategy cannot achieve a 1 kHz repetition rate because the software has to wait for the completion of raw data conversion, spectra processing, and (most time consuming of all) data saving before starting the next cycle of data acquisition. Even when file saving is offloaded to a thread in parallel, data conversion and processing starts to slow the software down above ~100 Hz repetition rate because background applications can occasionally occupy all the resources of the computer’s central processing unit (CPU) in Windows 7. Thus, the CPU may delay action on time-sensitive hardware functions such as arming the digitizer to acquire signal before every laser pulse.

ChiMS achieves rapid data acquisition by minimizing the digitizer idling time while offloading as many tasks as possible to parallel threads. This strategy is denoted in the pink block in Figure 32(bottom) as the “repetition rate optimized method” and it is designed to both rearm the digitizer as soon as data transfer is finished and convert raw
digitizer output while the digitizer is recording signal for the next cycle. The general digitizer operation sequence starts with the armed hardware waiting for a trigger to acquire a spectrum. The program will run a digitizer status query in a loop with no wait timer (a compromise due to the lack of a sub-millisecond wait timer in Window 7 versions of LabVIEW). The main DAQ core switches to data transfer as soon as data acquisition finishes, then immediately re-arms the digitizer before performing data conversion, minimizing the chance of missed data acquisition cycles.

![Flowchart comparison of common simple method (top) versus repetition rate optimized method of data acquisition (bottom).](image)

ChiMS’s triple data buffer structure fully utilizes the multicore CPUs that are ubiquitous in modern computers by employing parallel processing to decouple the data flow dependency between acquisition, processing and saving. The ChiMS triple data buffer design consists of raw integer conversion buffer in the main DAQ core, floating number signal accumulation buffer in the processing core and a final buffer in the master subprogram for data analysis. Utilization of the data flow driven model\textsuperscript{131} of LabVIEW allows computational work on the three buffers to be distributed across the multicore CPU.
to allow execution in parallel, improving main DAQ core responsiveness and allowing higher overall data acquisition rates in ChiMS.

4. Motion control core.

The motion control core is used to drive the motion stages used predominantly for scanning sample position with respect to desorption laser beam, a requirement for MS imaging experiments. A common, but slow method for sample scanning is to repeat the sequence of moving then stopping the motion stage and firing the laser. Continuous motion scan imaging allows much higher repetition rates, but requires the highest position update rate possible. The device driver was completely rewritten to optimize the overall ChiMS position update rate to 170 Hz. Prior to this update, a jitter in position referencing resulted in pixel misalignment during data processing.

The motion control core has both a non-blocking and a blocking loop and is based on the fundamental communication design of some motion stages controllers that will not respond to the computer if they are running a blocking command. Commands are sent to the non-blocking loop first, which then decides whether to execute the command itself or pass the command to the blocking loop.

Two types of motion stage controller are currently supported (Corvus controller series, PI miCos and ESP series, Newport). Both share the same module interface with hardware differences hidden by the HAL (see above), facilitating replacement without modification of software.

5. Processing core.

ChiMS has also a data processing core that receives and processes data acquired from the main DAQ core. Simple data process can be performed with the data processing
core, including digital filtering, binning and signal averaging. The digital filter has three functions: zero offset adjustment, pseudo pulse counting, and baseline electronic noise removal (See Appendix for details).

6. Master subprogram for MS imaging.

ChiMS can only generate and display simple mass spectra without higher level control. A dynamic master subprogram application programming interface was therefore devised for more complex experiments such as MS imaging, depth profiling, and laser beam profiling. The MS imaging master subprogram presented here is one example of how dynamic master subprograms work in ChiMS. Parameters such as scan speed, average number of spectra per pixel, and overall image size are predefined through user input before the start of an experiment. However, the master subprogram application programming interface also allows modification of parameters during the experiment (i.e., in cases such as laser beam profiling, see below), where instrumental parameters are not known in advance of data collection and instead must be determined on the fly via calculation or feedback loop.

The dynamic master subprogram is provided as a template consisting of three major subroutines (known as subVIs in LabVIEW): sequence control, data sorter and secondary parameter setter. The sequence control subVI manages higher level motion of the sample and sets other instrumental parameters by calling the secondary parameter setter subVI. The data sorter subVI averages raw spectra into pixels, integrates peak areas based on a user-defined list of m/z values of interest, generates images and saves sorted images to disk in a standard format (Analyze v7.5 format, supported by the BioMAP image processing
software, [http://www.maldi-msi.org/](http://www.maldi-msi.org/). The data sorter subVI also allows users to view the image while data acquisition is still in process.

Figure 33 shows the overall topology and execution sequence of the MS imaging master subprogram. The master subprogram begins with the sequence control subVI moving the motion stage to the start location of the line scan, then it waits for all ready signals from various experimental components (i.e., lasers, motion stages, and laser attenuator). Next, the sequence control subVI triggers the lasers and executes a line scan at the user pre-defined velocity until reaching the end of the line on the sample. During this process, the sync out signal of the fs laser triggers all equipment at 1 kHz via the delay generator, while the digitizer acquires signal that it passes along to the processing modules (as described above). Simultaneous with these events, the data sorter subVI gathers signal filtered by the processing core and averages it into pixels, based upon the encoded motion stage position. Both subVIs run in a loop until the completion of each line scan, then the sequence control subVI first checks if the full image completed, then either starts the next line scan or terminates imaging. Finally, the data sorter subVI assembles all the acquired data into pixels, uses them to create one line of an image, and saves this line to disk. Preliminary images are generated in between line scans from the list of user-defined peaks of interest to provide a rapid image preview in the user interface while the overall experiment is still running.
Figure 33. Diagram of topology and execution sequence of master subprogram for MS imaging. Top and bottom boxes represent the sequence control subroutines (subVI) and data sorter subVI, respectively. The secondary parameters setter subVI is incorporated as a single from in the sequence control subVI. (Dotted line represents an alternative routine.)

Performance-wise, the MS imaging master subprogram acquires one 32k sample mass spectrum every 1 ms, then converts the raw 8-bit integer data to 32-bit single-precision floating point numbers for sorting to the image buffer. This process consumes hundreds of MB/s memory bandwidth. Both the Intel Xeon E3 or Intel Core i5 quad core processors can handle all these computations and higher repetition rates provided they are allowed by the hardware.


Precision laser beam profiling via the knife edge method is used to evaluate lateral resolution in MS imaging. Laser beam focus size and focus location must be measured within submicron precision to successfully acquire ion images at the highest spatial resolution possible. Thus, the laser beam profiling master subprogram was created which represents an additional application for ChiMS beyond mass spectrometry. Details of the knife edge measurement have been described in a previous chapter.
The benefits of automated beam profiling arise from the automatically determined variable scan velocity and length. Each beam radius measurement generates one transmission curve against scanning direction, then the curve fitting subVI calculates beam radius and location. Scan velocity and location calculated for each scan are used as guidance to assign parameters for the subsequent scan. By auto-fitting the parameters for each scan, idling time is minimized and useful data points are maximized. This strategy allowed very rapid automated laser beam profiling while producing higher quality data resulting from collection of extra data points in the transition region.

8. Alternate modes, auxiliary tools, configuration saving, and portability.

ChiMS is readily available to run on several instruments without modification of LabVIEW source code. One alternative instrument setup was used to demonstrate software portability. Briefly, a laser front end was designed for a customized ion source coupled to a field deployable mass spectrometer with an atmospheric pressure interface (MTE50, MassTech).\textsuperscript{134} The laser front end includes a motion stage with controller (ESP300, Newport) and a low cost multifunctional data acquisition device (Analog Discovery, Digilent) connected to an energy meter (J4-09, Molelectron) for laser pulse energy measurement. ChiMS was used for laser beam profiling and also sample rastering by the motion stage, with the acquisition of mass spectra handled by the software provided by the mass spectrometer vendor.

The Auxiliary DAQ core was created for slow data acquisition tasks, such as monitoring high voltage power supply, vacuum pressure, and laser power (from analog output on power meter). The data acquired from the auxiliary DAQ core is fed into a control that is hidden on the front panel. The processing core regularly pulls data from the hidden
control and merges them with high speed signal from the main DAQ core, then sends the merged signal to the processing core. The data flow and hardware are not critical, so they are omitted on Figure 30.

ChiMS also supports saving to and loading from configuration files which can set instrument operation mode at the start of the program. This makes the instrument less prone to issues caused by incorrect hardware parameters, a common problem with customized instruments.

The ChiMS package also includes some auxiliary tools for mass calibration, text file viewing and batch file processing. A file format converter is also available to generate MS imaging files from raw binary data files. These are described further in Appendix.

D. Conclusions and ChiMS availability

ChiMS is open-source software developed for controlling custom-built imaging and depth profiling mass spectrometers and associated instrumentation. Optimized device drivers were incorporated to accommodate the highest laser repetition rates essential for high speed imaging. ChiMS is capable of running MS imaging experiments at 1 kHz repetition rate and the associated instrument has been used to demonstrate <2 μm lateral resolution (see below). The application of ChiMS has also been extended to depth profiling and laser physics experiments that include optical delay line zero delay searching and laser attenuator calibration.

A dynamically controlled master subprogram allows ChiMS to process and analyze signals together with feedback loop capability to algorithmically adjust instrument parameters during experiments. One example of dynamic control is automated laser beam profiling, which requires change of scan region and velocity during the experiment. These
parameters cannot be known before the experiment and must be calculated after each scan, so the devised master subprogram allows this auto-tuning. This strategy could be extended to automatic optimization of ion optical potentials for maximum ion transmission and mass resolution.\textsuperscript{135} Although tandem MS is not implemented on the instrument used here, the interface can be used to programmatically select a precursor peak and run tandem MS, once such a capability is available.

The ChiMS software package can be downloaded at https://github.com/ycui7/ChiMS. ChiMS is licensed under LGPL v3 license.\textsuperscript{136}
IV. ELEMENTAL ANALYSIS BY ATOF

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A. Equipment and sample preparation

The first phase instrument configuration used here is based on Chapter II.A, which was equipped with an ion funnel interface for low vacuum operation, but which was disabled when high vacuum experiments were carried out. The mass analyzer was a linear TOF, which is detailed in published literature.\(^{137}\)

All metal samples were used as received without any processing. Semiconductor wafers were used as received except for methanol washing. A resolution test target (Negative 1951 USAF Wheel Pattern Test Target, R3L1S4N, Thorlabs, Newton, NJ) was also used without further preparation. A tantalum oxide film was prepared on tantalum foil anodically using a standard method.\(^{138}\) All materials and the resolution test target were adhered to the sample plate inside the mass spectrometer with conductive copper tape.

B. Ablation under high vacuum, lateral resolution and depth profiling

Figure 34 shows the laser ablation mass spectra of five elemental metal samples (from bottom to top – Ta, Mo, Cu, Ag, and Au). The surface of each sample was cleaned by repetitively raster scanning under the focused laser beam, after which mass spectra were
recorded with ~6 keV DC bias on the sample plate. All expected metal ions appear in the spectra, with a mass resolution of ~200. The isotopic distributions were also well represented by the relative intensities of the atomic ions. The Mo\(^+\) peak intensities correlated well with the natural isotope abundances of \(^{92}\)Mo (14.84\%), \(^{94}\)Mo (9.25\%), \(^{95}\)Mo (15.92\%), \(^{96}\)Mo (16.68\%), \(^{97}\)Mo (9.55\%), \(^{98}\)Mo (24.13\%), and \(^{100}\)Mo (9.63\%). Reasonable agreement was also observed for the Cu and Ag isotopes.

Oxygen ions, observed in almost all spectra, resulted from the surface oxide layer that continually reforms at the background pressure of 10\(^{-6}\) Torr. Residual carbon was also observed on some samples, as were Cu\(^{2+}\) and Ta\(^{2+}\) obtained from their respective oxidized metal foils (not shown). Na\(^+\) and K\(^+\) were observed from most samples, as is common in direct laser desorption, because of the low ionization energies of Na and K atoms. Some cross-contamination was observed from deposition of metal that was laser ablated from adjacent samples.

Figure 35 shows laser ablation mass spectra of undoped Si and GaAs wafers, recorded with the laser focused at a fixed position on each sample, recorded at a 1 kHz data acquisition rate. The sample was fixed during data acquisition because the mass resolution was found to be higher without sample scanning. Si\(^+\) and GaAs\(^+\) together with their clusters up to Si\(_7\)^+ and Ga\(_m\)As\(_n\)^+ (m+n=4) were observed. Larger clusters were observed at lower intensity.

Figure 36 shows that Cr\(^+\), Fe\(^+\), Ni\(^+\), and Mo\(^+\) were observed in the laser ablation mass spectrum of stainless steel 316, as expected, because all four elements are major components of this alloy. An Al metal standard (Al5182AS, Alcan) was also analyzed, as shown in Figure 36, to demonstrate the potential for elemental quantification with this
instrument. Al\(^+\) and Mg\(^+\) were observed in the spectrum of the Al alloy standard as well as other major alloy components, including Li\(^+\). Mn\(^+\) appeared with a signal-to-noise ratio >10, although it comprises only ~0.3\% of the alloy content. These results indicate that quantification of elemental content from the laser-ablated ion intensity is feasible. Hydrocarbon fragment ions were also observed in the Al alloy mass spectrum.

Figure 34. Laser ablation mass spectra of (from top) Ta, Mo, Cu, Ag, and Au foils.
Figure 35. Laser ablation mass spectra of (from top) GaAs and Si wafers.

Figure 36. Laser ablation mass spectra of stainless steel and Al alloy. (top) Stainless steel 316 and (bottom) Al 5182 alloy standard.
Figure 37. Mass spectrometric ion images of a USAF 1956 test target. Ion intensity bars shown at right with linear integer scales corresponding to ion detector voltage (-1 = 0 mV, 300 = 46 mV, and 12 = 1.8 mV). (a) \( \text{Cr}^+ \) and (b) \( \text{Cr}_2^+ \).

The USAF test target used to demonstrate the lateral resolution of the instrument was a Cr-coated glass substrate plated with negative resolution patterns. Figure 37 (a) is a MS image of \( \text{Cr}^+ \) signal from the test target, taken with 1 \( \mu \)J laser pulses with a 32×16 \( \mu \)m\(^2\) elliptical spot size on the surface. Scanning was done in a line-by-line pattern by
successively scanning the x-axis of the motion stage and then incrementing the y-axis position. The total acquisition time for this image was 80 min. The image was constructed by setting a nominal pixel size at 5×5 μm². All the spectra recorded while the centroid of the laser beam was within a given pixel were averaged together.

Besides the main Cr⁺ peak, the Cr₂⁺ cluster peak was also integrated to construct the image shown in Figure 37(b). The intensity of Cr₂⁺ was only ~5% of the Cr⁺ peak, yet both the Cr⁺ and Cr₂⁺ images have similar appearance: the group 6 pattern elements are clearly resolved in both images, corresponding to 10 μm lateral resolution. Some vertical strips in group 7 are also resolved, corresponding to ~5 μm lateral resolution. The horizontal resolution was lower than the vertical because of the 60° incident irradiation angle. No effect of redeposition of Cr on adjacent glass areas was observed.

The lateral resolution observed was somewhat smaller than the laser spot size, which can be explained by the property that only the center part of the laser beam (TEM₀₀) exceeded the ablation threshold. This effect is a direct consequence of the highly nonlinear ablation process induced by ultrafast near-IR laser pulses, which has been observed in numerous prior studies.²⁴, ⁵⁰, ⁵¹ This ability to form craters smaller than the beam diameter has the potential for a dramatic improvement in analytical lateral resolution,²⁴, ⁵², ¹³⁹, ¹⁴⁰ and is discussed later in this thesis. A similar effect was also observed in nanosecond laser desorption.¹⁴¹

It is vital to determine the laser focal point, the laser spot size, and the Rayleigh range because ultrafast laser ablation is very sensitive to laser fluence. The laser spot size was measured as described in Chapter II.A.2. The beam was fitted to a Gaussian function with the beam radius treated as an adjustable parameter.²⁴ The laser beam profile was
measured at different z-axis positions (moving through the focus), so that the laser spot radius vs. z-axis position was acquired. These results showed that proper sample positioning was crucial while working with the 10× microscope objective lens because of the tight focal range and the correspondingly short Rayleigh range of ~75 µm. Ideally, the thickness of a sample should be measured prior to analysis to a precision of ~20 µm, so that the measured focus can be located as close as possible to the sample surface.
Figure 38. Ion signal vs. laser shot number at various pulse energies for a Ta oxide film. The data were obtained by ablating a ~330 nm Ta2O5 film deposited on Ta foil.

Figure 38 shows the depth profile of a ~330 nm thick Ta2O5 film deposited on Ta foil, measured by continuously ablating a fixed area on the sample at a laser repetition rate of 1 kHz. The laser ran for ~1 s to drill each hole, and each spectrum from the digitizer was stored separately, with no averaging, so that ~1000 spectra were recorded for each hole.
(Figure 38 displays data from the first 500 laser shots only). To improve the statistics, the measurement was repeated on 100 separate spots on the foil, then the data from different spots were averaged according to the laser shot number. Finally, the peak areas corresponding to each element were plotted vs. the number of laser shots, corresponding approximately to the hole depth.

No attempt was made to calibrate the number of laser shots with the actual depth profile of the Ta$_2$O$_5$ film. Nevertheless, an estimate of the potential depth resolution can be made by assuming a drop in K$^+$ signal (which appeared mostly from the surface) to 10% of its initial value. This assumption leads to complete Ta$_2$O$_5$ film removal after ~60 laser shots for 2.3 µJ laser energy, the highest used in this study. This result indicates that at this fluence (0.6 J/cm$^2$), ~6 nm of Ta$_2$O$_5$ film was removed per laser shot. The data clearly demonstrates that less material was removed per laser shot at lower laser fluences. By comparison, 30 nm was removed from metallic titanium per laser shot at 1 J/cm$^2$ fluences using 80 fs, 800 nm laser pulses.$^{142}$

C. Ion funnel and elevated pressure

Variable-pressure experiments were performed by introducing He into the source chamber through a leak valve. The six orders of magnitude of differential pumping between the sample and detection regions of the chamber allowed ~0.3 Torr of He gas to be introduced into the ion source chamber without the collision rate in the TOF drift region or the detector reaching problematic levels.

A Ta sample was used to test the performance of the ion funnel at elevated pressures. Previously, ions could pass through the ion funnel and be extracted into the TOF.
However, continued efforts failed to achieve acceptable sensitivity and mass resolution at elevated pressures. Suspected reasons could be the axial extraction scheme do not work well with ion funnel, indicating that an orthogonal extraction scheme is needed to achieve sufficient extraction.

D. Laser ablation of clusters and molecules

Figure 39 shows the results from laser ablation on Ta surface. A long series of Ta clusters show up in the MS spectrum. The spectrum was taken with pseudo pulse counting mode, so that the lowest limit of detection can be achieved. The plot is in log scale, showing the large dynamic range of all clusters (saturation is observed at low mass range). Several small molecules were also tested in LDI mode, including NPM, C_{60}, C_{70}, and some fluorescence dyes (not shown). Figure 40 shows the LDI spectrum of C_{60} and NPM. It is obvious that all of the tested molecules fragmented heavily under LDI conditions. However, some molecular ions survived the ablation process and were preserved as intact molecular ions.
Figure 39. Ta clusters by LDI, showing clusters up to Ta$_{20}^+$ in log scale.

Figure 40. LDI spectrum of C$_{60}$ and NPM molecules. Figure shows significant fragmentation by LDI. M$^+$ denote the molecular ion and F$^+$ denote the fragment ions.
V. MOLECULAR ANALYSIS ON INTACT BIOFILM BY FS-LDPI

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A. Equipment and sample preparation

The instrument configuration used in this chapter was mostly based on the second phase instrument described in Chapter II(B) and II(C), with only slight differences. These differences include the lack of the shielding tube and the presence of only one set of deflector assembly instead of two. Due to the availability of LiF and quartz lenses at the time, the VUV generation cell was operated at only one third output of the final configuration. This older configuration also had lower ion transmission in the TOF than the final configuration of the instrument, and hence represented a non-optimized signal quality. Comparison with ns-LDPI-MS was performed by analysis of samples using a separate custom-made instrument equipped with a ~5 ns diode-pumped solid state (DPSS) Nd:YLF (Explorer, Spectra-Physics) desorption laser, a Nd:YAG (Tempest-10, New Wave Research, ESI, Portland, OR) ionization laser to pump a 10.5 eV VUV frequency tripling cell filled with pure Xe, and a custom reTOF used for mass analysis. Sample positioning and scanning were done by a two-dimensional vacuum-compatible motion stage, similar to that employed in the fs apparatus, capable of submicron precision. Starting Chapter V,
fs-LDPI will be used as the brief replacement for the longer acronym of fs-LD-ns-VUV(118 nm)-SPI.

Baker’s yeast *Saccharomyces cerevisiae* (Red Star Yeast Company) monoculture biofilms were grown either on polycarbonate membranes (GE PCTE filter membranes, 09-732-18, Fisher Scientific) in sessile media or on indium tin oxide (ITO) coated glass or medical titanium (Ti-6Al-4V, 9081K111, McMaster) in a drip flow biofilm reactor. Yeast – *Escherichia coli* (ATCC 25922, American Type Culture Collection, Manassus, VA) coculture biofilms were grown on polycarbonate membranes. A chronic wound model biofilm system was comprised of medical isolates of the bacteria *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Clostridium perfringens*. This polymicrobial mixture was grown on polycarbonate membranes placed on brain heart infusion (BHI) agar media. Membrane biofilms were attached to stainless steel MALDI sample plates by copper tape or blotted for analysis by LDPI-MS, as noted below. Drip flow reactor biofilms were analyzed directly.

### B. Comparison of fs-LDI-MS and fs-LDPI-MS

Figure 41 shows a comparison between fs-LDI and fs-LDPI mass spectra of yeast biofilms grown on polycarbonate (PC) membranes. The fluence of the desorption laser was ~0.1 J/cm² for fs-LDI mass spectra, ~2 - 3× above the ion formation threshold, chosen to achieve a reasonable signal-to-noise ratio without the dramatic degradation of mass resolution observed at higher fluences. The desorption fluence for fs-LDPI was ~0.3 J/cm², ~2× above the desorption threshold. Fs-LDPI employed a pulsed deflector to reject ions formed by direct LD, while the same deflector was simply grounded in fs-LDI mode.
Figure 41 clearly shows that fs-LDPI-MS generated much more molecular information than fs-LDI-MS above m/z 300 (see below). The peak patterns are notably different for the two detection methods. The fs-LDPI signal was \( \sim 20\times \) higher than for fs-LDI. The most intense peaks in fs-LDI-MS are due to \( \text{Na}^+ \) and \( \text{K}^+ \) ions.

C. **Comparison of ns vs. fs laser desorption**

The bottom two spectra in Figure 42 of the same type of yeast biofilms grown on PC membranes show the difference between ns and fs laser desorption neutrals that are photoionized by 10.5 eV radiation. Both spectra show distinct peak patterns, which may be due to laser pulse length dependent differences in desorption mechanism\(^{24}\) and/or internal energy transfer.\(^{103}\) Separate studies probed the extent of energy transfer during fs-LDI and fs-LDPI-MS.\(^{144}\) The mass detection range was similar in both ns and fs-LDPI, ranging up to m/z 800.
Figure 41. Comparison between fs-LDI and fs-LDPI-MS of yeast biofilms. Biofilms were grown on polycarbonate (PC) membranes, with the fs-LDI-MS signal scaled by 20x. The VUV source was turned off to collect fs-LDI-MS.
Figure 42. Comparison of ns- and fs-LDPI-MS of yeast biofilms. Biofilms were grown on PC membranes, labeled “PC membrane (ns)” and “PC membrane (fs)”, respectively, shown in the bottom two spectra. Fs-LDPI-MS of drip flow yeast biofilms grown on Ti and ITO glass surfaces are shown in the top two spectra.
Figure 43. Ion images from fs-LDPI-MS of blotted co-cultured yeast - *E. coli* biofilm.
(a) total ion current, (b) m/z 93, (c) m/z 283, (d) m/z 258, (e) m/z 414, and (f) optical image. Color bar on right indicates signal levels.
D. Comparison of different substrates

Figure 42 displays fs-LDPI-MS of yeast membrane biofilms as well as drip flow biofilms grown on Ti and ITO glass. Some common peaks were observed, but overall spectra differed with substrate, especially at the lower mass range. Titanium gave the lowest signal of the three yeast biofilms by fs-LDPI, even though electrically conductive substrates improve ion extraction in the instrument. Peaks around m/z 400 displayed different ratios, which were possibly caused by metabolite differences. The biofilm grown on the ITO glass substrate displayed two unique peaks slightly above m/z 110 that could derive from indium and an organic species complexed with this constituent of ITO glass.

E. MS imaging

The optical micrograph at the bottom of Figure 43 of yeast - *E. coli* coculture biofilms grown on membranes displayed three distinct regions attributed to “pure” yeast culture, “pure” *E. coli*, and a mixed region. MS images of the cocultured biofilms were recorded from a sample area of 7 × 3 mm² with desorption laser fluence of 0.5 J/cm², with two spectra recorded and averaged for each pixel. The imaging process took one hour to complete and was rate limited by the 10 Hz laser used to generate VUV radiation. The top five panels of Figure 43 display the MS images for total ion count, m/z 93, m/z 283, m/z 258, and m/z 414. The m/z 93 appeared mainly in the mixed region while m/z 283 only existed in yeast, and m/z 258 only in *E. coli*. The m/z 258 peak was previously attributed to a metabolite and was used to image different strains of *E. coli* by ns-LDPI-MS. The m/z 414 appeared mainly in the mixed region and also in the yeast region with lower abundance.
F. Depth profiling

Previous studies showed the feasibility of using <100 fs, 800 ns laser desorption for depth profiling by continuously sampling a fixed spot area.\textsuperscript{24, 57} The feasibility of depth profiling by fs-LDPI-MS was examined here using a chronic wound model membrane biofilm. This 3D biological model was developed previously from three bacterial species isolated from a medical infection that segregate vertically in a biofilm into aerobic (top) and anaerobic (substrate) regions.\textsuperscript{143} A depth-profiling experiment was performed in which the lasers ran for a total of 20 s (i.e., 200 laser shots), drilling a hole down into the intact biofilm sample, where each fs desorption laser shot was followed after \( \sim 10 \mu s \) delay by a VUV laser pulse. Each spectrum from the digitizer was stored separately, with no averaging, so that \( \sim 200 \) spectra were recorded for each hole. To improve the statistics, the measurement was repeated on 50 separate spots on the biofilm. All the data were sorted and averaged according the number of shots at each point. Finally, the normalized peak areas corresponding to each peak were plotted in Figure 44 vs. the number of laser shots, corresponding approximately to the depth.
Figure 44. Depth profile of a polymicrobial chronic wound model membrane biofilm. Figure shows normalized peak areas of five representative peaks at m/z 85, m/z 103, m/z 136, m/z 301, and m/z 581 vs. depth as represented by the number of laser shots. Raw data points were smoothed by 10 point window averaging then plotted as continuous curves. Absolute peak areas are in parenthesis.

Three major profiles were observed from the depth profile of the chronic wound model biofilm, together with some peaks that showed no intensity difference vs. depth. Figure 44 shows five peaks out of ~15 total observed, which displayed different intensities vs. depth. Because the absolute intensity of these peaks varied significantly, the data were normalized, and a 10 point window average smoothing was applied.

The chronic wound model consists of three medically isolated microbes which spatially segregate based on metabolic potential. *P. aeruginosa* resides at the aerobic top of the biofilm, where m/z 103, 136, and 301 are the most intense in the Figure 44 depth
profile. The middle region of the biofilm consisted largely of cell debris, where m/z 301 remained intense. *C. perfringens* and *S. aureus* resided near the anaerobic bottom of the biofilm where m/z 85 and 551 were most intense (near laser shot 50). All peaks decreased beyond laser shot 80, indicating that the biofilm was mostly removed. Figure 44 accordingly displays data from only the first 100 laser shots. While these results support the feasibility of depth profiling by fs-LDPI, correlating analyses are required to confirm that the different profiles measured do in fact match the microbial spatial distributions.

**G. Lateral resolution and other instrumental issues**

The instrument was also equipped with an objective capable of delivering a 7×8 μm focal spot. A lateral resolution of ~10 μm was previously demonstrated using an inorganic test target with a laser focus size of 32×16 μm caused by the nonlinear ablation event. Analyses of biofilms by fs-LDPI-MS, however, did not produce such high lateral resolution with the equipment setup used here. One reason is the relatively short Rayleigh range of ~100 μm for the micro-focused fs laser beam. Such short Rayleigh ranges were problematic for the biofilms studied here given that they displayed surface roughnesses >100 μm, thereby preventing uniform focusing of the laser onto the surface. The nonlinear dependence of the ablation efficiency on laser intensity accentuates this problem. The sample surface roughness problem should be solvable with better sample preparation techniques. Another reason for the lack of high lateral resolution might be that the micro-focused laser beam concentrated the energy into a very small volume, causing most desorbed species to fragment, thereby reducing the molecular ion intensities.

The fs-LDPI-MS images shown in Figure 43 were collected using a 200 mm focal length achromatic doublet lens to focus the ultrafast laser onto the sample at a 30° angle of
incidence. The beam profile produced with this lens was found to display ~25 μm diameter focus when measured by the scanning knife edge method. The focusing optics worked well with the current biofilm and other biological samples as its Rayleigh range was on the mm scale, precluding the aforementioned focusing problems.

An artifact that can potentially cause false identification in LDPI mode arises from the two lasers firing in sequence with 5 - 100 μs delay which may cause interference between ion packets, even if most of the direct ions are electrostatically rejected by the ion optical deflector. Both lasers generate an ion packet with an intervening delay comparable to the laser firing delay. The typical ion time-of-flight was 1-100 μs, so heavier direct ions produced by the fs desorption laser pulse could arrive at the detector together with lighter photoions produced by VUV ionization. This phenomenon can cause multiple broad peaks to appear in the low mass region of the LDPI mass spectra. These false peaks may be identified, however, by changing the delay between the two lasers to shift the LDI peaks with respect to those from LDPI. Alternatively, blocking the VUV beam eliminates photoions and leaves behind the undesirable LDI peaks. However, LDI does not typically generate many heavy ions, so these false peak artifacts do not frequently arise in LDPI-MS. Furthermore, all the LDPI data presented in this chapter were checked to eliminate LDI artifacts. Nevertheless, only the first pulsing method, described in Chapter II, was used. Hence, there is a small chance some peaks from LDI entered the photoionization spectra.

Sub-100 fs laser pulses display a remarkable ability to machine materials with high precision while causing minimal damage to the remaining sample.\textsuperscript{51, 53, 54, 56} Furthermore, ablation of intact biofilms and animal tissue with fs laser pulses previously demonstrated
the feasibility of depth profiling via MS re-analysis at the same spot.\textsuperscript{24, 57} The combination of VUV single photon ionization with fs laser desorption is shown here to permit MS imaging and depth profiling of intact biofilms deposited on various biomaterials without any addition of a matrix. In contrast, MALDI-MS imaging (as most commonly performed with UV ns lasers) does not permit depth profiling and requires the addition of a matrix, which can affect sample chemistry and reduce effective lateral resolution by diffusion.\textsuperscript{5, 8, 10} Nevertheless, a more thorough comparison with MALDI-MS and other methods awaits further studies, as this work constitutes an early presentation of results for the constructed fs-LDPI-MS instrument.

Mid-infrared ns lasers induce vibrationally resonant desorption, which in principle allows depth profiling and additionally avoids the need for matrix addition.\textsuperscript{5, 67} However, the mid-IR laser desorption efficiency fluctuates with a sample’s water content,\textsuperscript{145} so that the desorption efficiency is likely to vary dramatically at the interface between an aqueous biofilm or tissue and a non-aqueous biomaterial.

Femtosecond laser desorption without postionization has been shown to be effective for quantitative elemental analysis.\textsuperscript{146, 147} Although elemental analysis was not pursued in this work, both fs-LDI-MS and fs-LDPI-MS have been demonstrated with the reTOF LDPI instrument. Quantitative analysis of small molecular species on a biomolecular interface was also previously demonstrated by ns-LDPI-MS imaging.\textsuperscript{91} It follows that spatially resolved, quantitative elemental and molecular analysis of biomaterial interfaces and intact biological samples should be possible with the MS imaging instrument described here by using appropriate fs laser fluences.
There are also some shortcomings to MS imaging using the current instrument. Although fs-LDI-MS imaging can rapidly collect images at a 1 kHz rate, fs-LDPI-MS imaging is limited here by the 10 Hz repetition rate of the VUV-generating Nd:YAG laser. However, Nd:YAG lasers with a 200 Hz repetition rate are commercially available and might be deployed to increase the fs-LDPI-MS imaging rate by 20x.

Another shortcoming of the current instrument is the lack of high mass resolution and tandem MS capabilities which precludes actual chemical identification of the MS peaks reported here. High resolution and tandem MS capabilities, despite their absence from most commercial SIMS instruments, are the hallmark of modern biological MS studies.

SIMS remains the MS imaging method of choice for highest lateral resolution and depth profiling, although these capabilities come at the cost of molecular fragmentation that is sometimes quite severe. Insufficient lateral resolution is a problem for all methods in laser desorption-based methods for MS imaging. Generally, lateral resolution in MS imaging performed by ns UV or mid-IR lasers is limited to ~50 μm, although ~5 μm lateral resolution has been reported for selected samples. ~10 μm lateral resolution has been demonstrated in elemental analysis of inorganic test targets by fs-LDI-MS, and the highly nonlinear nature of the desorption event supports arguments that yet higher lateral resolution should be possible with fs-LDPI-MS and fs-LDI-MS. Three phenomena have limited the collection of higher lateral resolution MS images from biofilms by fs-LDI and fs-LDPI-MS. First is the problem of sample roughness; the highly nonlinear dependence of fs laser desorption on laser intensity requires that the sample roughness be well below the desired lateral resolution. Second is the problem of low postionization yields in fs-
LDPI-MS, which limits the pixel size. Third is poor ion transmission in the instrument.

Some of these issues are addressed in the next Chapter.
VI. LIMIT OF LATERAL RESOLUTION OF FS LASER SAMPLING

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A. Introduction

Laser desorption (LD)-based mass spectrometry imaging is under intense study for spatially resolved molecular analysis of intact biological samples, biomaterials interfaces, and electronic devices. Matrix assisted laser desorption ionization (MALDI) with UV lasers is the premier LD-based method in MS imaging capable of preserving molecular structure with typical lateral resolutions approaching ∼25 μm. Lateral resolution approaching 5 μm has been achieved in MALDI using high numerical aperture optics, aspheric optics with Gaussian laser beams, imaging in microscope mode, and back of sample irradiation of thin slices. Near field optical strategies have also been examined for this goal. However, MS imaging of single cells and other submicron sized structures require lateral resolution beyond 5 μm that remains elusive by MALDI specifically and LD generally. Secondary ion mass spectrometry can achieve submicron lateral resolution, but so far only with primary ion sources that tend to enhance molecular fragmentation.

Ultrashort pulse length lasers operating in the near infrared (IR) region show promise for LD-based MS imaging. The most common configuration operates using a
Ti:sapphire laser to create ~75 fs, 800 nm pulses which can generate useful molecular ion signal without addition of a matrix. Recent experiments have shown that fs-LD can enable internal energy transfer in vacuum\textsuperscript{144} or at atmospheric pressure\textsuperscript{155} comparable to that of MALDI performed at the corresponding background pressure. Near-IR fs laser pulses can be used to directly desorb either intact molecular ions\textsuperscript{60, 144} or neutrals for postionization by VUV radiation\textsuperscript{144} or electrospray.\textsuperscript{155}

Near-IR fs-LD has previously demonstrated 10 to 30 µm lateral resolution MS imaging of intact plant leaves\textsuperscript{60} and biofilms, respectively. However, the optimum lateral resolution of fs-LD MS imaging should approach that of the submicron sized features that have been micromachined into biological and synthetic structures by ultrashort laser pulses.\textsuperscript{52} One caveat to the submicron goal for fs-LD MS imaging is the balance between lateral resolution and molecular fragmentation since abundant atomic ions are observed at the high laser irradiances that can be generated by tightly focused fs-LD beams. Combining fs-LD with laser postionization (fs-LD-ns-VUV-SPI, fs-LDPI will be used in this Chapter as a brief replacement of the longer acronym) allows for a considerable increase of molecular ion signal while operating with lower laser irradiances, yielding the added benefit of reduced molecular fragmentation.\textsuperscript{144} For such cases, especially when desorption is performed under vacuum, the time delay between the fs-LD and postionization laser pulses becomes an important factor influencing the extent of molecular fragmentation.

This Chapter presents several experimental results in support of the fs-LDPI approach for high lateral resolution MS imaging. First, the lateral resolution for MS imaging of molecular species by ~75 fs, 800 nm pulsed LD is determined here for a
simulated organic electronic device under vacuum. Next, the dependence of intact molecular ion survival on both desorption laser fluence and delay between desorption and photoionization laser pulses is observed for a small molecule desorbed from an organic multilayer (originally devised as a model of a bacterial biofilm). When considered in light of recent results in the literature, these experiments demonstrate the potential for high lateral resolution MS imaging by fs-LDPI.

B. Experimental details

Experiments were performed with the second phase fs-LDPI-MS operating in microprobe mode using ~75 fs, 800 nm, nominally p-polarized laser pulses from a Ti:sapphire laser for fs-LD of samples under vacuum of ~10^-7 Torr. Photoions were formed by 10.5 eV VUV (~8 ns, 118 nm) single photon ionization of gaseous neutrals formed by fs-LD. The repetition rate of the overall fs-LDPI-MS measurement was limited to 10 Hz by the Nd:YAG laser pumping a Xe/Ar cell, where the 355 nm third harmonic output was frequency tripled to produce 10.5 eV VUV photons. For imaging, a computer controlled in-vacuum XYZ translation stage was used to raster scan the sample with submicron precision as observed by an integrated optical microscope. An objective (NA 0.28, Mitutoyo, Japan) was used to focus the desorption laser beam for imaging experiments at the highest lateral resolution while an achromatic doublet lens (200 mm focal length, Thorlabs, NJ, US) was used for non-imaging experiments at optimal signal to noise ratio.

Two types of samples were prepared for these measurements. First, micron-scale square patterns of pentacene (P0030, TCI of America, Portland, OR, USA) were deposited onto an underlying Si wafer by sublimation from a heated ceramic crucible (LTE 11000K, 1 cc, Kurt J. Lesker, Pittsburgh, PA, USA) at 160 - 190°C through an electron microscopy
grid (16.5 μm pitch: 11.5 μm hole width and 5 μm bar width, G1500HS, SPI, West Chester, PA). Second, polyelectrolyte multilayers composed of ten layers each of chitosan and alginate were adsorbed onto an Au-coated Si wafer, then doped with a small molecule analyte, 2-methoxy-4-amino-5-chlorobenzoic acid (MACA, GMW 201.6).

C. Results and discussion

Figure 45 shows optical and MS images of pentacene deposited through an electron microscopy grid onto the Si wafer. The center of the optical image displays the region that was analyzed by fs-LDPI-MS, and depletion of the pentacene by fs-LD is clearly visible. The energy of the desorbing fs-laser was ~20 nJ/pulse, and the time delay between desorption and photoionization laser pulses was set to 25 μs in order to maximize ion signal. MS imaging was performed by moving the translation stage continuously at a rate of 5 μm/s along the X-axis, collecting two mass spectra per pixel, and by 1 μm steps along the Y-axis. The MS image was constructed from the signal for intact pentacene ions at m/z 278±0.5 with an effective pixel size of 1 μm². The mass spectrum of an integration of one hole area is plotted in Figure 47. The 16.5 μm grid pitch is marked on the MS image. An MS image line scan is also shown in Figure 45 for the integrated signal from the yellow shaded region of the MS image. The fs-LD probe size, corresponding to the lateral resolution, was estimated by extracting the 25%-75% intensity levels from the MS line scan by the established knife edge method, and yielded a <2 μm value, less than half of the ~4 μm diameter (1/e²) of the 800 nm laser beam (see Figure 45). VUV laser postionization allowed fs-LD to be recorded at lower laser fluences so that desorption occurred from spots considerably smaller than the laser beam focal spot (see Figure 48). This mode employs the phenomenon of nonlinear optical absorption, where only the
intensity above a certain threshold is absorbed by the material leading to its explosive ablation.\textsuperscript{156} This phenomenon is particularly pronounced in ultrashort laser pulse interactions with solids\textsuperscript{156, 157} where it has enabled submicron lateral resolution of fs laser micromachining of inorganic,\textsuperscript{158} organic, and biological material.\textsuperscript{52, 139, 140, 159}
Figure 45. MS Image and optical image of test target, with Y axis line profile.
A micron-scale square pattern of pentacene deposited through an electron microscopy grid onto a Si wafer by sublimation from a heated ceramic crucible - imaged by the fs-LDPI-MS instrument (middle) and by an integrated optical microscope after MS analysis (top). Grid dimensions were: 16.5 µm pitch, 11.5 µm hole width, 5 µm bar width. One MS image pixel corresponds to 1 µm sample translation step. Line scan for MS image area (obtained by signal integration of the yellow shaded region) demonstrates lateral resolution of ~2 µm (bottom). A percentile filter smoothing of original data was used to determine the average signal level corresponding to 100% intensity for extracting and comparing 25%-to-75% levels for estimating lateral resolution.
Figure 46. Beam profiling / knife edge scan results at various axial positions. Figure shows the minimum radius of the ~75 fs, 800 nm laser beam is ~1.9 μm and the Rayleigh range is ~10 μm, recorded with <20 nJ pulse energy. A 400 nm fs laser beam was also measured to be ~1.5 μm radius. The radius of the beam $\omega$ was determined as described previously and is defined as the region with laser fluence $\geq 1/e^2$ of the maximum.
Figure 47. Mass spectrum of pentacene from fs-LDPI-MS. By integrating over an entire single pentacene spot shown in Figure 45. The intense peaks below m/z 100 derive from intact ions of gas phase acetone, benzene and toluene used as calibration compounds and did not originate from pentacene.

Figure 48. Demonstration of laser beam diameter vs. sampling area. Diagram demonstrating principle of high lateral resolution imaging by fs-LD. Only the highest intensity, central region of the desorption laser beam above the ablation threshold for the sample material will lead to fs-LD via a multiphoton ionization mechanism. ¹⁶¹
However, use of this precision ablation in MS imaging is limited by the fact that low fluxes of desorbed/ablated material contain far fewer molecular and atomic species than are typically observed by LD-based MS instruments, drastically limiting the number of (even less abundant) ions available for mass analysis. For this reason, high lateral resolution fs-LD MS imaging was facilitated here by VUV single photon ionization of desorbed neutrals (fs-LDPI).\textsuperscript{73} Prior results found higher sensitivities for the detection of photoions by fs-LDPI than direct ions by fs-LD for species desorbed from intact bovine eye tissue.\textsuperscript{144} A crude estimate indicates that $<1$ femtomole of pentacene can be detected by this specific fs-LDPI-MS instrument, but additional study is required to more accurately determine the limit of detection.

It is important to recognize that the $<2$ $\mu$m resolution observed here is not a fundamental limit for the fs-LDPI technique because the specific MS instrument employed here suffered from excessive mechanical vibrations and suboptimal ion transmission. These factors collectively reduced instrumental sensitivity and prevented operation with the lower laser fluences required for higher lateral resolution. Other factors may have also degraded the lateral resolution, specifically the “fuzziness” of the pentacene pattern edges originating from the sample fabrication process.

5 $\mu$m size features on a patterned photoresist were previously distinguished by LD using the higher intensity central portion of a $\sim30$ $\mu$m diameter, UV ns pulselength laser beam (performed in the absence of added matrix).\textsuperscript{141} However, UV ns-LD can induce photochemical reactions in samples even in the linear intensity (low irradiance) regime, which limits the ability of ns-LD to perform MS analyses of biological samples - unless matrix is added to facilitate MALDI. Photochemistry is deleterious because it can
contribute to molecular fragmentation during MS imaging. It can be argued that these were some of the original motivations for the development of MALDI. By contrast, the possibility of laser-induced photochemical alteration of many microbial and fungal biofilms, most mammalian tissue, and many other biological samples is minimized by their transparency in the linear intensity regime for the 800 nm wavelength. Experiments applying near-IR fs-LD MS with VUV postionization to the pentacene patterned sample revealed mass spectra with almost no ion fragments, allowing the high signal to noise MS image shown in Figure 45 to be constructed entirely from the intact pentacene ion.

A 400 nm fs beam, which was acquired by inserting a doubling crystal and IR blocking filter into the beam, was also briefly used to test the lateral resolution by near-UV desorption. As shown in Figure 49 the lateral resolution in both cases are quite similar, slightly less than 2 µm. It seems 800 nm desorption has marginally better lateral resolution along the Y axis, while 400 nm desorption performs marginally better along the X axis. Beam profiling results in Figure 46 shows 400 nm beam exhibits a plateau around the focus, which may have resulted from either optical aberration or limits of the measurement method. Specifically, the size/thickness of the knife edge may limit the minimum measurable beam waist. It was worth noting that the 400 nm fs beam has a polarization direction perpendicular to 800 nm beam, which results from Type 1 SHG. However, it has not been confirmed whether polarization played a role here.
Figure 49. Line profile along X and Y axis at various desorption wavelength. (top) fs 400 nm desorption SPI (bottom) fs 800 nm desorption SPI. X axis line profiles are offset by 80 for both wavelength condition.

Recent experiments using fs-LDPI observed an increase in fragmentation of a thermometer molecule with increased overlap between adjacent laser spots, lower desorption laser fluence, and shorter delay time between the firing of the desorption and photoionization lasers. In order to clarify the interplay between these parameters and molecular fragmentation, some of these issues were revisited here in non-imaging mode.
on a sample that approximates intact biological material, a chitosan-alginate polyelectrolyte multilayer onto which a metabolite analogue was adsorbed. Figure 50 shows the fs-LDPI-MS of 2-methoxy-4-amino-5-chlorobenzoic acid desorbed from the multilayer at laser pulse energies of 0.11, 0.17, and 2.3 J/cm², all recorded with a larger ~25 μm diameter fs-LD beam and a delay of 25 μs between desorption and photoionization laser pulses. The threshold fs-LD energy for formation of ion signal was ~0.08 J/cm² (data not shown). Only the intact molecular ion at m/z 201 was observed for this chlorobenzoic acid ion at a fs-LD pulse energy of 0.11 J/cm², with the expected 3:1 ratio of ³⁵Cl:³⁷Cl isotopes for m/z 201:203. Almost no fragmentation was observed at 0.11 J/cm² and 0.17 J/cm², but the latter shows ~35× higher signal. Yet higher precursor signal was observed at fs-LD fluence of 2.3 J/cm², but at the cost of the additional appearance of fragments at m/z 184 attributed to [M-OH]⁺ and m/z 170 attributed to [M-OCH₃]⁺. Furthermore, the appearance of the Au⁺ atomic ion at the highest laser fluence of 2.3 J/cm² indicates that the entire multilayer was disrupted by the fs-LD event. It should be noted that the spectra in Figure 50 represent the sum of at least 50 individual mass spectra recorded from separate spots on the sample.

Figure 51 shows the fs-LDPI-MS of 2-methoxy-4-amino-5-chlorobenzoic acid on the multilayer at 5, 25, and 99 μs delay between firing of the two lasers, at a fixed fs-LD pulse energy of 0.17 J/cm² and with the same larger fs-LD beam (and also summing 50 spectra). While strong intact molecular ion signal was observed for all three delays, molecular fragmentation decreased as delay increased. Essentially no fragmentation was observed at the longest delay of 99 μs, albeit at a significant reduction in detectable ion signal, and maximum signal was observed here at ~25 μs. It was recently reported that 20
- 40 μs delays presented the optimal balance between maximizing signal and minimizing fragmentation in fs-LDPI for both these multilayers and bovine eye tissue. These results indicate that variation of the desorption to postionization delay can be used to fine tune fragmentation, facilitating compound identification in a fashion similar to that employed via postsource decay in MALDI and other time-of-flight MS experiments.

Figure 50. fs-LDPI-MS of MACA adsorbed onto PEM at various fluences. Spectra recorded at fs-LD fluences of 0.11, 0.17, and 2.3 J/cm², all with a fs-LD to postionization laser pulse time delay of 25 μs.
Figure 51. fs-LDPI-MS of MACA absorbed onto PEM at various PI time delays. All recorded at fs-LD fluence of 0.17 J/cm² and fs-LD to postionization time delays of 5, 25, and 99 μs.
D. Conclusions

The use of ultrashort near-IR laser pulses for desorption in conjunction with VUV laser postionization imparts a unique synergy which yields fundamental advantages for MS imaging of a simulated electronic device and should be applicable to biomedical samples. First, the increased number of detectable ions in a single laser desorption event enabled by laser postionization of desorbed neutrals permits using lower fs-LD irradiance so that the effective size of the laser microprobe can be made considerably smaller than the actual diameter of the desorbing laser beam spot – thanks to a highly nonlinear, multiphoton ionization-induced ablation process. Second, the use of lower laser desorption irradiances results in noticeably reduced molecular fragmentation, which can become virtually undetectable under certain combinations of desorption and postionization conditions. Furthermore, the damage to the remaining undesorbed target material is minimized under such conditions, facilitating accurate depth profiling and thus three-dimensional MS imaging. Third, the variation of the time delay between desorbing and postionizing laser pulses offers an efficient tool to control and fine tune the molecular fragmentation, which can help improve accuracy of the molecular species identification.

Prior work showed that useful molecular ion signal can be generated by fs-LDPI without introduction of the matrix compounds, nanoparticles, metal additives, or nanostructures required by other LD-based MS imaging methods. When considered in light of the universal nature of ultrashort pulse laser desorption and the ability of VUV single photon ionization to detect a wide range of molecular and atomic species, it is clear that fs-LDPI-MS will be effective for the near-surface analysis of a wide range of inorganic, organic, synthetic, and biological solids. The experiments
presented here demonstrate <2 μm lateral resolution for fs-LDPI-MS imaging, which in many cases should permit single cell imaging. Efforts are needed to advance this resolution to the submicron level by further improving the instrument sensitivity (so that measurements at even lower desorbing laser irradiances can be conducted) and by reducing mechanical vibrations. However, the high surface roughnesses typical of many biological samples will induce the objective-focused laser beam - with its small, ~10 μm Rayleigh range - to desorb widely varying amounts of material from pulse to pulse from such rough samples. The determination of the lateral resolution for fs-LDPI-MS imaging must therefore be examined on actual biological samples in conjunction with the collection of additional morphological information.
VII. FS 267 NM MULTIPHOTON IONIZATION

Despite the near universal sensitivity of 10.5 eV SPI to organic species, the repetition rate of a commercial Gaussian beam pump laser is limited to 30 Hz due to crystal thermal properties.\textsuperscript{162} An alternative approach to high speed imaging is demonstrated by fs 267 nm multiphoton ionization. Traditional ns 266 nm 4\textsuperscript{th} harmonic Nd:YAG output has been demonstrated for detecting aromatic small molecules, but the high laser energy imparted to the molecules often leads to extensive fragmentation.\textsuperscript{163} Reports have found that the fs laser can significantly suppress fragmentation during the multiphoton ionization process, with a large portion of molecular ions being preserved.\textsuperscript{116,117} Thus, a fs 267 nm generation setup has been built and was described in a previous chapter.

Figure 52 shows the gas phase MS from fs 267 nm photoionization of a gas mixture. In this graph, the same gas mixture, used in 118 nm mode, was leaked into the ion source at $2 \times 10^{-6}$ Torr pressure. An aromatic toluene peak is shown in 267 nm PI spectrum, but the aliphatic octane is not shown, while both toluene and octane peaks appear in the 10.5 eV SPI spectrum. This is due the lack of a UV-absorbing functional group in aliphatic octane, which lowers the two photon ionization cross-section. A control spectrum with no leaked gas mixture is also presented, showing some residual gas in the vacuum chamber could be ionized by fs 267 nm laser with very high efficiency, at a base pressure below $1 \times 10^{-7}$ Torr.
Figure 52. Photoionization mass spectra of gas mixture, with ns 118 nm and fs 267 nm ionization lasers.
Comparison between ns 118 nm SPI vs fs 267 nm MPI showed MPI generates higher level of fragmentation. The 267 nm MPI background mass spectrum shows peaks resulting from residual gas in the vacuum chamber. The high background noise in 118 nm SPI was due to low number of average of the spectrum.
Br$_2$-Tyr was used to demonstrate ns-LD-fs-MPI at various ionization pulse energies and LD to PI delays. Br$_2$-Tyr was sprayed onto a stainless steel sample plate by an automated chemical sprayer until a visually uniform film was observed. Figure 53 shows the mass spectrum of Br$_2$-Tyr in ns-LD-fs-MPI MS. Figure 54 shows the peak assignment of Br$_2$-Tyr fragments, which is kept consistent with prior work.$^{92}$ Figure 55 shows the influence of fs 267 nm pulse energy on signal intensity. The molecular ion and several fragments are plotted. It is clear that the signal intensity corresponding to ionization efficiency increases quite dramatically at low laser pulse energy, but levels out above ~15 μJ.

Figure 53. MS spectra of Br$_2$-Tyr in ns-LD-fs-MPI mode.
Figure 54. Peak assignment of Br₂-Tyr LDPI-MS fragments.

Figure 56 show the fragmentation trend against PI pulse energy, by plotting the ratio of fragment intensities over that of the molecular ion. The M⁺ curve, of course, is a straight line as the reference. The fragments ion can be categorized into three groups according to Figure 56. Group 1, consisting of fragment III, V, and VI, have an increasing trend as the pulse energy increases. Group 2, consisting solely of fragment II, experiences only a ratio drop, implying it is a meta-stable ion, which fragments into others when ionization energy increases. Group 3, consisting of fragment IV, has a maximum ratio around 10 μJ pulse energy. Fragment I was not plotted due to the low peak intensity and signal-to-noise ratio. The hypothesis, here, is fragment IV is an intermediate fragment with complex kinetics. The rate of formation of fragment IV surpasses the rate of secondary fragmentation below 10 μJ pulse energy. The relationship between formation and secondary fragmentation flips after 10 μJ pulse energy, hence, the maximum ratio of fragmentation IV is formed. Further experiments are needed to prove the mechanism of why a local maximum exists in Figure 56, work which has not been done in this dissertation.
Figure 55. Br$_2$-Tyr ns-LD-fs-MPI peaks intensity vs ionization pulse energy.
(top) log scale, (bottom) linear scale. Peak assignments refer to Figure 54.
Several other LDPI modes with different combinations of desorption lasers and ionization lasers were also briefly tested. Figure 57 shows three different modes, utilizing three modes: (blue) ns-LD(349 nm)-ns-VUV(118 nm)-SPI, (red) fs-LD(267 nm)-ns-VUV(118 nm)-SPI, (black) ns-LD(349 nm)-fs-UV(267 nm)-MPI. Among the three combinations, fs-UV(267 nm)-MPI shows significantly higher fragmentation. Ns-VUV-SPI generates mostly a few major fragments as listed in Figure 54, while fs-UV-MPI generates overwhelmingly high fragments in the mass region less than m/z 100, which blocks potential application for very low mass molecular analysis without ultrahigh mass resolution capability. As shown by the top and middle curve in Figure 57, fs UV(267 nm) desorption seems to be more efficient than ns-UV(349 nm)-LD, generating less grass-like chemical noise. All three curves are normalized to fragment III, while the fs-UV(267 nm)-LD-ns-VUV-PI gave the highest signal and S/N. It was also shown before, that 267 nm UV desorption is more efficient than 349 nm in case of ns lasers. However, due to this
instrument’s problematic performance during these experiments which was before improvements to ion transmission were implemented, the seemingly more efficient fs desorption could be due to artifacts. Overall, the difference between 118 nm SPI vs. 267 nm MPI agree with the results published in the literature.\textsuperscript{93}

Figure 57. Comparison of various LDPI methods.
In conclusion, fs 267 nm beam was used for MPI of small molecules. Gas phase ionization was carried out briefly for testing MPI without complication from laser desorption. A Br$_2$-Tyr organic film on stainless steel plate was used for ns-LD-fs-MPI at various ionization fluences. Results show the fragmentation pattern changes with ionization fluence change. Alternative laser desorption with fs 267 nm was also briefly tested to compare with other LDPI methods.
VIII. CONCLUSION

This thesis is dedicated to the usage of ultrafast laser pulses on laser desorption mass spectrometry. Several LDI and LDPI methods were carried out to demonstrate the capability of ultrafast femtosecond pulse laser on sampling materials, both inorganic and organic.

In Chapter II, the instrumentation of the method is detailed in many aspects, including vacuum, ion optics, laser sources, electronics and mechanics. The instrument control software is also presented in an appendix as a follow up of hardware system assembly. The final version of the instrument is capable of ~2 µm lateral resolution in imaging mass spectrometry. Near-diffraction limited laser focusing was also achieved through several versions of laser delivery setups. Mass resolution of ~1100 was demonstrated with photoions from fs-LDPI.

In Chapter III, it is shown that fs-LDI is a useful method for elemental analysis. It is also good for imaging and depth profiling applications. The lateral resolution was initially demonstrated on a test target to ~10 µm, and improved to ~2 µm later on. Depth resolution was not quantified, but sufficient to show step-wise the penetration of a 300 nm thick Ta₂O₅ film. Common Al alloy and stainless steel sample were used to demonstrate the capability of detecting low abundance elements in these alloys. Several molecules were used, and results shown molecular ions and significant fragmentation.

Chapter IV demonstrated the fs-LD-ns-VUV-SPI-MS method, which used fs 800 nm beam for desorption and 118 nm VUV beam for single photon ionization for the first time. Several biofilms were prepared on various substrates and compared with alternative
LDPI methods. Imaging and depth profiling showed fs-LDPI can be used to analyze intact biological samples.

Chapter V examined the limit of lateral resolution of fs near-IR desorption and molecular preservation in the fs-LDPI-MS method. Lateral resolution of ~2 µm was demonstrated on an organic electronics device sample, comparable to that observed for elemental analysis by fs-LDI. It was also shown that lower laser fluence and longer photoionization time delay helps preserve the molecular ion. Thus the imaging experiment condition was guided by these results.

Chapter VI briefly studied fs UV MPI and compared it with VUV SPI. The results were roughly consistent with the literature. Higher fragmentation was observed in fs MPI but a fair amount of molecular ions are detected. Fs MPI also displayed less signal intensity compared to VUV SPI, at least with the current 267 nm generation setup which produces 40 µJ max.

In short, ultrafast laser pulses are very useful tool as either molecular or elemental probes. They are capable of micrometer scale lateral and depth resolution. The combination of fs laser desorption and VUV single photon ionization can produce molecular imaging MS at ~2 µm lateral resolution. Combined with depth profiling, they should ultimately allow 3D imaging MS.
APPENDIX

A. Technical Details of ChiMS

Event Driven User Interface (UI). The UI loop is where all user events are handled. It receives user input from the keyboard, calculates necessary parameters based on current instrumental conditions and passes them on to the hardware module cores. The user event loop generally does not engage in direct hardware communication. The user event loop also prevents UI lock-up that can occur while waiting for communication results. The user event loop can send an emergency stop directly to hardware, bypassing module cores, if so directed or in the case a certain module core is in a blocking condition.

All instrumental parameters are categorized in two groups, high level and low level. Controls (similar to the variables concept in text based programming language) on the front panel hold most instrument parameters, including both high and low level. When a high level control changes value, the user event loop will catch the event. It will then calculate and set the corresponding low level control values, and then generate low level user events. These low level user events are responsible for sending the low level instrument settings to the corresponding device module core. Later, these cores handle the actual hardware communication. For flexibility, the user can change low level controls directly and independently. This is a temporary solution which nevertheless leaves high level control values inconsistent with actual instrument parameters. However, this strategy need not modify the program and it allows rapid experimental prototyping.

General Aspects of High Level UI. The high level user interface of ChiMS plots the same data as two separate graphs with different binning options or scales (i.e., linear or
Graph display is limited to 20 frames/s to save CPU resources and because typical users cannot perceive faster frame changes. The size of graphs can also fit to the size of the program window to allow optimal viewing on large monitors. Two file saving options are available below the plots, recording and single spectrum saving. Laser power is displayed under the graph for the user to monitor laser system status and stability. Custom laser systems are common in many physical chemistry experiments, but such lasers are often quite complicated and prone to drift. The ability to monitor laser output, commonly simplified to just power/pulse energy, is quite important for the success of such experiments. On the right side of the front panel, tabs are used to separate user controls of different instruments or sessions. Each major component gets one tab and high level experimental parameters share one tab.

**Details of Digital Filters.** Several types of digital filters are incorporated into ChiMS: pseudo pulse counting, baseline electronic noise removal, and binning. Either pseudo pulse counting or baseline electronic noise removal is critical when working with low intensity signals. Binning was also implemented to improve graphical displays for signals processed through pseudo pulse counting and baseline electronics noise removal.

Pseudo pulse counting is used to simulate pulse counting, but is simplified for lower consumption of CPU resources. Instead of measuring the time of rising edge by constant fraction discrimination, the software simply set every data point to unity if it is above a preset threshold with all other data points set to zero. This convenient method mimics pulse counting, but suffers from degraded time resolution. It also introduces other artifacts such as reducing the dynamic range of 8- or 12-bit ADCs and ignoring multiple ion events within
short time windows. The estimated time resolution for pseudo pulse counting is \( \approx 2 \times \) the sampling period of the ADC.

Baseline electronic noise removal is used to remove electronic noise while preserving analog information. In this mode, each signal data point below a preset threshold is set as zero while all others are left intact. This removes baseline electronic noise while preserving multiple ion events with short time windows, preserving the full dynamic range of an ADC.

**Low Speed DAQ Cores and Auxiliary Instrument Control.** Low speed DAQ cores are auxiliary modules of ChiMS used for monitoring the analog output of various equipment such as laser power meters, ion optical potentials from high voltage power supplies, vacuum pressure gauges, and other non-time critical instrumental parameters. Several minimum viable control modules are also included for simple component tasks such as turning on/off lasers through serial communication. Currently supported devices includes two types laser energy/power meter, Spitfire delay generator II (Spectra-Physics) and Surelite laser (Continuum).

**File Saving Core.** ChiMS saves data in several mass spectrometry file formats. UIC text format consists of an instrument parameters header and signal as two column text for convenient of ASCII file handling. mzML format is implemented for data exchanging with other software, such as mMass. A stand-alone mzML format converter is also included in the package. Raw binary format is implemented for disk streaming of raw signal at the highest transfer rate for subsequent offline processing. Because the binary format is linked to the type definition of the signal segment, a binary file reader with simple version control is incorporated so that old data can be retrieved when signal segment data type is changed.
A typical usage of raw binary files is to allow the instrument to run in prototype mode without ChiMS having to slow down to run any of its data analysis cores.

**Program Configuration Loading/Saving.** Loading configuration files helps minimize misconfiguration that can occur in customized instruments while facilitating instrumental diagnosis and reducing experimental preparation time. Json (JavaScript object notation) is used for the configuration file format. Users can understand json strings without going through documentation, and modifying the configuration. A json for LabVIEW package is well designed and available on Lava.org free of charge. A small toolkit is also provided in the source code to generate a syntax-correct configuration file to avoid modifying the file by text editor.

**Auxiliary Toolkits.** Several auxiliary tools are included in ChiMS package. AnalysisTool is used for mass calibration, centroiding peaks, peak list extraction, and batch processing of text files. Simple statistics calculations with automatic determination of error bars can be performed by extracting instrument parameters out of filenames via regular expression pattern matching, then sorting data by a subVI. More complex batch processing can be done by modifying the data sorting subVI. Binary file viewer is used for individual viewing of spectra saved in binary raw file. File format conversion tool is available for generating BioMAP compatible files from raw binary file.

**DAQ Performance and Limitations.** Based on currently implemented repetition rate optimized routines high speed DAQ core in ChiMS, the repetition rate limitation also heavily dependent upon the type of communication bus. A common hardware limitation in many digital oscilloscopes is their data transfer throughput between a computer which can be limited by either use of the GPIB interface or inefficient implementation of a USB port.
(i.e., virtual COM port). While many high end digital oscilloscopes can easily collect data at rates at 5 GS/s and >250k waveform/s capture rate, they can only transfer those acquired spectra to a computer at rates of ~4 spectra/s (i.e., see datasheet of Tektronix oscilloscope http://www.tek.com/datasheet/mso5000-dpo5000/mso5000b-dpo5000b-series-datasheet-0). This significantly limits experiments with high repetition rate in pulsed mode. A traditional way to solve this problem is to sum/average spectra in onboard memory. While summing/averaging often solves the data transfer problem, it also loses any useful information from individual events. The ability to capture all spectra without averaging is extremely useful for application such as temperature-selected mass spectra in MALDI or ion mobility TOF.

Data throughput is critical in MS imaging and low repetition rates of some lasers (i.e., 10 Hz) require very long data acquisition times for an entire image. However, Ti:sapphire and other lasers allow imaging at rates of up to 5 kHz which would require a theoretical raw data throughput of ~0.5 GB/s. The limit of repetition rate is mostly determined by the combination of bandwidth and latency. Among the common communication buses, the decades-old GPIB standard is only capable of several MB/s and USB 2.0 is capable of 30-40 MB/s in practice. USB 3.0 has sufficient bandwidth, but similar latency as USB 2.0 which limits the repetition rate to ~300 Hz (without onboard memory for buffering), based on local testing (using the Analog Discovery card by Digilent).

The PCI bus does have sub-μs latencies which permit repetition rates >1 kHz, but the bandwidth is about only 100 MB/s, which means a long spectrum (>32 KS) cannot be used at high repetition rate. The latest PCI-e bus can provide bandwidth up to, but not
limited at 3.1 GS/s and sub-μs latency (see Gage brochure at http://www.gage-applied.com/digitizers/GaGe-Digitizer-CobraMaxCS-PCI-PCIe-Data-Sheet.pdf or Acquirs brochure at http://www.keysight.com/en/pc-1152071/high-speed-digitizers?nid=35502.0&cc=US&lc=eng). Manufacture supplied example code shows 8 kHz repetition rate is achievable in optimized C code (Gage Applied). Besides relying on a low latency bus, high repetition rates can also be achieved by using the digitizer’s onboard memory. This strategy will allow an old PCI bus digitizer to run at a >20 kHz trigger rate. However, the data transfer rate will still be limited by the PCI bus, which actually takes more time than actual data acquisition. In the end, the effective repetition rate is limited to the bandwidth of the PCI bus. Nevertheless, the latest PCI-e bus digitizers can match the data throughput from a 5 kHz laser and beyond.
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License Number: 3526181061400
Order Date: Dec 11, 2014
Publisher: AIP Publishing LLC
Publication: Review of Scientific Instruments
Article Title: Depth profiling and imaging capabilities of an ultrashort pulse laser ablation time of flight mass spectrometer
Author: Yang Cui, Jerry F. Moore, Slobodan Milasinovic, et al.
Online Publication Date: Sep 11, 2012
Volume number: 83
Issue number: 9
Type of Use: Thesis/Dissertation
Requestor type: Author (original article)
Format: Print and electronic
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Expected completion date: Mar 2015
Estimated size (number of pages): 150
Total: 0.00 USD

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