

Improving the Performance of Microscope Mass Spectrometry Imaging



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This thesis is dedicated to my mum who has always been and will always be there supporting me every step along the way since I was a toddler.

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Abstract

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Mass spectrometry imaging (MSI) is a powerful tool that provides mass-specific surface images with micron or sub-micron spatial resolutions. In a microscope MSI experiment, large sample surfaces are illuminated with a defocused laser or primary ion beam, enabling all surface molecules to be desorbed and ionised simultaneously before being electrostatically projected onto a position-sensitive imaging detector at the end of a time-of-flight mass analyser. Traditionally only the image of one mass-to-charge ratio can be obtained in a single acquisition, which limits its applicability. However, the development of event-triggered sensors, such as CMOS-based cameras, revives the microscope MSI method by allowing multi-mass imaging. Therefore, the challenges facing microscope have MSI shifted to improving its mass resolution, effective mass range, and mass accuracy. This thesis proposes effective solutions to each of them, and thus significantly improves the performance and applicability of microscope MSI.

To increase the mass range, two modified post-extraction differential acceleration (PEDA) techniques, double-field PEDA and time-variable PEDA, were used to demonstrate mass-resolved stigmatic imaging over a broad m/z range. In double-field PEDA, a potential energy cusp was introduced into the ion acceleration region of an imaging mass spectrometer, creating two m/z foci that were tuned to overlap at the detector plane. This resulted in two focused m/z distributions that stretched the mass-resolved window with $m/\Delta m \geq 1000$ to 165 Da without any loss in image quality; a range that doubled the 65 Da achieved under similar conditions using the original PEDA technique. In time-variable PEDA, a dynamic pulsed electric field was used to maximize the effective mass range of PEDA. By simultaneously focusing ions between 300 to 700 m/z using an exponentially rising voltage pulse, time-variable PEDA provides an effective mass range more than six times wider than the original PEDA method.

Although reflectrons are widely used to improve the mass resolving power of ToF-MS, incorporating them in a microscope MSI instrument is novel. A reflectron MSI instrument was designed and implemented. Simulations demonstrated that one-stage gridless reflectrons were more compatible with the spatial imaging goal of the microscope MSI instrument than the gridded

reflectrons. Preliminary experimental results showed that coupling the gridless reflectron with single-field PEDDA achieved a mass resolution above 8,000 $m/\Delta m$ while keeping a spatial resolution of 20 μm . In conclusion, the gridless reflectron was able to triple the mass resolving power without losing any spatial imaging power.

The poor mass accuracy hurdle was overcome by machine learning algorithms, which can construct clinical diagnostic models that recognise the peak pattern of biological mass spectra and classify them accurately without knowing the actual mass of each peak. After a proof of concept “experiment”, where the mass spectra of dye molecules were classified by various learning algorithms, three pairs of datasets (ovarian cancer, prostate cancer, chronic fatigue and their respective controls) were used to build classifiers that accurately distinguish blood samples from controls. Possible biomarkers were also discovered by evaluating the importance of each m/z feature, which may assist further studies.

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Chapter 1

Introduction

1.1 Overview

Mass spectrometry (MS) is a powerful analytical technique which is especially useful for identifying and quantifying unknown chemical species within complex environments as well as investigating the structural and chemical properties of a wide variety of molecules. Mass spectrometric analysis relies on the conversion of neutral molecules to gas-phase ions, which are separated based on their mass-to-charge ratios and detected commensurately with their abundance. The mass-to-charge ratio m/z , where m is the mass of an ion and z is its charge number, is by definition unitless. However, physically speaking mass units are defined as $u=1.660540 \times 10^{-27}\text{kg}$, and the charge z of the ion is q/e with q as charge and e as the elementary charge $1.6 \times 10^{-19}\text{C}$. In a mass spectrum, the x -axis typically corresponds to m/z , whereas the y -coordinate represents ion counts or relative abundances. Technically speaking, “mass-to-charge spectrum” would be a more correct term.

The sensitivity, speed, and universality of mass spectrometers have earned a prominent position for it in industry and academia. Life sciences are increasingly dependent on mass spectrometry for the analysis of proteins, peptides, and oligonucleotides [1]. It is also critical for pharmaceutical studies (drug discovery, combinatorial chemistry, pharmacokinetics, and drug metabolism) [2], as well as clinical medicine, including neonatal screening and haemoglobin analysis [3–5]. A myriad of useful bioinformation can be obtained with a mass spectrometer. For example, it is a fundamental tool for quantifying protein post-

translational modifications using m/z changes [6, 7]. In principle, high resolution mass spectrometers are capable of determining the formulas of small organic compounds by distinguishing the isotopes of the molecular ions [8]. Tandem mass spectrometers can elucidate the structure of biomolecules by mass selecting a parent ion, fragmenting it, and analysing the products [9–11].

Other analytical applications of mass spectrometry include forensic science (the analysis of explosives, hair, and controlled substances) [12], environmental sciences (monitoring pollutants in different media) [13], and geology (oil composition) [14]. It has also played an important role in a range of fundamental investigations in physics and chemistry, such as the studies of isotopes and the half-lives of radio nuclides [15], determinations of thermodynamic parameters [16, 17], molecular reaction dynamics [18, 19], and geochronology [20].

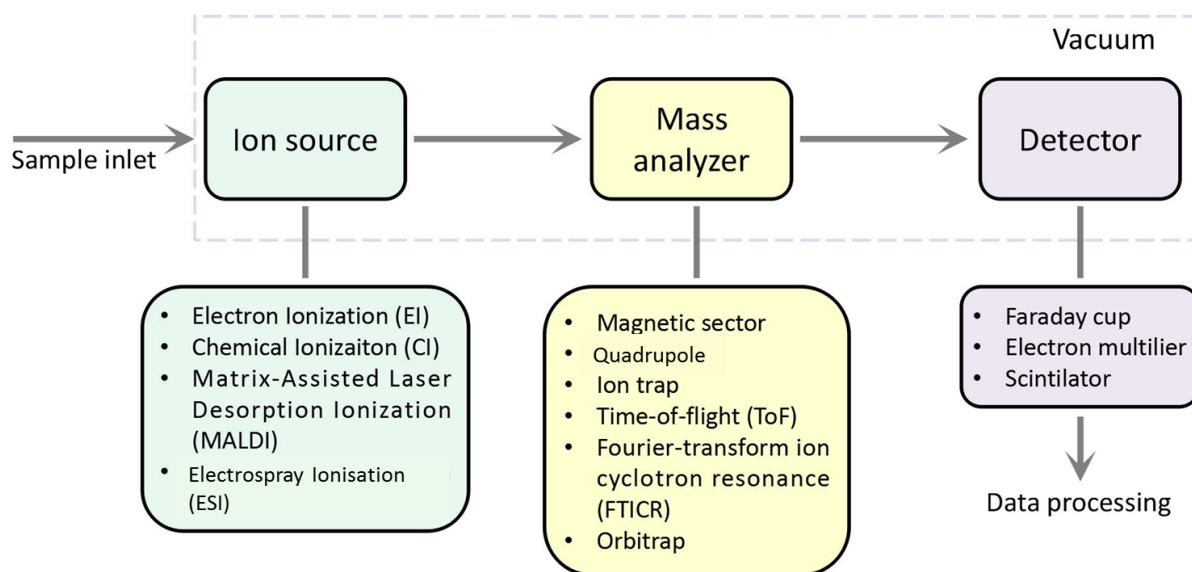


Figure 1.1: Basic diagram for a mass spectrometer: samples are ionised, separated according to their mass-to-charge ratio, and eventually counted by converting ion impacts into electrical signals for further processing.

The general setup of a mass spectrometer is given in Figure 1.1. Analytes are injected into the mass spectrometer and ionised by one of the methods listed in Figure 1.1. Electric or magnetic fields are then employed to manipulate the motion of the ions and isolate them according to their m/z . This process is called “mass analysis”. Sometimes, there may be more than one stage of mass analysis in a mass spectrometer: in such cases, a

precursor ion is typically selected by the first mass analyser before undergoes a spontaneous or induced fragmentation. Its product ions are then transmitted into a second mass analyser to perform further analysis. This is called tandem mass spectrometry, abbreviated MS/MS. The ions emerging from the mass analyser(s) are measured by a detector assembly that converts the abundance of the ions into readable signals. An oscilloscope or computer is generally used to process the signals and to display the results as spectrum. Conventionally, the ionisation sources, mass analysers, and detectors are kept within a high vacuum environment, but recently several ambient ionisation methods have been developed that enables ions to be generated under atmospheric conditions [21, 22].

As shown in Figure 1.1, there exist different types of ionisation sources, mass analysers, and detectors, some will be introduced in this chapter following a retrospective of mass spectrometry's development in Section 1.2. Mass spectrometry imaging (MSI) is a novel technique that uses mass spectrometry to map the spatial distribution of different chemical components over a sample surface. This thesis focuses on improving the performance of microscope MSI technique in different aspects such as mass resolution and mass range. Before the backgrounds of microprobe and microscope MSI are provided in Section 1.6, MALDI will be introduced in Section 1.3 because it is the ionisation source employed in the microscope MSI instrument. Also since ToF mass analyser is used in microscope MSI to differentiate ions based on their m/z , it will be described in Section 1.4. Various types of detectors will be introduced in Section 1.5 Then the ion optical aberrations that affect the spatial imaging capability of microscope MSI will be introduced in Section 1.7. The introduction chapter wraps up in Section 1.9 by offering a summary of the chapter and an outline of the entire thesis.

1.2 A brief history of mass spectrometry

Mass spectrometry is more than a century old and has contributed greatly to different scientific fields. German physicist Eugen Goldstein discovered positively-charged ion beams

known as anode or canal rays in 1886 [23,24]. His apparatus is shown in Figure 1.2(a). The cathode, which is at the middle of a glass tube, consists of a perforated flat disc and a metal tube. The anode is a wire placed above the cathode. When an electrical discharge passes through the residue gas at low pressure, luminous anode rays are generated and stream through the disc into the lower part of the vessel while the cathode rays travel in the opposite direction (see Figure 1.2(b)). Goldstein concluded that these anode rays are positively charged gas phase particles.

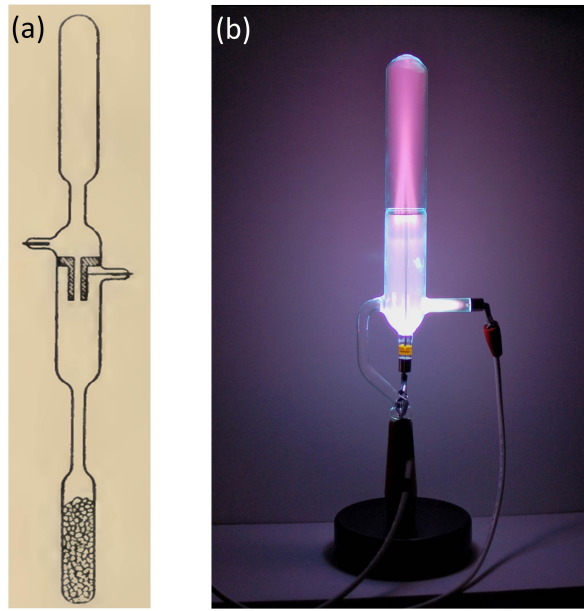


Figure 1.2: (a) Goldstein's anode ray apparatus: a gas-discharge tube with a perforated cathode (b) the anode rays passing through the perforated cathode cause a pink glow. It should be noted that the tube in (b) is actually upside down relative to (a). Adapted from ref. [23] and Wikipedia.

Inspired by Goldstein and other scientists, British physicist Sir J. J. Thomson constructed the “parabola ion spectrograph” at Cambridge University. This became known as the first mass spectrometer [23,24]. Thomson's apparatus is illustrated in Figure 1.3(a). Ions are created in a gas discharge cylinder and accelerated towards a cathode K . The cathode has a channel through it, inside which is an exceedingly fine-bore tube F that is fixed to collimate the ion beam. M and N are the poles of an electromagnet and P_1 and P_2 are parallel electrode plates. The ion beam therefore passes through combined magnetic and electric fields before striking a photographic screen S . As a result, the

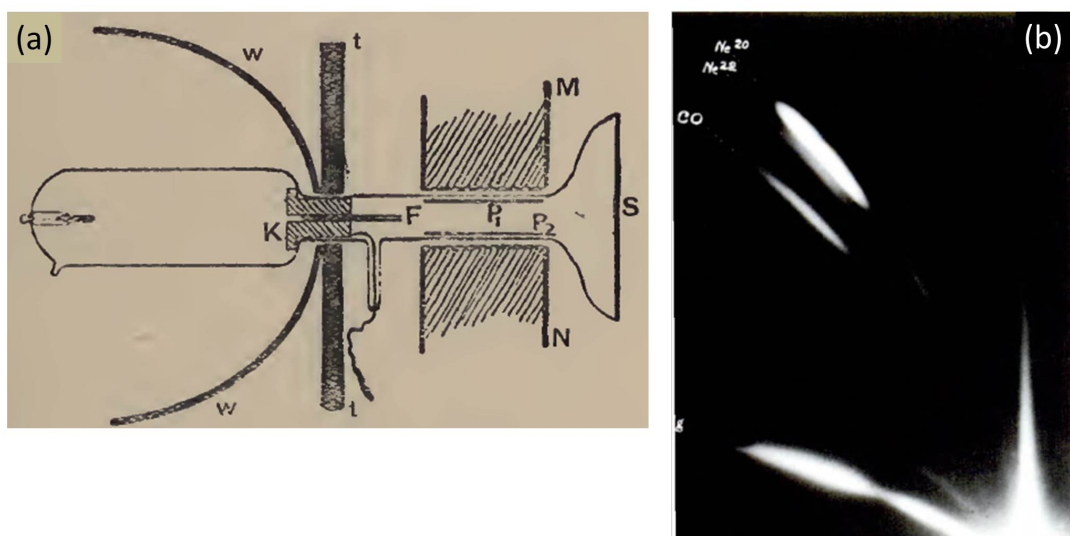


Figure 1.3: (a) Sir J.J. Thomson's "parabola ion spectrograph" apparatus and (b) the famous spectrum from 1913. In the upper left corner, the isotopes ^{20}Ne and ^{22}Ne are visible. Adapted from ref. [23].

mass spectrum collected with this instrument consists of a series of parabolas, as shown in Figure 1.3(b). Each parabola corresponds to one m/z . The kinetic energy of each ion of a particular m/z determines its position along the parabola. With this instrument, Thomson discovered isotopes 20 and 22 of neon in 1913, proving that stable elements were not all monoisotopic. From then on, mass spectrometers were widely used to investigate the isotopes of chemical elements and their abundance ratios [25,26].

In the late 1930s, Alfred Nier built a simple, low cost, yet high resolution mass spectrometer based on a sector field magnet [26]. In this apparatus, the analysed ion currents were measured with an electrometer tube, which revolutionised the photographic measurement techniques that had been widely employed in the earlier mass spectrographs.

In the 1940s, the demand for gasoline, synthetic rubber, and war materials encouraged petroleum and chemical industries to develop mass spectrometers for the analysis of hydrocarbon mixtures from petroleum. This created an alternative to the conventional chemical and distillation procedures which were often time-consuming, inaccurate [27], and required large amount of samples [28,29]. An early commercial mass spectrometer built in the 1940s is shown in Figure 1.4 (adapted from ref. [29]). In the Manhattan Project, a magnetic sector mass spectrometer called "Calutron" was used to enrich ura-

nium for the nuclear bomb “Little Boy”, which was then dropped onto Hiroshima on August 6, 1945 [30].

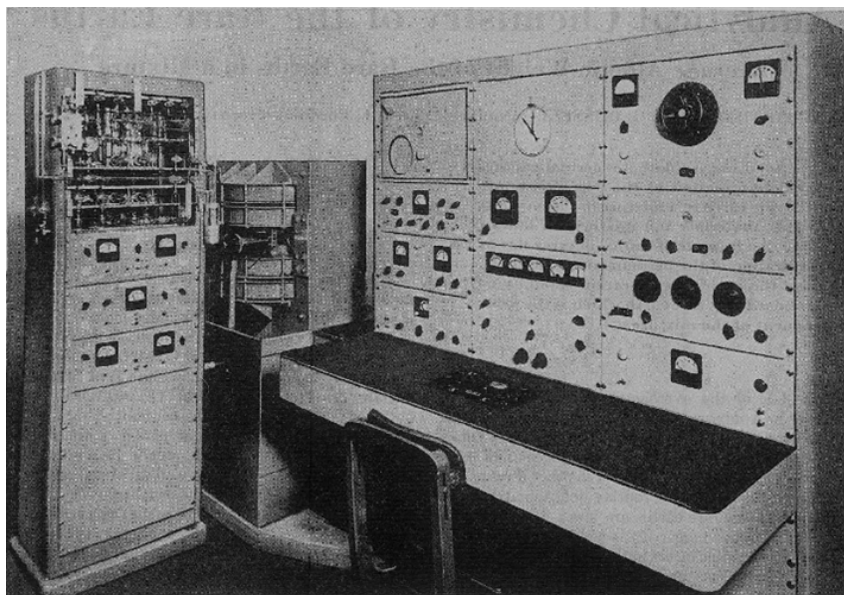


Figure 1.4: An early commercial model of a mass spectrometer, manufactured by the Consolidated Engineering Corporation, Pasadena, US, in 1943. In the centre background, an electromagnet that creates a uniform magnetic field is used to separate ions based on their m/z . The sample inlet system and evacuation apparatus locates on the left to the electromagnet. The operators table and the cabinet that encloses the automatic recording apparatus and power packs are on the right. Reprinted from ref. [29].

The applicable scope of mass spectrometry was considerably extended after being coupled with gas chromatography (i.e., GC-MS) in the late 1950s [31]. Complex mixtures of chemicals could then be separated as a gas chromatogram based on their interaction with the coating of a capillary column, and quantitatively determined by a mass spectrometer. Applications of GC-MS include drug detection, forensic science, environmental monitoring and cleanup, as well as astrochemistry (such as the component analysis of soil samples collected during Mars missions [32]). Progress in mass analysers, such as the advent of quadrupole and ion trap MS in 1953 [33], time-of-flight (ToF) MS in 1955 [34], and Fourier transform ion cyclotron resonance (FTICR) in 1974 [35], further broadened MS applications (more details on mass analysers will be provided in Section 1.4).

During the 1970s, harsh ionisation techniques such as Electron Impact (EI) were used in most mass spectrometers. These were generally too energetic and would break up

large biomolecules (such as proteins, nucleotides, complex carbohydrates) into smaller fragments. As a result, for a long time only low mass compounds (typically < 1000 Da) that were sufficiently volatile and thermally stable could be handled by commercially available mass spectrometers with good sensitivity.

In response to the need for studying complex biological substances, the 1980s saw the emergence of “soft ionisation” techniques, including fast atom bombardment (FAB), field desorption (FD), electrospray ionisation (ESI), and matrix-assisted laser desorption/ionisation (MALDI). The last two techniques, MALDI and ESI, proposed by Tanaka in 1988 [36] and Fenn in 1989 [37], respectively, were hailed as significant breakthroughs for mass spectrometry in the life sciences. Over the past decade, the development of new ionisation methods together with the improvements in mass analysers have been the primary driving force behind the remarkable growth of biological applications of mass spectrometry. Today, samples can be analysed in solid, liquid, or gas states at concentrations in the attomolar range (10^{-18}) [38, 39]. Developments in sample separation (HPLC-MS and CE-MS), ion fragmentation techniques (MS/MS), and bioinformatic tools, enable unprecedentedly accurate identification and characterisation of biological molecules across different medical and bio-related disciplines [40–44].

The importance of mass spectrometry can also be seen from the fact that a number of scientists who contributed to the birth and development of mass spectrometry (Thomson in 1906, Wien in 1911, Aston in 1922, Paul in 1989, Fenn in 2002, and Tanaka in 2002) or used mass spectrometry to aid important discoveries (*e.g.*, the 1996 chemistry award for the discovery of the nanomaterial buckminsterfullerene by Curl, Kroto, and Smalley) have been awarded Nobel prizes. Mass spectrometry is a very active research area undergoing constant improvement and refinement, where fascinating stories are taking place every day.

1.3 Ion sources

As pointed out above, the ion source is an important component of a mass spectrometer where neutral analytes are vaporised and ionised prior to entering a mass analyser. A variety of ionisation techniques are currently in use, which can be categorized into two groups, ‘hard’ and ‘soft’, based on the amount of internal energy transferred to the analytes during the ionisation process. Hard ionisation techniques supply excessive energy to the analytes and lead to extensive fragmentation, whilst soft ionisation techniques transmit minimal energy to them and produce molecular ions.

As one of the most traditional ionisation methods developed for mass spectrometry, electron ionisation (EI) is a hard ionisation technique. A high energy beam of electrons is issued from a heated filament and electrostatically accelerated towards the sample. When a collision happens between a high energy electron and a gaseous molecule, one or more electrons of the molecule are knocked away leaving behind a molecular ion. The molecular ion may split into neutral pieces and smaller fragment ions due to the excessive energy from the collision, which limits the detection efficiency of the molecule. Chemical ionisation (CI) is a softer ionisation method, which uses the primary ionisation of a gaseous molecule, such as methane, to protonate the analytes. Nevertheless, both EI and CI are limited to gas-phase ionisation and only suitable for analytes that are volatile, thermally stable, and of small masses (< 1000 Da).

Electrospray ionisation (ESI) is one of the soft ionisation methods capable of ionising non-volatile, labile, and large biological molecules [37]. During ESI, a solution containing analyte is infused into the ion source through a capillary which is held at high voltage. An aerosol of charged droplets is emitted that gradually shrinks in size due to evaporation of solvent. Eventually, ions will be created as a result of Coulombic repulsion. A detailed description of the ESI process will be given in Chapter 3 as part of an introduction to a home-built electrosprayer used for sample preparation. ESI has a very high sensitivity that allows detection of analytes at picomolar scale. Also, ESI can create multiply charged

species of biomolecules, allowing very large molecules appear at relatively lower m/z .

A new ionisation technique based on ESI, called Desorption Electrospray Ionisation (DESI) is rapidly gaining popularity. DESI is an atmospheric pressure ionisation method described for the first time in 2004 by Takats and co-workers [21]. In DESI, an aerosol of charged droplets created by ESI impinges the sample surface, from which small organic molecules and macromolecules are extracted, ionised, and transported into the inlet of a mass spectrometer as desolvated ions.

1.3.1 Matrix-Assisted Laser Desorption/Ionisation (MALDI)

MALDI is another dominating soft ionisation technique. It was developed independently but concurrently with ESI. MALDI has been widely used to detect intact biomolecules such as proteins, peptides, oligonucleotides, synthetic polymers and large inorganic compounds.

Unlike ESI, which uses solvated samples, MALDI samples are introduced into the ion source in the solid phase. The analytes are first dissolved in an appropriate solvent and blent with an excessive amount of small, laser-absorbing organic molecules, creating a so called matrix. The mixture is then deposited on a substrate and air-dried. As the liquid solvent evaporates, the analytes co-crystallise with matrix. In typical MALDI sample preparation, the molar ratio of analyte to matrix is between 10^{-2} (small analyte molecules) and 10^{-4} (large analyte molecules). Analyte molecules are incorporated into matrix crystals in a homogeneous distribution and completely isolated from each other. The analyte-doped matrix crystal is then brought into the vacuum environment of the ionisation source. The next step of MALDI is ablation/desorption, where a bulk volume of the sample is removed by laser. UV lasers including N_2 lasers ($\lambda = 337$ nm) and Nd:YAG lasers ($\lambda = 266$ or 355 nm) are commonly employed in MALDI. IR lasers like Er:YAG lasers ($\lambda = 2.94$ μm) or CO_2 lasers ($\lambda = 10.6$ μm) are also used. Typical laser fluences for UV-MALDI are around 20 mJ/cm^2 with a few nanosecond pulse duration (IR-MALDI usually needs one magnitude higher laser fluence than UV-MALDI). This corresponds to

a very high density of excitation energy, sufficient to break all of the intermolecular bonds, which leads to ablation of the excited crystal volume.

The underlying mechanism that enables the charged matrix and analytes to be formed in MALDI is still under debate. A number of models have been proposed:

(1) Photo-ionisation model [45, 46]: Analytes initially exist in the matrix as neutral molecules. During the primary ion formation process, matrix photo-ionisation occurs through photon absorption and efficient energy pooling between excited neighboring matrix molecules. A plume is produced by laser ablation, where gas phase proton transfer or electron transfer occurs between analytes and matrix molecules. Analyte ions are therefore only generated in the secondary ion formation process.

(2) “Lucky survivor” [47]: Analytes are incorporated into the matrix crystals in a partially solvated form, so they retain their solution charge state and carry a positive excess charge that is balanced by counter ions (typically trifluoroacetate or matrix anions). During laser ablation, the crystal lattice breaks up into small clusters upon desorption. It is statistically impossible that there is a positive or negative charge in some of these clusters due to a deficit or excess of counterions. As the plume expands, neutral matrix and solvent molecules are released from the clusters along with counter ions, which become free acids or bases after they recombine with analyte (de)protonation sites. Most of the analyte charges are neutralised in this process, except in ‘lucky’ cases with the remaining excess charge.

(3) Thermal proton transfer [48, 49]: Photon energy becomes thermal energy, leading to a rapid increase of the temperature in the laser-irradiated volume. As the temperature rises, the sample starts to melt before desorption happens. Thermally-induced proton transfer reactions then take place in the liquid phase before the matrix and analyte molecules are desorbed from the liquid as both neutrals and ions to the gas phase. As the laser plume expands into space, the concentrations of these chemical species drops quickly and the reactions slow down and eventually stop.

There are other models that were proposed to explain the ionisation process of MALDI

as well, such as those described in ref. [50–55]. Each model has its own merits in explaining observed phenomenon in experiments and it could be that some if not all of these processes contribute to the production of analyte ions to some degree.

Since the mechanism of MALDI has not yet been fully elucidated, choosing a correct matrix for a specific analyte is empirical. There are some common requirements for a good matrix candidate, such as strong absorbance at the laser wavelength, mass that does not overlap with analyte, vacuum stability, or ability to promote analyte ionisation, solubility, and a lack of chemical reactivity. A list of common UV-MALDI matrices is given in Table 1.1.

Analyte	Matrix abbreviation
Peptides/proteins	CHCA, DHB, SA
Oligonucleotides	THAP, HPA
Carbohydrates	CHCA, DHB, THAP
Synthetic polymers	IAA, DIT, DHB
Organic molecules	DHB
Inorganic molecules	DCTB
Lipids	DIT

Table 1.1: Commonly used UV-MALDI matrices (CHCA = α -cyano-4-hydroxycinnamic acid, DHB = 2,5-dihydroxybenzoic acid, SA = sinapic acid, THAP = 2',4',6'-trihydroxyacetophenone monohydrate, HPA = 3-hydroxypicolinic acid, IAA = trans-3-indoleacrylic acid, DIT = dithranol, DCTB=trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile). Adapted from ref. [1].

MALDI-MS has a very low detection limit, which can reach attomolar concentrations. Compared with ESI, MALDI is more tolerant to the modest presence of contaminants. Moreover, MALDI usually forms singly charged species, which greatly facilitates the identification of the molecular ions of unknown compounds. However, MALDI suffers from high shot-to-shot variation and strong dependence on the sample preparation protocol. In practice, the quality of MALDI spectra can be considerably affected by where the laser beam ablates the sample and there are so called “sweet spots” on the sample that produce an optimal spectrum [56].

1.4 Mass analyser

The mass analyser, where ions are differentiated according to their m/z values, is the heart of a mass spectrometer. Similar to ion sources, there are many types of mass analysers available today and they function based on different principles, which leads to different sizes, prices, and performance. Although in the early days of mass spectrometry, magnetic fields were commonly employed, currently the majority of mass spectrometers rely on the creative usage of static or dynamic electric fields.

Table 1.2 compares the characteristics of eight most widespread mass analysers (the quadrupole ion trap, ToF, ToF reflectron, Qq-ToF, magnetic sector, FT-ICR, and Orbitrap). The most important parameters in the table are mass accuracy, mass resolution, and mass limit. Mass accuracy gauges the discrepancy between the actual m/z of an ion and the value measured by the mass analyser, which is usually presented in parts-per-million (ppm). Mass accuracy is to a large degree determined by the stability and mass resolution of the analyser. The mass resolution, or mass resolving power, of a given ion signal is defined as the mass spacing at which neighbouring spectral peaks can be unambiguously separated from each other. Very often the mass resolution is measured as $m/\Delta m$ where Δm is the width of a mass peak at its half maximum. A detailed discussion of mass resolution and mass accuracy will be given in Chapter 2 and Chapter 3. The mass limit characterises the upper bound of m/z that can be effectively measured. Other important descriptive parameters of a mass analyser include its sensitivity, detection limit, and S/N ratio, but these parameters often vary significantly depending on the sample. Each analyser has its own advantages and disadvantages, so hybrid mass spectrometers including various types of analysers have been constructed to achieve higher performance.

Although an elaborated description of all these mass analysers and their hybrids is beyond the scope of this chapter, the principle of quadrupole and ToF analysers will be briefly introduced.

A quadrupole consists of four parallel cylindrical electric rods arranged in the form of

	Quadr -upole	Ion trap	ToF	ToF refle -ctron	Qq -ToF [57]	Magn -etic	FTICR	Orbitrap
Mass limit	4000	6000	$> 10^6$	10,000	66,000	20,000	30,000	50,000
Resol -utions (at 1000 Da)	2000	4000	5000	20,000	$< 10^4$	10^5	5×10^5	10^5
Accuracy (ppm)	100	100	200	10	≈ 5	< 10	< 5	< 5
Ion sampling	Pulsed	Conti -nuous	Pulsed	Pulsed	Pulsed	Conti -nuous	Pulsed	Pulsed
Pressure (Torr)	10^{-5}	10^{-3}	10^{-6}	10^{-6}	10^{-7}	10^{-6}	10^{-10}	10^{-10}

Table 1.2: Comparison of common mass analysers. Adapted from ref. [1].

a square. A DC potential U_{DC} is applied to a pair of opposing rods with an AC radio-frequency (rf) component U_{AC} of angular frequency w ($U_+ = U_{DC} + U_{AC} \cdot \cos wt$). On the other pair of rods, the DC component is negative and the AC component is 180° out-of-phase ($U_- = -U_{DC} - U_{AC} \cdot \cos wt$). When a positively charged ion enters the space inside the rods and moves along the central axis of the quadrupole assembly, it will be drawn towards a negatively charged rod. But the rod will change its polarity before the ion collides with it and then the ion switches its direction and moves towards the central axis. Consequently, ion beams will travel down the axis following a helical trajectory. For a combination of U_{DC} , U_{AC} , and w , only those ions of a particular m/z (also known as “resonant ions”) can reach the end of the quadrupole and end up at the detector. All other ions (“non-resonant ions”) will strike one of the rods and be neutralised. Quadrupoles serve as filters for ions within a narrow range of m/z . By scanning U_{DC} and U_{AC} , one can collect ions with different m/z when the quadrupole allows their transmission. The upper mass limit of a quadrupole system is mainly determined by the maximum amplitude of the rf voltage U_{AC} [57].

1.4.1 Time-of-flight analyser

The idea of time-of-flight (ToF) mass spectrometry was proposed in 1946 [58] and revived at the end of 1980s. Compared with other mass analysers, the principle of ion separation in a ToF analyser is incredibly simple, and only relies on a 1–2 m field-free flight tube. The layout of a ToF system is illustrated in Figure 1.5. At the beginning of an experiment ($t = 0$), ions are produced from a sample source, typically using a pulsed laser. The ions are accelerated along the ToF axis by the strong electric field between the sample holder and a grounded grid. Ions of different mass-to-charge ratios will therefore enter the field free flight tube with the same kinetic energy and at roughly the same time. Therefore, the ions will then separate according to their velocities and arrive at the detector located at the end of the drift tube at different times. Ions of larger m/z arrive later than those of smaller m/z .

The m/z value of an ion is therefore determined by measuring its flight time in the field-free drift region. The equations that correlate m/z with total time-of-flight (t_f) can be derived as follows

$$PE = qU_{rep} = zeU_{rep}, \quad (1.1)$$

where PE is the electric potential energy of the ion, and U_{rep} is the high voltage applied on the sample holder. The kinetic energy of the ion KE is

$$KE = \frac{mv^2}{2}. \quad (1.2)$$

Since $KE = PE$ at the entrance of the field-free region, the velocity of the ion leaving the source v is

$$v = \sqrt{\frac{2zeU_{rep}}{m}}. \quad (1.3)$$

In the field-free drift tube, the ion travels towards the detector with a constant velocity

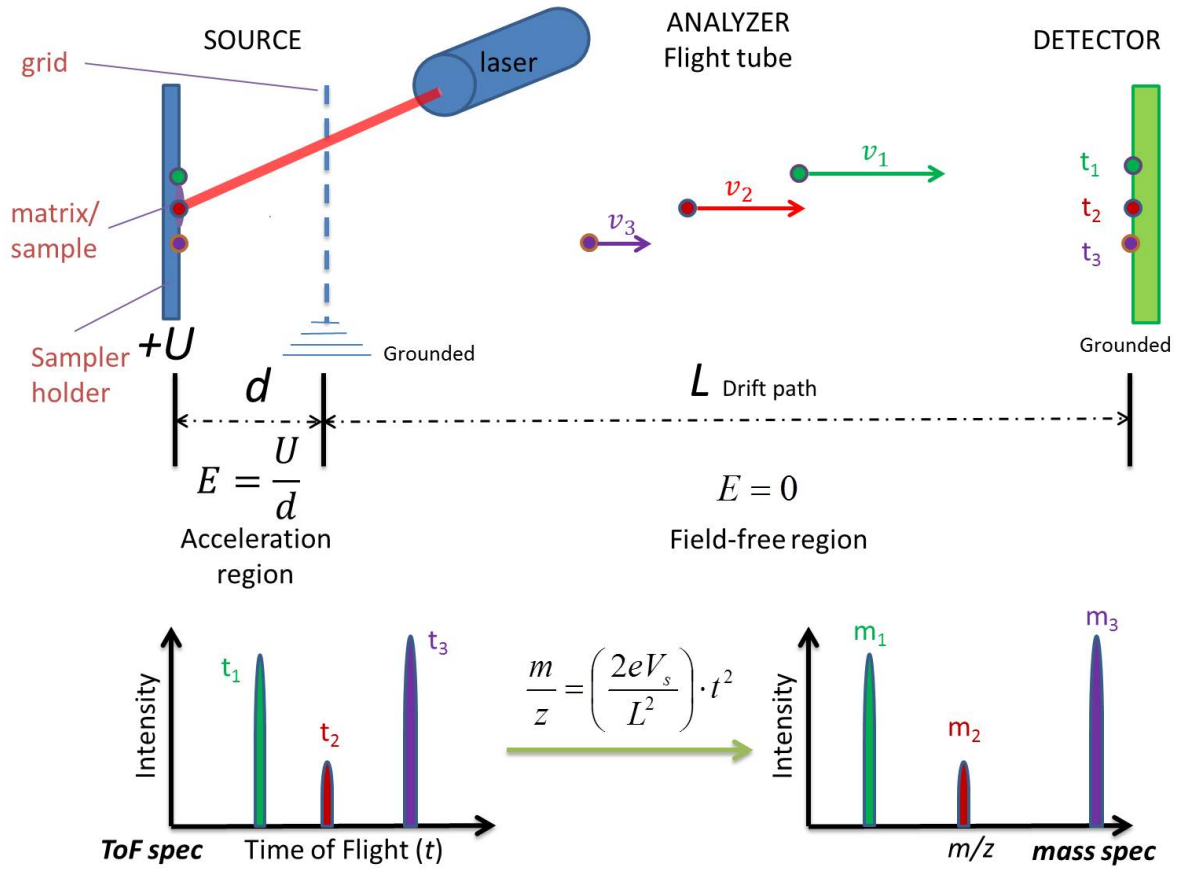


Figure 1.5: Illustration of ToF-MS. Ions of different m/z will travel at different velocities in the flight tube when accelerated by the same electric field. Lighter ions will arrive earlier than heavier ions. The ToF spectrum of each ion can be converted into mass spectra through a calibration function.

along the ToF axis. If the distance of the drift path is L , then the total time-of-flight t is

$$t = \frac{L}{v}. \quad (1.4)$$

Substituting v with Equation (1.3), one has

$$m/z = \frac{2eU_{rep}t^2}{L^2}. \quad (1.5)$$

What one collects experimentally is a spectrum in which the ion current signal is plotted against arrival time at the detector (as shown in Figure 1.5). Mass spectra are obtained using Equation (1.5) as a calibration function to convert flight times to m/z . More details about calibrating a ToF mass spectrometer will be provided in Chapter 3. In

principle, no matter how heavy an ion is, a ToF analyser is able to distinguish its signal as long as it is produced at the ion source. This means there is no upper mass limit to ToF mass spectrometer. As a result, ToF systems are ideally suited to soft ionisation sources, especially MALDI sources where very narrow ion packets are generated with short laser pulses. Moreover, since one does not need to scan over a wide mass range to build a mass spectrum, the analysis speed of a ToF system is significantly faster than a quadrupole. ToF analysers also feature high transmission efficiencies and therefore high sensitivities.

An expression for mass spectrometer resolution can be obtained by differentiating both sides of Equation (1.5):

$$\frac{1}{z}dm = \left(\frac{2eU_{rep}}{L^2}\right)2tdt. \quad (1.6)$$

By rearranging Equations (1.5) and (1.6), one has

$$\frac{m}{dm} = \frac{t}{2dt}. \quad (1.7)$$

The mass resolution R of a ToF mass spectrometer can be expressed as follows

$$R = \frac{m}{\Delta m} = \frac{t}{2\Delta t}, \quad (1.8)$$

where m and t are the mass and flight time of the ion respectively, and Δm and Δt denote the widths measured at half maximum of the mass and ToF peaks. Besides the response time of a detector, the source of the flight time spread Δt is primarily a distribution of initial kinetic energies when ions are desorbed in a plume from the sample surface.

In order to improve the mass resolving power of a ToF instrument, one may try to enlarge t by lengthening the flight path L or using a low U_{rep} . However, both methods come at a cost of sensitivity. Besides, increasing t also increases Δt . In practice scientists have developed techniques to decrease Δt instead.

The total kinetic energy of an ion is the sum of its initial energy U_0 and the energy imparted by U_{rep} . For a given ion, the distribution of initial kinetic energy will lead

to a distribution of ion velocity v in the field free drift path, which broadens Δt and degrades the mass resolving power of the system. Modern ToF mass analysers address this issue with a pulsed-ion extraction method called “delayed extraction” (DE) [59, 60]. The mechanism of DE is illustrated in Figure 1.6. In a DE-ToF system, ions are formed

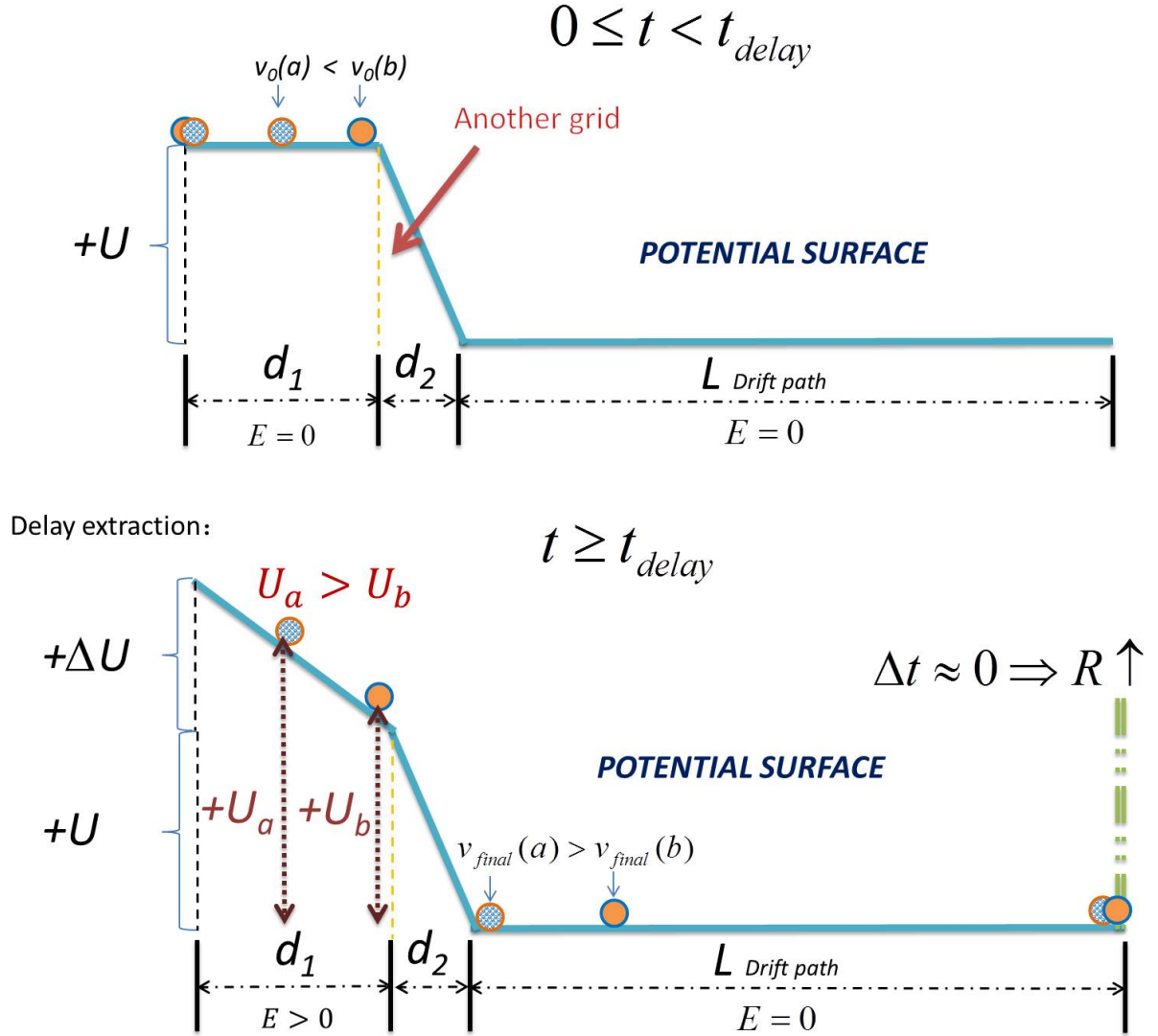


Figure 1.6: Illustration of DE. The ToF spread (Δt) due to the initial velocity distribution of an m/z can be compensated for using a pulsed potential $+\Delta U$ on the repeller at t_{delay} .

in a weak initial electrical field. For a given m/z , ions with greater U_0 will be closer to the grounded electrode than slower ones at the moment when a high voltage pulse is applied to the sample holder. After the electrical field is switched on, the slower ions will gain more kinetic energy than the faster ones, so the initial kinetic energy difference is corrected.

When the ions enter the field-free drift path, the originally slow ions will be traveling faster than the originally fast ions and eventually catch up with them at the temporal focus, the specific position of which can be controlled by changing the pulse potential and timing. If the detector plane overlaps with the temporal focus, the mass resolution will be improved by nearly a magnitude. Unfortunately, DE has a few drawbacks. The first is the mass dependency of the time-of-flight focusing effect of DE, which means the improved mass resolution can only be obtained for a narrow mass range. One has to properly adjust the parameters of DE, such as the pulsed potential or the delay time, to switch the optimal mass range to cover the targeted analyte ions. The mass resolution enhancement by DE also gradually degrades above $m/z = 30,000$ due to peak broadening caused by fragmentation and adduct ion formation [61]. Moreover, since the use of DE considerably complicates the flight time equations, higher-order polynomials, along with more reference mass peaks, are needed to calibrate an instrument.

Another method to compensate the initial velocity/kinetic energy distribution of ions generated by MALDI is the reflectron, which is an ion mirror that reflects the ion trajectories using a static electric field. More details regarding reflectrons will be presented in Chapter 5.

1.5 Detectors

A detector is an essential component of mass spectrometers, where ions are counted after they are separated by the mass analyser. Incident ion beams impact the detector and generate signals by inducing electric currents. Common detectors include the Faraday cup, the electron multipliers, and the microchannel plates (MCPs). The Faraday cup [1] is a simple metal cup, where secondary electrons will be ejected when it is impacted by ions of high kinetic energies. The number of ions striking the cup can be measured using the flow of electric current due to the production of electrons. A electron multiplier [1] is a series of dynodes. When an incident charged particle hits the first dynode, more than one

secondary electrons will be emitted and therefore trigger a repeated secondary emission along the string of dynodes, amplifying the ion signal significantly. Compared with the Faraday cup, the electron multiplier offers an exceptionally high gain and sensitivity.

A MCP has a large array of 5–20 μm diameter channels. When a large positive potential difference ($\approx 1\text{ kV}$) is applied between the front and back faces of the MCP, each channel can be seen as a electron multiplier with a “continuous dynode” and each incident ion is converted into a shower of electrons. By coupling the MCP with an two-dimensional organic scintillator, the positional information of each ion strike can be conserved: due to the confinement of the channels, a group of electrons created by the same ion strike lights up a smaller area on the scintillator and recorded by a CCD camera. This detector assembly is especially useful in velocity map [19] and spatial map imaging experiments where both ToF and positional information are required. Compared to the detection methods that directly accelerate the ions towards scintillators, there are mainly three advantages of using the MCPs [62]: (1) the ion signal is significantly amplified, (2) the scintillator screens have much longer lifetime because electrons cause much less damage than ions of comparable energy, and (3) the organic scintillators have approximately one order of magnitude larger conversion efficiency for an electron than for a heavy positive ion.

Traditionally, in an experiment, the CCD camera is time-gated on one ToF peak and acquires an ion image corresponding to one single m/z . Recently, however, the development of revolutionary fast sensors [63], such as the delay-line anode, Timepix, and PImMS, allows the ToF and the position of each ion event for the entire m/z spectrum to be simultaneously obtained.

For the delay-line detector [64], electrons emitted from the back face of the MCP stack are detected directly without the scintillator. When an electron cloud hits the wire that serpentine along the x and y axes of the anode, the resulting current signal propagates in two directions along the wire before reaching the time-to-digital converters at each end. The runtime difference at each end is used to calculate the position of the original impact.

Therefore, the delay-line detector can measure both the arrival time and position of an ion event. The dead time between successive measurements is 10–100 ns depending on the number of anodes incorporated. However, the delay-line anode is limited by its low count rate, which allows just one (or a few) particles to be resolved simultaneously.

TimePix [65] and PImMS [63] are both CMOS (complementary metal-oxide semiconductor) cameras and their basic operating principles are similar. Each pixel of the TimePix and PImMS sensor contains one or more counters, which operate independently, time stamping each individual event.

In the case of the TimePix sensor which has 256×256 pixels [65], there are three main operation modes of its pixel: (i) event counting mode, where the number of incident particles is counted; (ii) ToF mode, where the time-of-arrival of an incident particle is recorded; or (iii) time over threshold mode, where the duration in which an event remains above a predefined threshold is stored. For time-resolved imaging applications, the TimePix sensor should work in mode (ii) in which time-stamping is performed with a precision up to 1.56 ns.

Pixel Imaging Mass Spectrometry (PImMS) is a fast, time-stamping, multi-hit pixel sensor [63]. There are 72×72 pixels in the first generation PImMS sensor and 324×324 pixels in the second generation. Each pixel has four 12-bit time-stamp registers, which means it can perform multi-mass imaging in a single ToF acquisition cycle. The PImMS sensor is capable of operating with a time precision of 12.5 ns, which corresponds to a frame rate of 80 Mfps with a conventional camera. The advantage of a time-stamping detector is avoiding the storage of a huge amount of null data which is usually useless. Rather than capturing whole frames like CCD cameras, PImMS sensor only stores ion events if a predefined threshold is exceeded by the signal on individual pixels.

The motivation for this thesis, which focuses on microscope mode imaging, is all driven by the multi-mass imaging sensors now becoming available. The application of the PImMS camera in microscope MSI will be introduced in more details in Section 1.6 of this chapter and the detector assembly used in experiments will be described thoroughly in Chapter

3.

1.6 Mass spectrometry imaging

Mass spectrometry imaging (MSI) is widely used to obtain mass-specific surface images with micron or sub-micron spatial resolutions [66–69]. Since its maturation in the late 1990's, MSI has frequently been coupled with MALDI [45], secondary ion mass spectrometry (SIMS) [8], and DESI [21] to generate and image ions from a wide range of samples. Its unique capability of correlating chemical information to a spatial coordinate on a sample has led to the uptake of MSI in omics-based life sciences, where it has been applied to image polymers, metabolites, and other biomarkers [70–75]. Figure 1.7 shows an example image obtained by MSI of lipids contained in a $13 \times 10 \text{ mm}^2$ area of rat brain tissue [76].

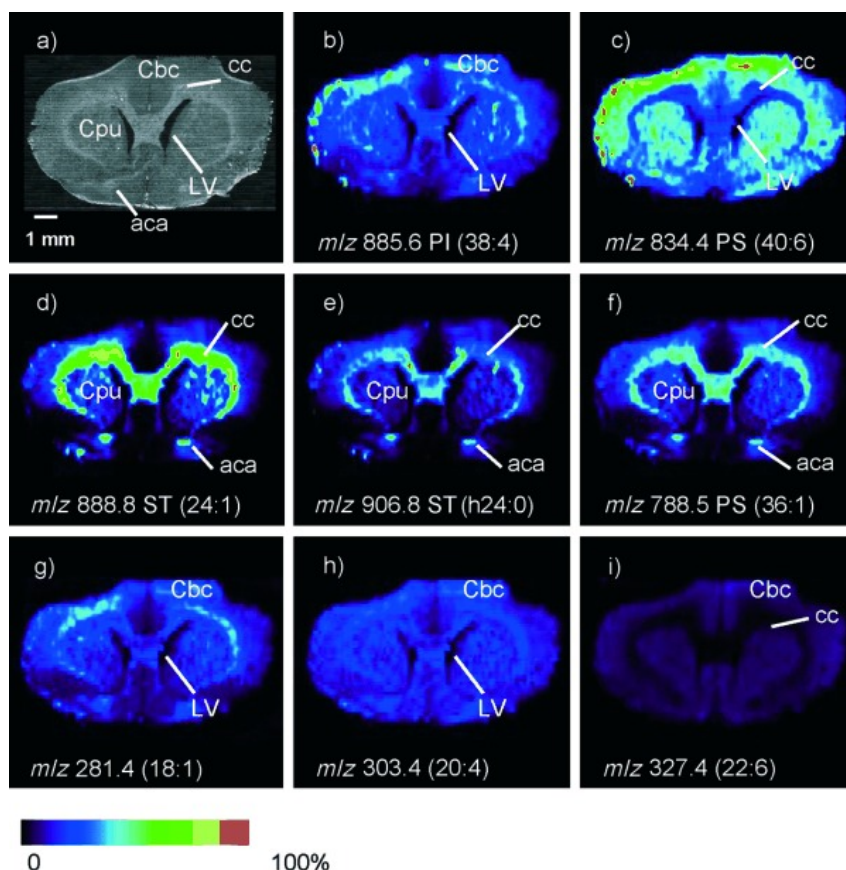


Figure 1.7: Selected molecular ion images of lipids in a $13 \times 10 \text{ mm}^2$ area of rat brain tissue obtained by DESI MSI. Adapted from ref. [76].

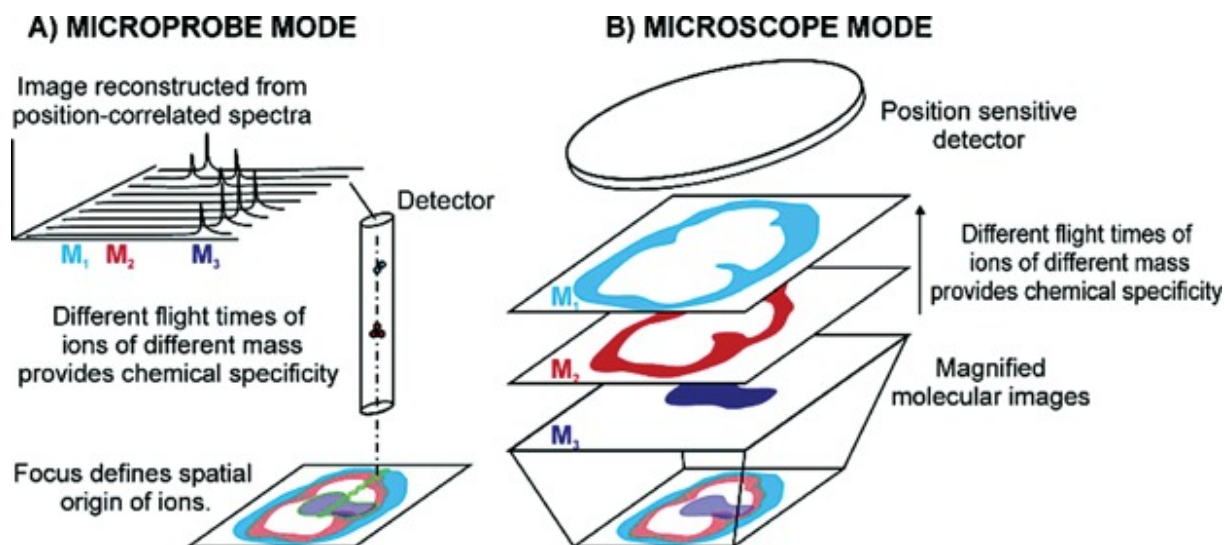


Figure 1.8: Examples of the two modes of MSI. In microprobe MSI (A) mass spectra are acquired from an array of designated positions to reconstruct a molecular image pixel by pixel. Microscope iMSI (B) directly collects magnified images of the ion distributions using a position sensitive detector. Reprinted from ref. [77].

Contemporary imaging mass spectrometers are typically microprobe instruments where a focused laser, primary ion beam, or electrospray source is used to desorb and ionise molecules at a surface spot. Microprobe MSI is illustrated in Figure 1.8(A), mass spectra are recorded with their locations by raster scanning the probe across the entire surface, enabling ion images of analytes (such as drugs, lipids, peptides, proteins) to be reconstructed by plotting the intensity of specific ions at different probe positions. Developments in mass analysers can be directly applied to MSI instruments to strengthen their mass resolution, mass accuracy, sensitivity, and other capabilities. Moreover, unlike other visualisation techniques (*e.g.* fluorescence, radioimmunoassays and others), labeling is usually not required in MSI experiments. Like other scanning imaging techniques such as electron microscopy [78], the image quality or spatial resolution of MSI is determined by the size of the probe spot. The consequent drawback of this approach is that the time required to collect an image is inversely proportional to the area of the probe beam. Analysing a sample with high resolution is therefore time consuming and may take several hours. This could be problematic if the sample degrades over time. Table 1.3 characterises the typical parameters of the three major MSI ionisation techniques (SIMS, MALDI, and DESI) as

well as other techniques that are mostly derivative.

	Ionisation source	Type of ionisation	Analytes	Spatial Resolution	Mass range
SIMS	Ion gun	Hard	Elemental ions, small molecules, lipids	50 nm – 10 μ m	0 – 1000 Da
DESI	Solvent spray	Soft	Small molecules, lipids, peptides	100 μ m	0 – 2000 Da
nano-DESI	Solvent bridge	Soft	Small molecules, lipids, peptides	10 μ m	0 – 2000 Da
MALDI	UV Laser beam	Soft	Lipids, peptides, proteins	2.5 – 20 μ m	0 – 10 ⁵ Da
LAESI	mid-IR Laser beam, solvent spray	Soft	Small molecules, lipids, peptides, proteins	200 μ m	0 – 2000 Da
LA-ICP-MS	UV Laser beam	Hard	Elemental ions	10 – 100 μ m	0 – 250 Da

Table 1.3: Comparison of typical parameters among MSI techniques. Adapted from ref. [79]

Microscope MSI has the potential to record well-resolved ion images faster than microprobe methods. In this approach, sample surfaces are illuminated with a defocused laser or primary ion beam, enabling all surface molecules to be desorbed and ionised simultaneously. [80–82] The spatial distribution of ions is then electrostatically projected onto a position-sensitive imaging detector at the end of a time-of-flight mass analyser. As shown in Figure 1.8(B), this process is analogous to stigmatic microscopy, and has the advantage of decoupling spatial resolution from the probe beam size while retaining resolutions of 1–20 μ m.

Although the principles of ion microscopy have been well established since the 1960’s [83], their uptake compared to microprobe instruments has been limited due to the difficulty in imaging more than one m/z (or a range of m/z) during an experimental cycle. The advent of event-triggered sensors, such as CMOS-based cameras [84–86], has overcome this problem by removing the need to apply time gates to the detector; ion images

can now be recorded for every resolved m/z in a defined mass range during a single experiment [63, 87, 88].

In 1992, B. W. Schueler [89] reported the stigmatic imaging mass spectrometer shown in Figure 1.9(a). The sample surface was bombarded by a pulsed primary ion beam and the analytes were emitted as secondary ions, which were electrostatically extracted and spatially focused by an immersion lens. The ion image was then electrostatically transferred into three sequential 90° hemispherical electrostatic analysers (ESAs) that minimised flight time dispersions due to the initial kinetic energies of the ions. Eventually, the ion image was projected onto a fast position-sensitive resistive anode encoder (RAE). The field of view of the instrument was $300\text{ }\mu\text{m}$ and the image resolution was $1\text{--}5\text{ }\mu\text{m}$. A mass resolution $\approx 15,000\text{ }m/\Delta m$ was obtained for C^2F^{5+} secondary ions.

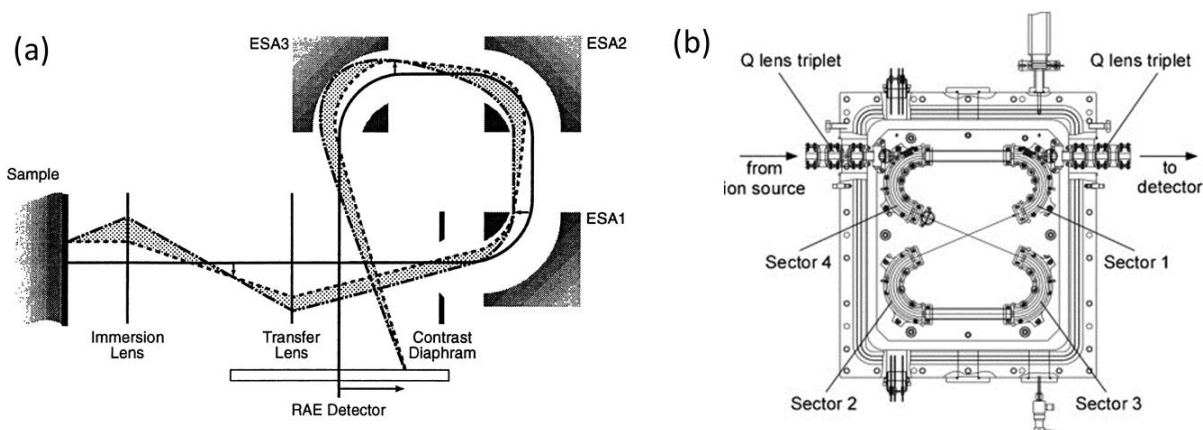


Figure 1.9: Layouts of the microscope MSI instruments: (a) the direct imaging ToF mass spectrometer developed by B. W. Schueler and (b) the multi-turn TOF mass analyser (MULTUM-IMG) developed by M. Toyoda. Reprinted from ref. [89] and [90], respectively.

R. M. A. Heeren and coworkers obtained the first macromolecular ion microscope images by performing MALDI with a modified commercial TRIFT II (triple focusing time-of-flight) mass spectrometer in 2004 [77] (the TRIFT II instrument was very similar to the one used by B. W. Schueler). Ion images of substance P (mass 1348 Da) and insulin (mass 5733 Da) were acquired using 2,5-DHB as matrix. The field of view was $150\text{--}300\text{ }\mu\text{m}$ in diameter and the spatial resolution was about $4\text{ }\mu\text{m}$. The same instrument was used to analyse a rat brain section [91]. They also reported the first application of a

hexanode delay-line detector in microscope MSI [64]. In 2010, the Heeren group used the Medipix Pixelated detector [92], which showed spatial resolutions of $<6\ \mu\text{m}$ with SIMS and of $8\text{--}10\ \mu\text{m}$ with laser desorption ionisation. Later in 2011, the Timepix detector was used [93]: mass-resolved spatial images of different m/z species were captured in a single measurement by coupling it with the TRIFT instrument.

A multi-turn TOF mass analyser (MULTUM-IMG) was developed by M. Toyoda *et al.* in 2008 [90]. As shown in Figure 1.9(b), MULTUM-IMG consisted of four toroidal electric sectors, where ions could travel in a closed orbit for hundreds of turns following a figure eight trajectory. In principle, if the instrument satisfies the “perfect focusing” condition, ions of the identical m/z entering the multi-turn section simultaneously will return to the same position simultaneously when exiting, regardless of their velocities and incident angles. Hence, the ToF resolution can be improved by cycling the ions through the turning sectors: the widths of the ToF peaks stay constant while the flight times increase with more turns. With the MULTUM instrument, a mass resolution of $130,000\ m/\Delta m$ was obtained after 500 turns, the positional information could also be conserved during the cycles. However, the ion images projected on the detector were severely distorted after one turn, and became blurry after three turns.

1.6.1 Previous work on microscope MSI using linear ToF-MS techniques

M. Brouard and coworkers [87] built an MSI instrument by modifying a conventional velocity map imaging (VMI) setup as shown in Figure 1.10. On this instrument, microscope mode MSI results were obtained for the first time with a PImMS camera.

A large area of $5\times 5\ \text{mm}^2$ was spatially imaged with a mass resolution of $m/\Delta m = 67$ (PMT) or 48 (PImMS) and a spatial resolution of $23\ \mu\text{m}$ (CCD camera) or 99 (PImMS). The better mass resolution with a PMT is due to its shorter time interval than PImMS and the better spatial resolution with a CCD camera is because it has more pixels (768×576). The multi-mass imaging capability of the PImMS sensor was demonstrated in Figure 1.11

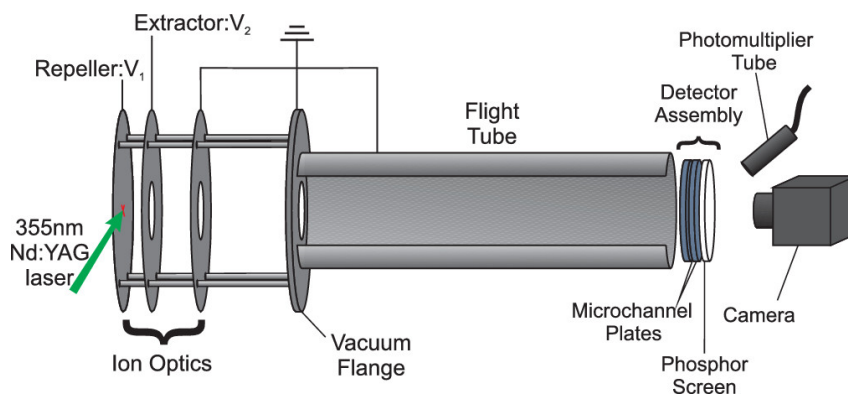


Figure 1.10: Schematic of the spatial imaging instruments used in ref. [87].

where ion images belonging to two different substances (Auramine O and Rhodamine 590) were obtained. The dyes were deposited over the substrate as alternating stripes. Figure 1.11 clearly shows how in one single measurement spatial distributions of different mass peaks can be acquired using the PImMS camera.

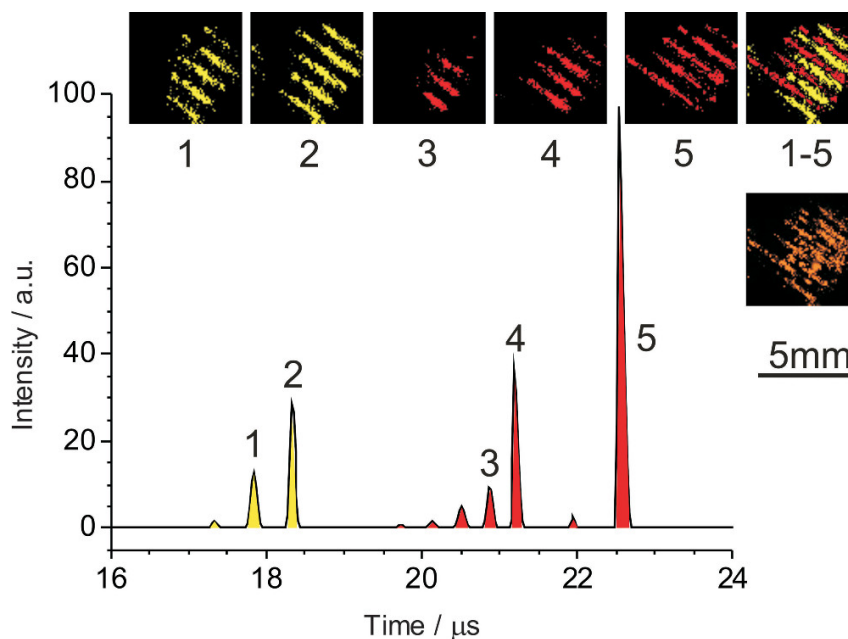


Figure 1.11: ToF spectrum and ion images collected by the PImMS sensor; Peak 1 and 2 are fragments of Auramine O (yellow), peak 3–5 are from Rhodamine 590 (red). An image that includes the entire ToF range is presented on the right of the ToF spectrum (orange), and compared with an overlay of the images 1–5 above it. All data shown in this figure were obtained during a single measurement. Reprinted from ref. [87].

In a later work, a commercially available linear TOF mass spectrometer, the LT2 Plus (SAI Ltd, Manchester, UK), was modified and combined with the PImMS camera

Instruments	Mass resolution ($m/\Delta m$)	Spatial resolution (μm)	Ion transmission rate (%)	Mass range (m/z)	Ion optics rescaling	Imaged sample size (mm^2)
Modified VMI rig	67 (PMT) 48 (PImMS)	23 (CCD) 99 (PImMS)	≈ 100	1-67	No	5×5
Modified LT2 Plus	800-1500 (PMT) 500-1000 (PImMS)	20-60 (PImMS)	≈ 100	200-800	No	unknown
Velocity corrected Extraction PEDa	1000-2200 (PMT)	20-30 (CCD)	≈ 100	350-730	Yes	5×5

Table 1.4: Summary of previous work [87, 94, 95].

to record microscope MSI data [94]. Experimental data was collected then processed through post-acquisition and cluster analysis algorithms. When an ion impinged the microchannel plates, $10^5 - 10^6$ electrons were produced during the signal amplification process, which activated a small area on the scintillator and were recored by the PImMS camera as a “cluster” of events that spread across multiple pixel coordinates and time codes (x, y, τ) . By finding all the events that originate from the same ion hit, the cluster can be centroided into a single (x, y, τ) coordinate that corresponds to the original ion impact. More details of reducing a cluster of events captured by a camera into one single event will be introduced in Chapter 3. Therefore the size of the data set was reduced and, more importantly, the mass and spatial resolution were improved. The DE technique was employed at the ion source to compensate for the initial velocity distribution of ions with the same m/z . While still maintaining an optimum spatial resolution of $25 \mu\text{m}$, this new system provided a mass resolving power above the isotopic limit ($\Delta m_{50\%} \leq 1m/z$) for ions between 200 and 800 m/z , which was one magnitude better than the home-built instrument. A downside of the DE technique was that at the very beginning of the desorption/ionisation process, there was no extraction field and the ion plume could expand freely. As a result, spatial information was partially lost since ions could travel

transversely until the extraction field was applied after a certain time delay. So in the latest development, the home-built instrument was further modified to employ the post-extraction differential acceleration (PEDA) technique [95]. The principle of PEDA is very similar to DE, the only difference is that an extraction field is always present to control the trajectories of desorbed ions, instead of allowing the ablation plumes freely disperse into the vacuum. A voltage pulse is then applied to the extractor electrode when the ions are in the region between it and the grounded electrode (see Figure 1.10), rather than to the repeller as in DE. PEDA will be described in more detail in Chapter 4. With the PEDA instrument, an optimum mass resolution above 2,000 $m/\Delta m$ was achieved along with a spatial resolution at 25 μm . The achievable mass resolution, spatial resolution, and mass range of the aforementioned instruments are listed in Table 1.4.

1.7 Spatial imaging and aberration

The spatial resolution of a microscope MSI instrument is mainly limited by a variety of ion optical aberrations. Three of them will be described in this section: spherical aberration, chromatic aberration, and Petzval field curvature.

Spherical aberration [96] is a very common phenomenon that occurs in both light and ion optical lenses. As shown in Figure 1.12(a) it results in an imperfection of the produced image. When charged particles are focused by a lens, the particles that pass through the edge of the lens bend more strongly (a smaller focus length) than those that pass through the lens closer to its center. So, when particles are emitted from a point source P on the optical axis, if the distance between the point source and the lens is larger than the focus length, the charged particles will be refocused into a circle, termed the circle of least confusion rather than to a point. The diameter of the circle of least confusion (d_s) is given by,

$$d_s = C_s \beta^3 \quad (1.9)$$

where C_s is a lens-specific factor. β is the diameter of the lens aperture. Consequently,

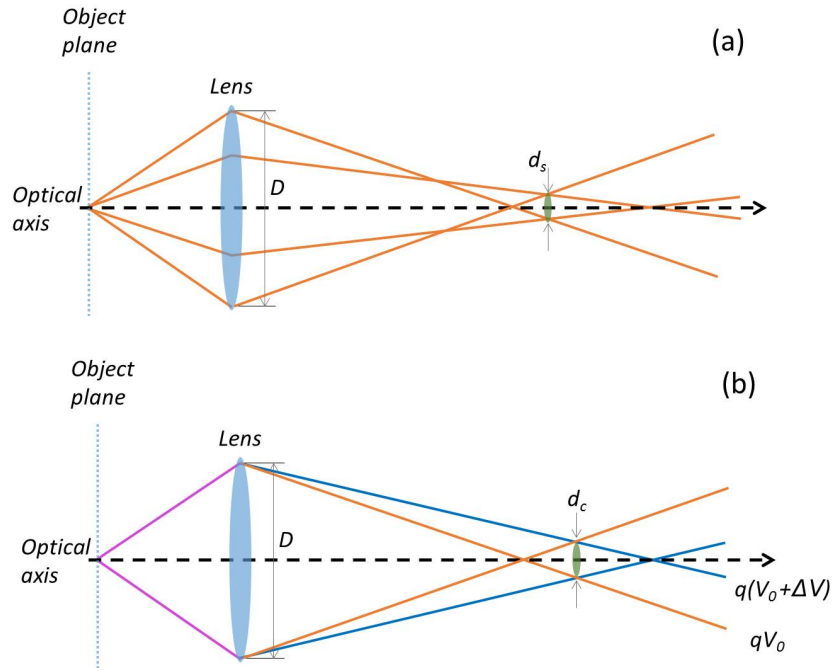


Figure 1.12: Illustration of (a) spherical and (b) chromatic aberrations.

Equation (1.9) indicates that a point on the sample surface is imaged on the detector as a circle with a radius of d_s , which limits the spatial resolution of the instrument.

Chromatic aberration [96] is an effect observed in traditional optics caused by the fact that the refractive index of every optical glass lens varies with wavelength. Short wavelengths (high single photon energy) are refracted more than long wavelengths (low single photon energy). Chromatic aberration also occurs in ion optics, where charged particles of different kinetic energy are focused by an ion lens differently as shown in Figure 1.12(b). But in contrast with the light optics, charged particles of small kinetic energies are actually deflected to a larger extent than those with high kinetic energy. Consequently, for an ion beam with a distribution of kinetic energies, their optimal image planes do not overlap in space and there is again a circle of least confusion where chromatic aberration is at its minimum and where the detector should be located. The diameter (d_c) of the circle of least confusion in the case of chromatic aberration can be calculated by,

$$d_c = C_c \beta \frac{\Delta V}{V_0}, \quad (1.10)$$

where C_c is a coefficient factor which depends on the type of ion lens, $\Delta V/V_0$ corresponds to the relative energy spread of the charged particles. Therefore the spatial resolution of a point source declines in proportion with the aperture size β .

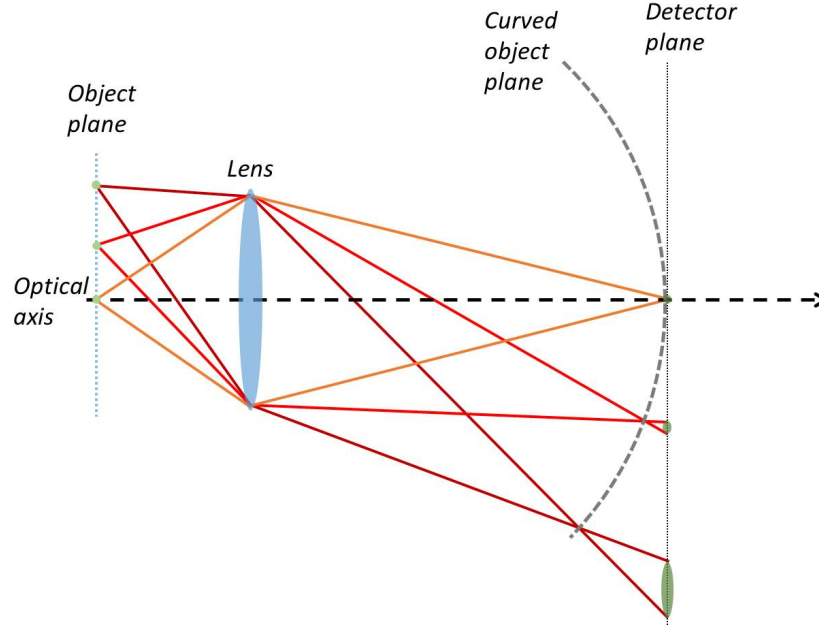


Figure 1.13: Illustration of Petzval Field Curvature.

The Petzval Field Curvature Theorem (after Joseph Petzval, 1807-1891) [97] is an optical aberration of a thin lens whose focal plane is actually not planar, but spherical. This aberration also appears in ion optics. As shown in Figure 1.13, trajectories emitted from a flat object plane cannot be perfectly focused at an identical plane behind the lens: if charged particles are generated from the object plane at a larger displacement from the optical axis, they will be projected onto a image plane located closer to the lens and vice versa. Consequently, an ideal detector plane would be a curved plane, deliberately bent to compensate for the focus length difference. However, most detectors are flat. If the detector plane coincides with the image plane of paraxial trajectories, the peripheral field of view will be out of focus at the detector plane, degrading the overall spatial resolution of the ion optical system.

1.8 Microprobe vs. microscope MSI

The microprobe and microscope imaging modes will be compared in this section. The biggest advantage of microprobe MSI is the ultra-high mass resolving power. For instance, a hybrid ion trap-orbitrap mass spectrometer (Orbitrap EliteTM, Thermo Fisher Scientific) is able to deliver a mass resolving power above 240,000 $m/\Delta m$ at 400 m/z , enabling the detection of low-abundance compounds in complex environments. The space-resolving power of a microprobe MSI instrument is dictated by the size of its ionisation beam. With a transmission geometry MALDI source, R.M. Caprioli and coworkers achieved a 1 μm laser spot diameter with a minimal raster step size of 2.5 μm [98]. Nano-scale secondary ion mass spectrometers (NanoSIMS) reportedly offers a lateral resolution down to 50 nm [99,100].

In the case of microscope MSI, the mass resolution is usually less than 10,000 $m/\Delta m$ and the spatial resolution is no less than a few micrometers. However, as discussed earlier, microscope MSI is relatively fast, as discussed earlier, because the acquisition time of an image does not depend on the number of pixels to raster or the scan speed of the probe. For example, if a microprobe instrument is used to obtain an ion map with 20 μm spatial resolution over a 4×4 mm sample, the probe beam needs to be scanned over 40,000 20 μm pixels. If a state-of-art commercial instrument such as Rapiflex MALDI Tissue typerTM (Bruker) is employed, its scan speed is up to 50 pixels/second, which corresponds to an acquisition time of 800 s for each sample. If the microscope instrument described in ref. [94], which has a 4×4 μm field of view, is used to perform the same task (a spatial resolution of about 20 μm), the experiment acquisition time would be around 250 s with a laser repetition rate of 20 Hz, or 5 s if a laser of 1 kHz repetition rate is used. This two orders of magnitude shorter than the microprobe mode. In practice, one chooses between the microprobe and microscope MSI methods based on the requirements posed by specific applications and whether or not multi-mass imaging is required.

1.9 Summary

Mass spectrometry is a powerful tool that holds the promise of answering two fundamental questions in analytical science: what exists in a sample and how are they distributed in the sample. Following a general overview of mass spectrometry, the first section of this chapter presented the technique's history. The second section was devoted to the mechanisms and advantages/disadvantages of different ionisation sources, especially MALDI. In the third section, representative mass analysers, such as time-of-flight, were discussed. The detectors were introduced in the fourth section. The rest of this chapter introduced the mass spectrometry imaging technique, which is able to visualise the spatial distribution of molecules by their molecular masses. Two working modes, namely microprobe and microscope modes, were described and compared. Recent developments of implementing microscope mode MSI were reviewed. Due to the requirement of conserving the spatial information of ions ablated from a sample surface, many commonly used performance enhancing methods in conventional ToF mass spectrometers cannot be transferred to our instrument straight away. As a result, there are three challenges facing microscope MSI: (1) the effective mass range, (2) mass resolution, and (3) mass accuracy.

(1) The effective mass range is the range of m/z that can be effectively resolved by a mass spectrometer (*i.e.* the mass spectrometer provides a good mass resolution for ions with a m/z among the effective mass range). The PEDDA technique is highly mass-dependent and therefore has a very limited mass range: for example, when the optimally temporal-focused mass is 410 Da, the mass range where ions have a mass resolution above 1,000 $m/\Delta m$ is between 375 and 440 Da. So, to analyse ions out of this mass range, the instrument has to be tuned accordingly, which will be inconvenient in practice. (2) Moreover, although the PEDDA technique is employed to correct the ToF spread of ions with the same m/z but different initial velocities, the highest achievable mass resolution of the PEDDA instrument is still less than 3,000 $m/\Delta m$, which is not comparable to modern commercial mass spectrometers that provide mass resolution above

10,000 $m/\Delta m$. (3) The mass accuracy of a mass spectrometer gauges the difference between the actual mass and the measured mass of an analyte. The microscope MSI instrument has a mass accuracy (in the order of 1,000 ppm) much worse than a commercial instrument (better than 5 ppm), which makes it harder to identify unknown compounds.

The methods used to model and simulate the microscope MSI instrument will be introduced in Chapter 2. The novel designs developed *in silico* will then be tested experimentally using the experiment methods described in Chapter 3. In the rest of this thesis, new approaches will be proposed to improve various aspects of the microscope mode MSI system and to tackle each of the three challenges: (1) Chapter 4 introduces two variants of PEDDA (double-field and time-variable PEDDA) that considerably expands the effective mass range; (2) Chapter 5 investigates the compatibility of a reflectron and the microscope MSI, both the simulation and experimental results show that the reflectron is able to triple the achievable mass resolution of the instrument without losing its spatial imaging capability; (3) Chapter 6 elaborates how machine learning algorithms can help to circumvent the poor mass accuracy problem and illustrates the potential of using the microscope MSI as a high throughput diagnostic tool. Chapter 7 concludes the thesis by recapping the key achievements presented above.

Chapter 2

Modeling and Simulation

Designing an appropriate ion optical system to perform certain tasks is a key step in developing a microscope mode MSI instrument. In this chapter, two different approaches to design an ion optical system are introduced: transfer matrix optics and SIMION ion trajectory simulations. Both approaches model the entire ion optical system and determine the trajectories of charged particles traveling through it. Therefore the ion optical performance can be estimated based on the modeling results, which may guide further modifications. The transfer matrix method provides a very intuitive way to understand the function of each ion optical component as well as the outcome of combining them in tandem, which helps to illustrate the geometric formation of ion images through a sequence of co-axial electrodes. However, due to various approximations involved when calculating ion trajectories, experiments modeled by the transfer matrix method are less accurate than those determined by the commercial SIMION software package, a powerful field and particle trajectory simulator. In the first half of this chapter, the transfer matrix method will be introduced in details. A few important transfer matrices will be derived mathematically, particularly the transfer matrix of an aperture that explains the lensing effect of ring electrodes. Additionally, a typical three-electrode spatial imaging system as well as the five-electrode system used in the instrument to be described in Chapter 3 are both explored with the transfer matrix method. Key aspects of SIMION will be covered in the second half of this chapter, including simulation, important aspects of SIMION, and ways to extract useful information from simulated experiments.

2.1 Modeling Ion Optical System with Transfer Matrices

2.1.1 The Transfer Matrix Method

For a cylindrically symmetric optical system, a light ray can be described by a trajectory travels within a 2D plane. Thus only two variables are needed to describe the path of the light ray in the system (see Figure 2.1(a)), the distance and direction of the ray relative to the optical axis. As the ray propagates in the optical system, these two variables will be altered as they pass through a variety of optical elements that comprise the system, such as free-space, mirrors, lenses, and prisms. When designing a complex optical system, one has to trace the ray transmitting through these elements in a straightforward way. The transfer matrix method is a useful trick to perform this task.

The transfer matrix method is based on the paraxial approximation, which means that all rays are assumed to always travel only at a small distance and a small angle relative to the optical axis of a system. As shown in Figure 2.1(a), in a 2D coordinate system, input and output planes are two reference planes perpendicular to the optical axis. The position and angle (r_2, α_2) at the output plane of an optical element can be calculated from the position (r_1) and angle (α_1) at its input plane through two linear algebraic equations. Therefore, the optical effect of an element on an incoming ray can be fully characterised by a 2×2 transfer matrix:

$$\begin{bmatrix} r_2 \\ \alpha_2 \end{bmatrix} = \begin{bmatrix} m_{11} & m_{12} \\ m_{21} & m_{22} \end{bmatrix} \begin{bmatrix} r_1 \\ \alpha_1 \end{bmatrix}, \quad (2.1)$$

where r_2 and α_2 are the transverse displacement and direction respectively of the ray with respect to the optical axis at the output plane, and $M = \begin{bmatrix} m_{11} & m_{12} \\ m_{21} & m_{22} \end{bmatrix}$ is the transfer

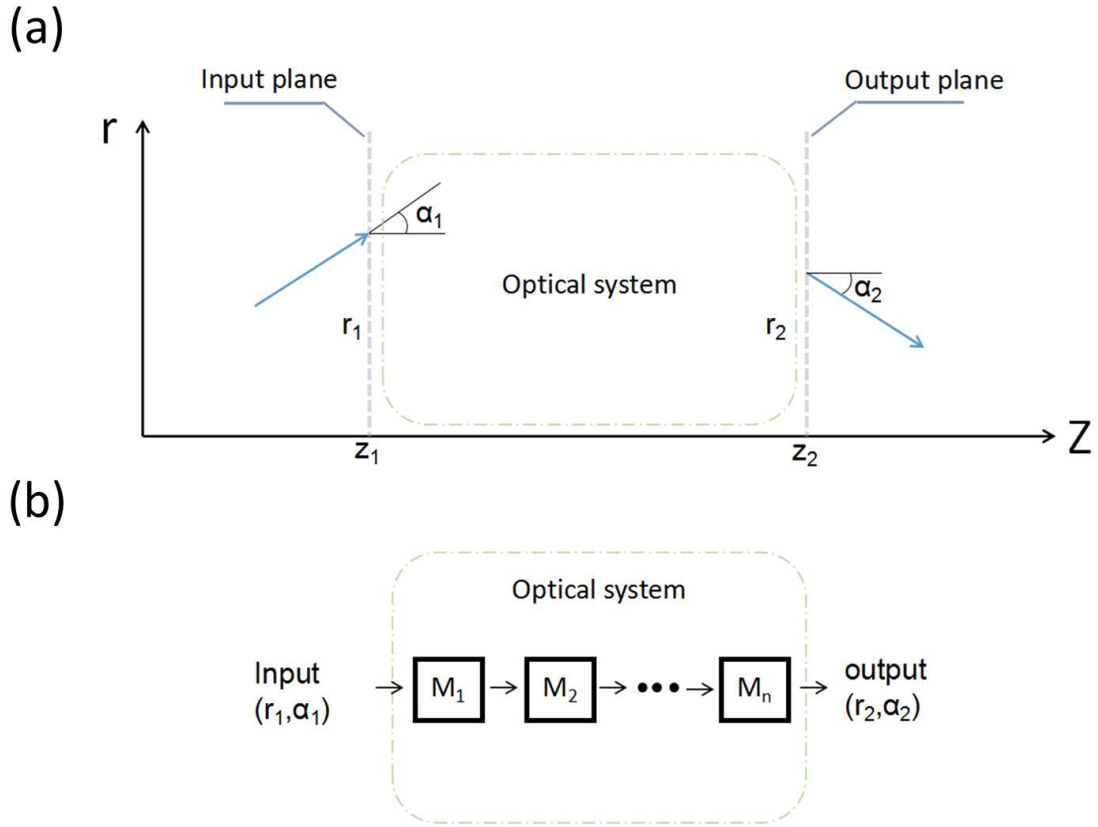


Figure 2.1: (a) For a cylindrically symmetric optical system, the displacement (r) and direction (α) of the ray with respect to the optical axis are employed to fully describe propagation of a light beam, (b) The $[r_2, \alpha_2]$ vector at the output plane of an optical system can be calculated by multiplying the transfer matrix of each optical component with the $[r_1, \alpha_1]$ vector at the input plane in an order that corresponds with the sequence of elements.

matrix that represents the optical element in the middle of the two reference planes. $\begin{bmatrix} r_1 \\ \alpha_1 \end{bmatrix}$ is a ray vector at the input plane while $\begin{bmatrix} r_2 \\ \alpha_2 \end{bmatrix}$ is a ray vector at the output plane. Equation 2.1 relates these two vectors by M .

Transfer matrices become very useful when characterising composite optical systems consisting of a series of elements. If two optical elements are placed next to each other along the optical axis, the combined optical effect can be represented by a new matrix which is the product of their respective matrices. Therefore the overall effect of combining

multiple optical elements can be estimated by a single transfer matrix, which is the result of multiplying the transfer matrix of each optical component in their corresponding order (see Figure 2.1(b)):

$$M_{total} = M_n \cdots M_2 M_1, \quad (2.2)$$

where M_1 represents the matrix of the first element experienced by the ray, and matrix M_n is the last optical element before the output plane. Applying M_{total} to an incident ray at the input plane of arbitrary displacement and angle $[r_0, \alpha_0]$ will generate the final displacement and angle $[r_n, \alpha_n]$ at the output plane of the whole system. The idea of employing matrix formalism in optics was proposed in early 1960s. The first books on transfer matrix method were published by O'Neill [101] in 1963 and Willem Brouwer [102] in 1964. Since then, matrix methods have been widely adopted to describe not only the geometric optics of paraxial light rays but also the propagation of electromagnetic or acoustic waves and of course the motion of charged particles in electromagnetic fields.

Ion optics are electrodes that control the formation, focusing, and deflection of charged particle beams. But unlike traditional light optics, ion optics are usually in a high vacuum environment, which prevents not only undesirable collisions between ions and neutral air molecules but also electrical breakdown between HV electrodes. The basic element of an ion optical system is the electrostatic lens, which is usually a circular electrode with an aperture at its centre, that assists in the transport of charged particles. Ion optics can control the propagation of charged particles with uniform or curved electric fields, analogous to the way a glass lens does in the transport of light in an optical instrument. As a consequence, ion optical systems can be designed in a similar way to traditional optical systems, meaning that the transfer matrix method is also applicable for ion optics. However, there is a small difference in the transfer matrix methods between ion optics and traditional optics: for the sake of simplicity in the derivation of formulas, rather than using α (in radians) to characterise the direction of a moving ion, the slope r' of the ion

trajectory is used instead

$$r' = \frac{dr}{dz} = \frac{dr/dt}{dz/dt} = \frac{\dot{r}}{\dot{z}} \quad (2.3)$$

therefore the vector that describes an ion's movement in a 2D plane becomes $\begin{bmatrix} r \\ r' \end{bmatrix}$.

2.1.2 Transfer Matrices for Uniform Electric Fields

The derivation of the two most important transfer matrices are briefly introduced here. The first matrix is the one used to describe a situation where charged particles pass through a uniform electrostatic field. As shown in Figure 2.2, an ion with kinetic energy qV_1 travels through a uniform field between the equipotential planes P_1 and P_2 which are also the input and output planes, respectively. The ion enters the field at zero displacement with a constant velocity and an angle α_1 relative to the optical axis and leaves at r_2 and α_2 . The ion also gains kinetic energy from this acceleration field, which increases to qV_2 at the exit.

The ion's motion follows a typical parabola trajectory, which can be calculated by Newton's law:

$$m\ddot{r} = 0, \quad m\ddot{z} = qE, \quad z_1 = r_1 = 0, \quad (2.4)$$

$$\dot{z}_1 = v_1 \cos \alpha_1, \quad \dot{r}_1 = v_1 \sin \alpha_1, \quad v_1 = \sqrt{\frac{2qV_1}{m}}, \quad (2.5)$$

where v_1 is the speed of the particle with a kinetic energy qV_1 , and m is its mass.

$$\dot{z} = \frac{qE}{m}t + v_1 \cos \alpha_1, \quad z = \frac{qE}{2m}t^2 + v_1 \cos \alpha_1 \cdot t, \quad (2.6)$$

$$\dot{r} = \dot{r}_1 = v_1 \sin \alpha_1, \quad r = v_1 \sin \alpha_1 \cdot t, \quad (2.7)$$

where r_1 is assumed to be 0 without loss of generality. By rearranging Equation (2.6), we

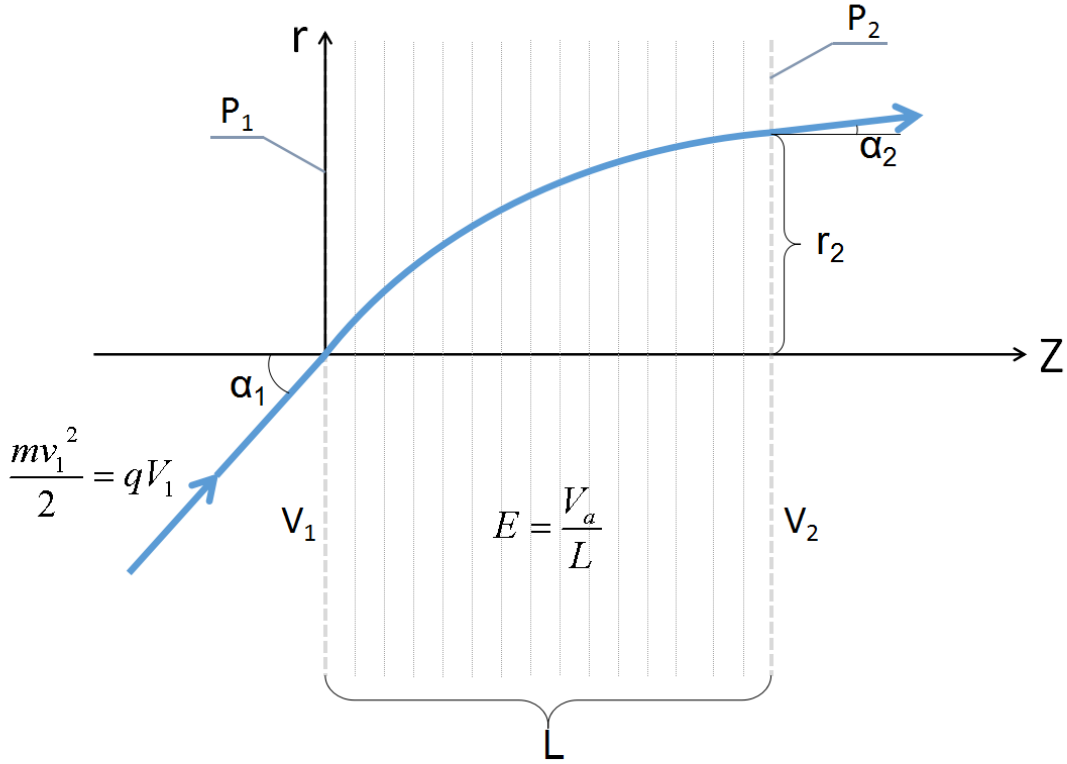


Figure 2.2: An ion with kinetic energy qV_1 is accelerated to qV_2 through an uniform field between the equipotential planes P_1 and P_2 . The ion enters the field at zero displacement and an angle α_1 relative to the optical axis and leaves at r_2 and α_2 .

can obtain a quadratic equation with t as an unknown variable:

$$\frac{qE}{2mv_1}t^2 + \cos \alpha_1 \cdot t - \frac{z}{v_1} = 0, \quad (2.8)$$

A meaningful solution of Equation (2.8) for t is:

$$t = \frac{mv_1}{qE} \left(\sqrt{\cos^2 \alpha_1 + \frac{2qE}{mv_1^2}z} - \cos \alpha_1 \right), \quad (2.9)$$

By substituting the t in Equation (2.7) with Equation (2.9), one obtains

$$r = \frac{2V_1}{E} \sin \alpha_1 \left(\sqrt{\frac{E}{V_1}z + \cos^2 \alpha_1} - \cos \alpha_1 \right). \quad (2.10)$$

Equation (2.10) describes the ion trajectory within the acceleration field. The electric field strength E is

$$E = \frac{V_2 - V_1}{L}. \quad (2.11)$$

By substituting the E in Equation (2.10) with Equation (2.11), we can find the distance from the optical axis at $z = L$:

$$r_2 = \frac{2L \sin \alpha_1}{V_2/V_1 - 1} \left(\sqrt{\frac{V_2}{V_1} - \sin^2 \alpha_1} - \cos \alpha_1 \right). \quad (2.12)$$

The slope $\dot{r}$ within the accelerating field is:

$$\dot{r} = \frac{dr}{dz} = \sin \alpha_1 \left(\frac{E}{V_1} z + \cos^2 \alpha_1 \right)^{-1/2}. \quad (2.13)$$

Again, by substituting E with Equation (2.11), one obtains the slope at the output plane $P_2(z = L)$:

$$\dot{r}_2 = \sin \alpha_1 \left(\frac{V_2}{V_1} - \sin^2 \alpha_1 \right)^{-1/2}. \quad (2.14)$$

When taking the paraxial approximation ($\alpha_1 \ll 1$) into consideration, Equation (2.12) and (2.14) can be further simplified with $\sin \alpha_1 \approx \tan \alpha_1 = \dot{r}_1$, $\cos \alpha_1 \approx 1$, $\dot{r}_1 \ll \frac{V_2}{V_1}$,

$$r_2 \approx \frac{2L \dot{r}_1}{V_2/V_1 - 1} \left(\sqrt{\frac{V_2}{V_1}} - 1 \right) = \frac{2L}{\sqrt{V_2/V_1} + 1} \dot{r}_1, \quad (2.15)$$

$$\dot{r}_2 \approx \sqrt{\frac{V_1}{V_2}} \dot{r}_1. \quad (2.16)$$

In a more general situation where $r_1 \neq 0$, one obtains

$$r_2 = r_1 + \frac{2L}{\sqrt{V_2/V_1} + 1} \dot{r}_1, \quad (2.17)$$

Therefore the transfer matrix for the uniform electrostatic field has the following form:

$$\begin{bmatrix} r_2 \\ \dot{r}_2 \end{bmatrix} = M \begin{bmatrix} r_1 \\ \dot{r}_1 \end{bmatrix} = \begin{bmatrix} 1 & \frac{2L}{\sqrt{V_2/V_1+1}} \\ 0 & \sqrt{V_1/V_2} \end{bmatrix} \begin{bmatrix} r_1 \\ \dot{r}_1 \end{bmatrix}. \quad (2.18)$$

There is a special case of the transfer matrix in Equation (2.18) for charged particles traveling through a field free drift space. In this case, $V_1 = V_2$, so the transfer matrix for a drift space is:

$$M = \begin{bmatrix} 1 & L \\ 0 & 1 \end{bmatrix}. \quad (2.19)$$

Another special case of practical importance is the extraction of charged particles emitted from a flat repulsive surface (see Figure 2.3). Such an electrode is called a repeller and is used to host samples on a conductive surface. Ions created by matrix-assisted laser desorption/ionisation (MALDI) or a focused primary ion beam (SIMS) are then accelerated towards an extractor electrode.

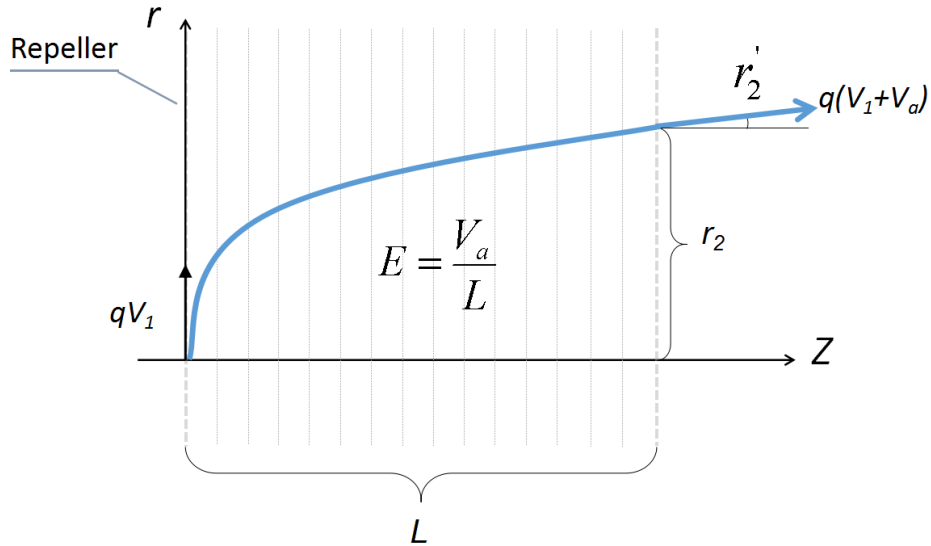


Figure 2.3: An ion is emitted from a repeller with an initial kinetic energy qV_1 and then accelerated to a higher kinetic energy $V_2 = q(V_1+V_a)$ by a uniform electric field $E = V_a/L$.

As mentioned earlier, Equation (2.12) and (2.14) can be further simplified using the paraxial approximation: the angle between the flight direction at input plane and the optical axis (α_1) is close to zero. But this approximation sometimes does not hold true for ions generated by MALDI or SIMS, which usually have a broad distribution of emitting angles. Some of the particles may leave the repeller at an angle up to 90° (perpendicular with the surface normal). If a charged particle leaves the surface with initial kinetic energy qV_1 followed by being accelerated by the potential difference (V_a) between the input and output planes, it will then exit the uniform acceleration field with kinetic energy $V_2 = q(V_1 + V_a)$. Equation 2.12 and 2.14 can be rearranged in this situation as

$$r_2 = \frac{2LV_1}{V_a} \sin \alpha_1 \left(\sqrt{\frac{V_a}{V_1} + \cos^2 \alpha_1} - \cos \alpha_1 \right), \quad (2.20)$$

$$r_2 = \sin \alpha_1 \left(\frac{V_a}{V_1} + \cos^2 \alpha_1 \right)^{-1/2}. \quad (2.21)$$

In practice, the potential difference V_a is far larger than V_1 , as a result one has $V_a/V_1 \gg 1 \geq \cos \alpha_0 \geq \cos^2 \alpha_0$. Therefore equation 2.20 and 2.21 can be approximately reduced to

$$r_2 \approx 2L \sin \alpha_1 \sqrt{\frac{V_1}{V_a}}, \quad (2.22)$$

$$r_2 \approx \sin \alpha_1 \sqrt{\frac{V_1}{V_a}}. \quad (2.23)$$

2.1.3 Transfer Matrices for An Aperture

The second useful matrix that will be discussed here is the one for an aperture. Figure 2.4 shows a situation where two uniform electric fields are separated by a circular electrode that has an aperture at its centre. If the strengths of these two fields are different, an inhomogeneous field will be created near to the aperture, that is the equipotential planes of the strong field bulge through the aperture into the weak field side. The curvature of

the equipotential surfaces imparts ring electrodes a power to converge or diverge charged particles passing through it. This is analogous to a glass lens, whose optical power depends on the radius of curvature of its spherical surface. As a consequence, an electrode with an open aperture can be seen as an electrostatic lens in an ion optical system. Ring electrodes are key to spatial imaging and velocity map imaging, and so mathematical derivations of the corresponding transfer matrix will be provided here to describe this lensing effect in detail. For the sake of simplicity and without loss of generality, the right

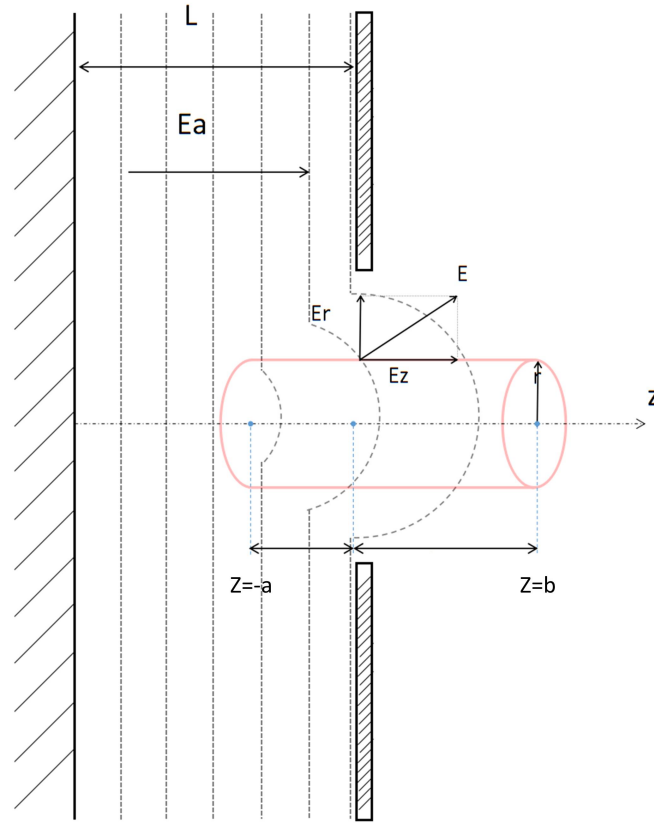


Figure 2.4: A circular electrode with an aperture at its centre separates two uniform electric fields of different strengths: the field strength on the left of the aperture is $E_a > 0$ while the one on the right is 0.

side of the aperture is assumed to be field free (or electric field strength of zero). When a charged particle travels near the aperture, it will be deflected away from the axis due to a radial component E_r . As shown in Figure 2.4 an imaginary cylinder goes through the

aperture along the optical axis with its left end locating at the region of the undisturbed uniform field (at $z = -a$) and its right end protruding into the field free space (at $z = b$). According to Gauss' law, the net electric flux through any hypothetical closed surface is proportional to the net electric charge enclosed within that surface. Since there is no charge existing in the cylinder, the net electric flux through it is zero. Hence,

$$-\pi r^2 E_a + 2\pi r \int_{-a}^{+b} E_r dz = 0, \quad \int_{-a}^{+b} E_r dz = r E_a / 2, \quad (2.24)$$

where E_a represents the uniform electric field on the left side of the aperture and the direction of this field is parallel with the optical axis; r is the radius of the circular face of the imaginary cylinder.

A charged particle obtains a radial momentum from E_r when passing through the aperture at the distance r from the axis, which is given as:

$$mv_r = \int q E_r dt = \frac{q}{v_z} \int_{-a}^{+b} E_r dz, \quad (2.25)$$

where dt was substituted with dz/v_z since $v_z = dz/dt$. Rigorously speaking, the charged particle also gets accelerated axially by E_a when traveling from $-a$ to b , but this increase of v_z is negligible compared with v_z itself. So v_z can be considered approximately as a constant.

Substituting the integral of E_r in Equation (2.25) with Equation (2.24), one obtains:

$$m\Delta v_r = \frac{qrE_a}{2v_z}. \quad (2.26)$$

If $\Delta\dot{r}$ represents the magnitude of deflection an charged particle experienced when crossing the ring electrode, it can be defined as

$$\Delta\dot{r} = \dot{r}_2 - \dot{r}_1 = \frac{v_{r2}}{v_{z2}} - \frac{v_{r1}}{v_{z1}} = \frac{v_{r2} - v_{r1}}{v_z} = \frac{m\Delta v_r}{mv_z}, \quad (2.27)$$

where $\dot{r}_1$ and $\dot{r}_2$ are the slopes of particle trajectory at the input and output planes

respectively, while v_{r1} and v_{r2} are the radial velocities of the charged particle, again at the input and output planes respectively. Here, the ring electrode is modeled as a “thin” lens, whose input and output planes overlap in space at the aperture plane. Thus, the trajectory appears to have a sharp kink at the aperture plane.

By substituting $m\Delta v_r$ in Equation (2.27) with Equation (2.26) and $mv_z^2/2$ with qV (*i.e.* acceleration potential), the particle kinetic energy, one obtains

$$\Delta\dot{r} = \frac{qrE_a}{2mv_z^2} = \frac{rE_a}{4V}. \quad (2.28)$$

Therefore the deflection of the trajectory $\Delta\dot{r}$ that an aperture imposes on a charged particle beam is proportional to the distance r from the axis at which the beam crosses the aperture, but independent of its original incident slope. This is exactly the property of an ideal lens in light optics. By analogy with the definition of focal length in light optics, the focal length of an electrostatic lens has a form as follows,

$$-f = \frac{r}{\Delta\dot{r}} = \frac{4V}{E_a}. \quad (2.29)$$

So far the field strength on the right side of the aperture has been assumed to be zero, however it is also very common that the aperture actually separates two regions of different non-zero field strengths. For this general case, the focus length of this aperture has a formula as follows,

$$-f = \frac{4V}{E_2 - E_1}, \quad (2.30)$$

where V corresponds to the kinetic energy of the particle at the aperture plane; E_1 and E_2 represent the field strengths on both sides of the ring electrode. The sign of the field strength is defined as positive if positively charged particles are accelerated in the field, otherwise negative if the particle is decelerated. The transfer matrix of such an aperture

is given as follows:

$$\begin{bmatrix} r_2 \\ \dot{r}_2 \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ -1/f & 1 \end{bmatrix} \begin{bmatrix} r_1 \\ \dot{r}_1 \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ (E_1 - E_2)/4V & 1 \end{bmatrix} \begin{bmatrix} r_1 \\ \dot{r}_1 \end{bmatrix}. \quad (2.31)$$

The derivations of the transfer matrices described above are reproduced from ref. [96].

2.1.4 Modeling the Experiment

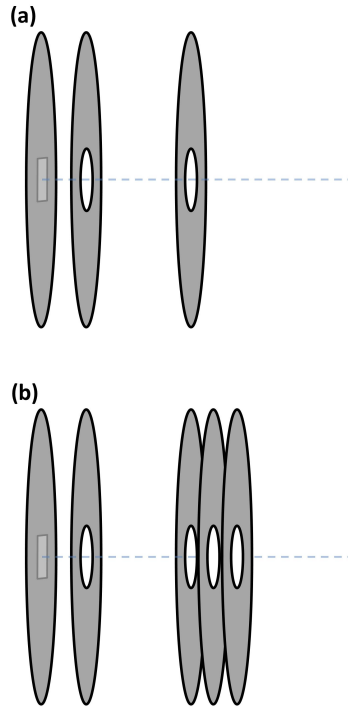


Figure 2.5: Two types of spatial imaging ion optics have been modeled by the transfer matrix method (a) three-electrode ion optical system and (b) five-electrode ion optical system.

A three-electrode ion optical system for spatial imaging is illustrated in Figure 2.5(a), which has been a standard since being proposed by W.C. Wiley and I.H. McLaren in 1955 [34]. The system is modeled by transfer matrix method in Figure 2.6. $[r, \dot{r}]$ vector at the input plane of the extractor electrode is calculated via Equation 2.20 and 2.21:

$$\begin{bmatrix} r_1 \\ \dot{r}_1 \end{bmatrix} = \begin{bmatrix} 2L_{0 \rightarrow 1} \sqrt{\frac{V_0}{U_0 - U_1}} \sin \alpha_0 + r_0 \\ \sqrt{\frac{V_0}{U_0 - U_1}} \sin \alpha_0 \end{bmatrix}, \quad (2.32)$$

where $L_{0 \rightarrow 1}$ is the distance between repeller and extractor, U_0 the voltage on repeller, U_1 the voltage on extractor, r_0 the emitting position of the particle, α_0 the emitting angle relative to the surface normal of repeller, V_0 corresponds to the initial kinetic energy,

$$V_0 = \frac{mv_0^2}{2q}. \quad (2.33)$$

The transfer matrix of the extractor electrode is derived from Equation (2.31),

$$M_1 = \begin{bmatrix} 1 & 0 \\ -1/f_1 & 1 \end{bmatrix}, \quad (2.34)$$

f_1 is the focus length of the aperture on the extractor,

$$f_1 = \frac{4(U_1 - U_0 + V_0)}{E_{1 \rightarrow 2} - E_{0 \rightarrow 1}}. \quad (2.35)$$

$E_{1 \rightarrow 2}$ and $E_{0 \rightarrow 1}$ are the electric field strengths on the right and left sides of the extractor respectively with

$$E_{0 \rightarrow 1} = \frac{U_0 - U_1}{L_{0 \rightarrow 1}}, \quad (2.36)$$

$$E_{1 \rightarrow 2} = \frac{U_1 - U_2}{L_{1 \rightarrow 2}}, \quad (2.37)$$

where U_2 is the voltage on the grounded electrode which equals to 0, $L_{1 \rightarrow 2}$ the distance between the extractor and grounded electrode. The trajectory of the charged particle between the extractor and grounded electrodes is calculated with the transfer matrix for the uniform electrostatic field Equation (2.18),

$$M_{1 \rightarrow 2} = \begin{bmatrix} 1 & \frac{2L_{1 \rightarrow 2}}{\sqrt{V_1/V_2+1}} \\ 0 & \sqrt{V_1/V_2} \end{bmatrix} \quad (2.38)$$

where $V_1 = U_0 - U_1 + V_0$ and $V_2 = U_0 - U_2 + V_0$, and

$$M_2 = \begin{bmatrix} 1 & 0 \\ -1/f_2 & 1 \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ \frac{E_{1 \rightarrow 2}}{4(V_0 + U_0 - U_2)} & 1 \end{bmatrix}. \quad (2.39)$$

After the charged particle leaves the ion optics, it drifts through a field free flight tube before terminating on the detector plane, the transfer matrix $TM_{2 \rightarrow 3}$ is given in Equation (2.19).

Therefore, the $[r, \dot{r}]$ vector at the detector is given as

$$\begin{bmatrix} r_3 \\ \dot{r}_3 \end{bmatrix} = M_{2 \rightarrow 3} \cdot M_2 \cdot M_{1 \rightarrow 2} \cdot M_1 \cdot \begin{bmatrix} 2L_0 \rightarrow \sqrt{\frac{V_0}{U_0 - U_1}} \sin \alpha_0 + r_0 \\ \sqrt{\frac{V_0}{U_0 - U_1}} \sin \alpha_0 \end{bmatrix}. \quad (2.40)$$

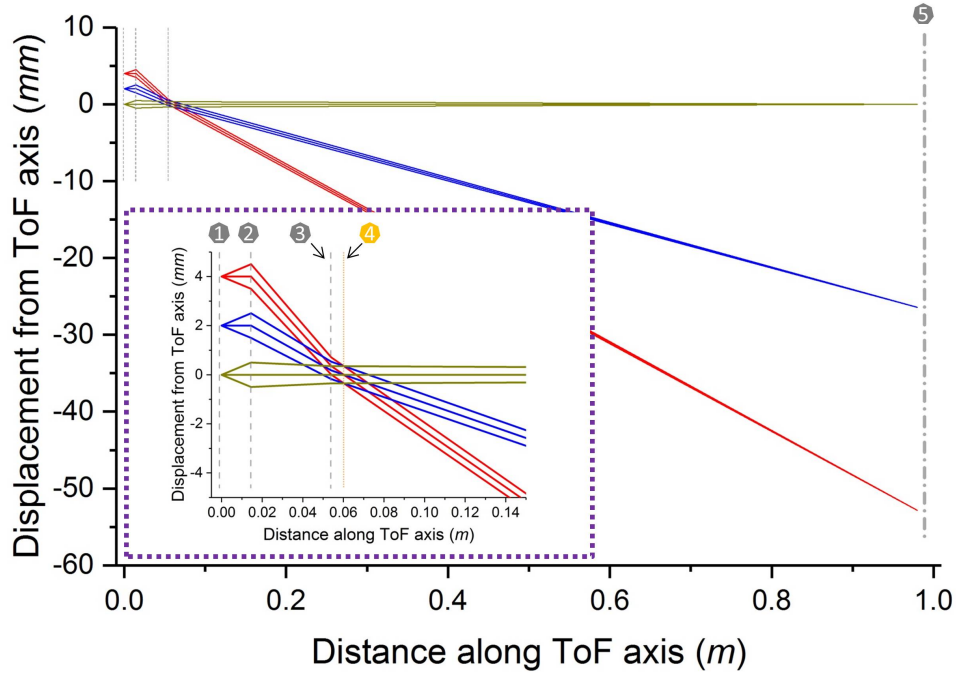


Figure 2.6: A three-electrode spatial imaging instrument is modeled with the transfer matrix method, the inset is an enlarged view of the ion optical system: (1) repeller, (2) extractor, (3) grounded electrode, (4) velocity-map imaging plane, and (5) detector plane.

For a charged particle generated from the repeller surface with a known initial distance from the axis r_0 , and known initial speed v_0 , and direction α_0 (angle between the

flight direction and repeller surface normal), its arriving position on the detector can be determined with Equation (2.40) by substituting all its variables with the corresponding dimensions and voltages of the instrument.

By connecting the starting point (r_0, x_0) on the repeller, the position on the extractor $(r_1, x_0 + L_{0 \rightarrow 1})$, the position on the grounded electrode $(r_2, x_0 + L_{0 \rightarrow 1} + L_{1 \rightarrow 2})$, and the final arrival point on the detector $(r_3, x_0 + L_{0 \rightarrow 1} + L_{1 \rightarrow 2} + L_{2 \rightarrow 3})$, we can estimate the trajectory of a charged particle in the ion optical system in Figure 2.6, the red, blue, and dark yellow lines represent particle trajectories starting from three separate point sources on the sample surface (r_0 equals to 4 mm, 2 mm, and 0 mm, respectively). From each point source, three distinct trajectories are emitted which correspond to particles with the same initial speed ($v_0 = 500$ m/s) but towards three different directions (r_0 equal to 30° , 0° , or -30°). At the detector plane, the ion image is magnified by about 13 times. It should be noted that the trajectories in acceleration regions $L_{0 \rightarrow 1}$ and $L_{1 \rightarrow 2}$ are only an approximation to the true trajectories, which are actually parabolic. The orange dashed line in Figure 2.6 is the so called velocity mapping imaging (VMI) plane [19], where ions with the identical velocity are focused to a single spot on the detector regardless of their initial position. VMI is a widely adopted method in the fields of molecular reactions, energy transfer processes, and photodissociation, where the internal energies and velocities of all products are of particular interest to scientists [19, 103, 104].

Experiments in this thesis were actually performed with a five-electrode ion optical system as shown in in Figure 2.5(b) (more details in Chapter 3 and 4). Two more electrodes were equidistantly added after the third electrode of the three-electrode ion optics. By holding the third and fifth electrode at ground and raising the fourth one to a defined positive potential, an einzel lens assembly was formed, which provided extra flexibility when adjusting the position of the spatial focusing plane. A five-electrode ion optical system becomes necessary when employing the PEDDA method because it ensures that both the temporal focusing plane of PEDDA and the spatial focusing plane of the ion optics overlap with the detector plane. This five-electrode system is also modeled

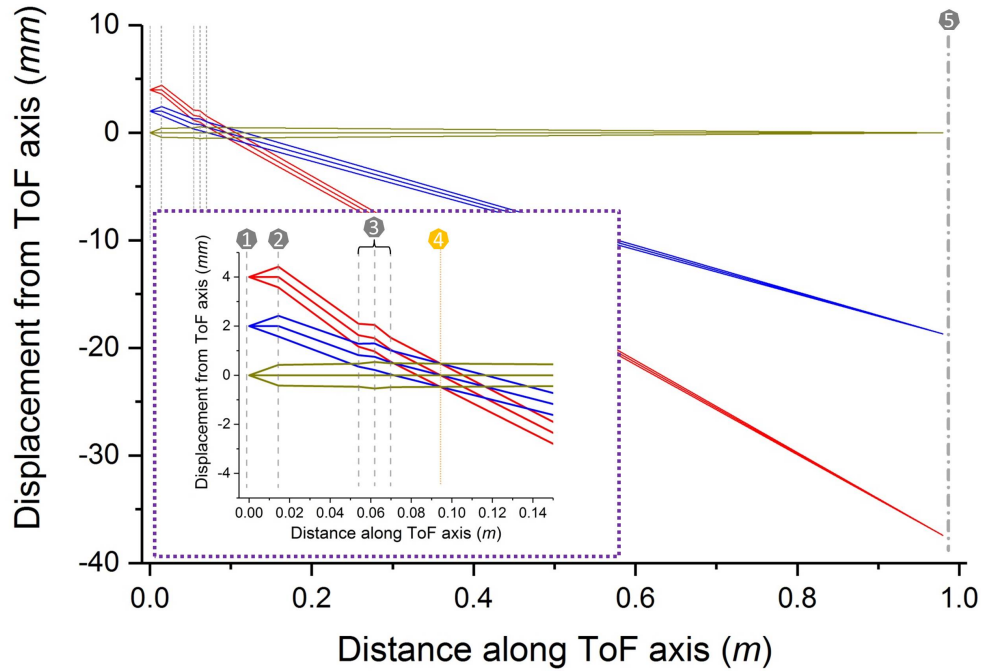


Figure 2.7: Our five-electrode spatial imaging instrument is modeled with the transfer matrix method, the inset is an enlarged view of the ion optical system: (1) repeller, (2) extractor, (3) einzel lens assembly, (4) velocity-map imaging plane, and (5) detector plane.

here with the transfer matrix method and its resulting trajectories are plotted in Figure 2.7. The magnification of this ion optical system is predicted to be 9.4, which agrees reasonably well with the experimental results (9.6 to 9.9) [95].

2.2 SIMION

SIMION is a commercial simulation package, capable of modeling ion/electron optics *in silico*. It was originally developed by David Dahl at the Idaho National Laboratory in 1973 [105]. Basically, SIMION does two essential jobs: the first is to calculate electric and magnetic fields in highly complex instruments, and the other is to calculate the motion of charged particles (ions or electrons) in these static or time-dependent electric and/or magnetic fields. All of the ion optics related experiments in this thesis were modeled and studied in SIMION before being developed in reality. SIMION is a powerful tool, which

can not only assist in the electrode design of ion optics, but also predict experimental results accurately. In the second half of this chapter, various aspects associated with SIMION simulations will be briefly introduced.

2.2.1 Potential Arrays

In SIMION, electrostatic and magnetic fields are represented by “potential arrays”. A potential array (PA) is a square (2D) or cubic (3D) grid of equally-spaced points (see Figure 2.8(b) and (c)). Each point in a PA defines the potential (*e.g.* voltage) and type (*e.g.* electrode, magnetic pole, or non-electrode and non-pole) of that location in space. For those points whose types are electrode or pole, their potentials are often known by the user and set as the boundary conditions for the array. Of course, an electrode of any shape in real life can be replicated in SIMION through a variety of methods. In our case, mathematical geometries are described in a text file that used to model the electrodes in a PA. This text file is called a “Geometry file” (an ASCII file with a “.GEM” extension) as illustrated in Figure 2.8(a).

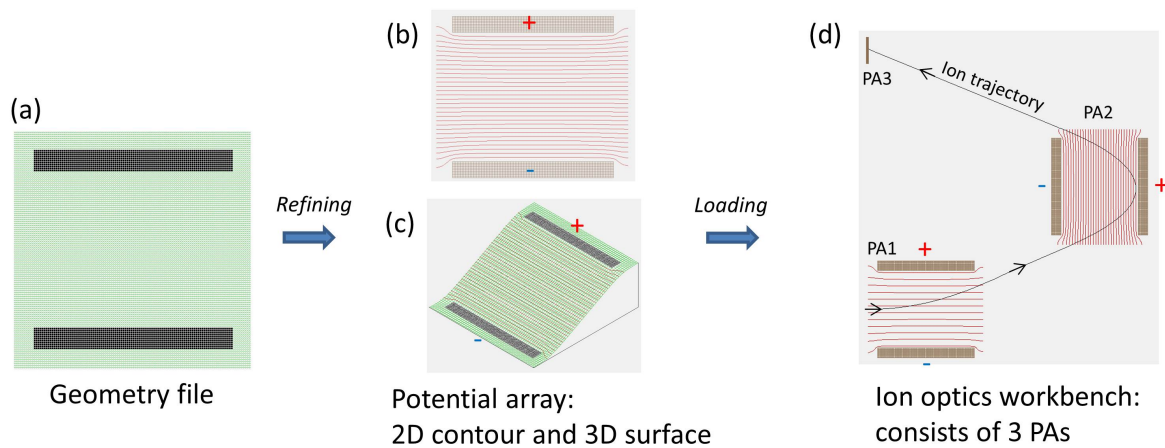


Figure 2.8: Building an ion optics workbench: (a) geometry files are used to define electrodes, (b) a refined potential array with 2D potential contour lines, (c) the same PA viewed as a 3D potential surface, (d) an ion optics workbench comprising three PA instances and a simulated trajectory of an ion moving in the ion optics.

A finer grid of space points in a PA results in a smoother electrode shape. However, this comes at high and sometimes unaffordable computational cost (each point requires 10

bytes of RAM, which means a $100 \times 100 \times 100$ 3D PA requires 10 megabytes of RAM). To tackle this challenge, three types of array symmetries (planar, cylindrical, and mirroring) are supported in SIMION when defining PAs, allowing smaller arrays to model larger areas (or volumes).

Potentials of all other points (non-electrode and non-pole points) within the array are numerically calculated by SIMION (or “refined” in SIMION terminology). More details about this refining process will be introduced later in the next subsection. For a charged particle, once the electrostatic potential and corresponding electric fields around it are known, the force on it and its movement in space can be calculated using Newton’s second law. There are various ways to visualise the PA in SIMION. For instance, in potential energy view (PE view), the vertical direction depicts the magnitude of potential corresponding to each point in the array as shown Figure 2.8(c). This is a very intuitive way to better understand ion motion in PAs (if we see ions traveling over potential surfaces as marbles rolling through hills and valleys). Equipotential planes of an electric field can be plotted in SIMION as well, as shown in Figure 2.8(b).

2.2.2 Solving Electrostatic Field and Ion Trajectory with SIMION

Ion optics harness the electrostatic and magnetic forces on charged particles to manipulate their trajectories. To explain this, some background theorems in electrodynamics will be briefly described in this section.

The first is Gauss’ law, which relates the electric field out of a closed surface to the net charge inside the volume enclosed by that surface. The total net electric flux through a closed surface is equal to the net charge enclosed times the reciprocal of the permittivity of the space:

$$\int_S \mathbf{E} \cdot d\mathbf{a} = \frac{Q}{\varepsilon_0}, \quad (2.41)$$

where S is a closed surface. $\mathbf{E} = \mathbf{E}(\mathbf{r}) = [E_x, E_y, E_z]$ represents the electric field at point $\mathbf{r} = [x, y, z]$, and $\mathbf{a} = \mathbf{a}(\mathbf{r}) = [a_x, a_y, a_z]$ is an infinitesimal area on the surface and the

direction of $\mathbf{a}$ is the surface normal. ε_0 represents the permittivity of free space. Q is the total net charge within the volume enclosed by S . Gauss' law is one of Maxwell's four fundamental equations which elegantly and concisely describe the interactions of electricity and magnetism.

As the size of S approaches zero, the divergence theorem in calculus allows Gauss' law to be reformulated as a differential:

$$\nabla \cdot \mathbf{E} = \frac{\rho}{\varepsilon_0}, \quad (2.42)$$

where ρ represents the charge density (charge per unit volume) at point $\mathbf{r} = [x, y, z]$.

The gradient relationship between electric field and potential is

$$\mathbf{E} = -\nabla V, \quad (2.43)$$

where $V = V(\mathbf{r})$ represents the potential energy at location $\mathbf{r} = [x, y, z]$. Since the electric field $\mathbf{E}$ is the negative gradient of the electric potential V , $\mathbf{E}$ can be readily computed from the PE surface or vice versa.

Combining Equation (2.42) and (2.43) produces Poissons equation, which relates a potential to the charge density distribution that gives rise to it:

$$\nabla^2 V = -\frac{\rho}{\varepsilon_0}. \quad (2.44)$$

If the charge volume density ρ is non-zero, we say the system has “space-charge”. Once ρ becomes great enough (*i.e.* ions are too close to each other), the electrostatic potential field will be considerably distorted by “space-charge effects”. Of course, the space-charge effects have a direct (usually negative) impact on ion trajectories but taking them into consideration is not a trivial job in SIMION. As electric fields and particle trajectories become mutually coupled, the simulations will be much more complex: if the electric fields in space depend on the space-charges, any change of the space-charge distribution

will affect the electric fields, which will in turn alter the charge distribution in an endless loop (since the motion of space-charges is driven by the forces from the electric field). In our experiment, only several dozens of ions are generated during each laser pulse (this is estimated by the number of ion events recorded by the camera in each frame), the space-charge effects are negligible for such small ion beam currents. As a result, all simulations done in this thesis ignores the space-charge effects to keep things simple. When ρ is 0, Equation (2.44) becomes Laplace's equation:

$$\nabla^2 V = 0, \quad (2.45)$$

Given a boundary condition, SIMION is able to solve the Laplace equation and calculate the electric potentials as well as the corresponding electric fields in space.

Laplace's equation can be uniquely solved under two types of boundary conditions: (1) the value of V is specified on all boundaries (the "Dirichlet boundary conditions") and (2) its normal derivative dV/dn is specified on all boundaries ("Neumann boundary conditions"). An example of Dirichlet boundary conditions is the surface of a perfectly conductive electrode. Typically, the electrode is held to a known potential by connecting them to a power supply or ground, so the Dirichlet boundary condition is satisfied. There are also a number of cases that can be treated as Neumann boundaries. For example, dV/dn at non-electrode points over a plane of mirror symmetry are set to zero in SIMION and dV/dn on open PA edges are also set to zero.

A solution of Laplace's equation is called a "harmonic function", which has the following properties: (1) for a harmonic function, the average value over a spherical surface equals the value at the centre of that sphere:

$$V = \frac{1}{4\pi r^2} \oint_S V da, \quad (2.46)$$

(2) solutions have no local maxima or minima, and (3) since the Laplace's equation is linear, the sum of two or more individual solutions is also a solution.

A numerical method called the “Finite Difference Method (FDM)” is used in SIMION to solve the Laplace’s equation, which is essentially an iterative averaging process that generates a solution using property (1). In a 2D or 3D electrostatic field represented by a very fine grid of points, one can find a potential surface that satisfies the Laplace’s equation to a good approximation by estimating the potential of each point as the average of its 4 (in 2D case) or 6 (in 3D case) nearest neighbor points. The estimated potential at each non-electrode point is updated using these averages, and the entire PA is scanned sequentially (point by point). Each scan through the PA is an iteration. Each successive iteration propagates the potentials of the electrode points further throughout the PA. This iteration process continues until an equilibrium is reached: in a single iteration the potential of no point in the PA changes by more than a predefined convergence limit. At this point the potentials of all non-electrode and electrode points have been estimated and are said to be “refined”. In the simulation, it is often necessary to modify the potentials on ion optics, for example to improve spatial focusing through trial-and-error or to apply a pulsed voltage on certain electrodes. This would be extremely time consuming using the Modify-Refine cycle to adjust voltages. To deal with this, SIMION offers a “Fast Adjust” method, making use of property (3), the additive solution property of the Laplace equation. So each electrode has a separate PA that needs to be adjusted. For every PA, the corresponding electrode potential is set, say, to 1 V and all other electrodes are set to zero. The set of PAs are then refined and saved. Composite fields are created by scaling the potential map of each PA properly (the scaling factor equals the voltage applied on the corresponding electrode if it is set to 1 V when the PA is being refined) and the individual potential contributions of all adjustable electrodes are summed. The composite potential is computed as follows:

$$P_n = k_1 \cdot P_{n1} + k_2 \cdot P_{n2} + k_3 \cdot P_{n3} + k_4 \cdot P_{n4} + k_4 \cdot P_{n5} + \cdots, \quad (2.47)$$

where n refers to a particular point in space, P_n is the composite potential at that point, scaling factor k_i equals the voltage applied on the corresponding electrode of the i^{th} PA,

and P_{ni} is the potential at point n determined using the i^{th} PA.

Ion trajectories with known initial velocities in electric and/or magnetic fields are determined by the Lorentz force law along with Newton's second law of motion. A particle of charge q flying with a velocity $\mathbf{v}$ in the presence of an electric field $\mathbf{E}$ and a magnetic field $\mathbf{B}$ experiences a force:

$$\mathbf{F} = q[\mathbf{E} + (\mathbf{v} \times \mathbf{B})]. \quad (2.48)$$

And its acceleration is thus:

$$\mathbf{F} = m\mathbf{a}. \quad (2.49)$$

Three steps of computations are conducted to calculate these ion trajectories. First, electrostatic forces upon the ion are computed based on its current position and velocity using Equation (2.48). Then, the current ion acceleration is calculated using Equation (2.49) and a standard fourth order Runge-Kutta method is employed as a numerical integration method to predict the position and velocity of the charged particle at the next time step. In addition, the time step continuously self-adjusts to maintain accuracy while minimising the number of necessary integrations.

2.2.3 Ion Optics Workbench

To simulate a complex system, we may need multiple PAs, each represents a different component of our instrument. For example, in our case, there are PAs for the ion optics chamber, field-free drift chamber, the detector, etc. Each components is defined and modeled in an independent way. This helps the user to take full advantage of symmetry that may be different for different components of the instrument. In SIMION, these refined PAs are loaded into a so-called ion optics workbench. SIMION's ion optics workbench (or just workbench) is an imaginary 3D rectangular volume of space (up to 8 cubic km) within the simulated universe. PAs (up to 200 instances) are assembled together in the workbench. Ions experience the electric field of each PA in the order represented by the

PA locations in space. The relationship between geometry files, PAs, and workbenches is summarised in Figure 2.8. The workbench is saved as an .IOB file, which is the main SIMION project file. An IOB file links all other files together, such as different PA instances, FLY2 files, and LUA files (which will be discussed later). In the IOB file, the dimensions (length, width, height) of the simulation universe enclosing the system are defined, and the scaling, rotation, and translation of all potential arrays are specified (therefore different instrument geometries can be investigated easily). IOB files also apply voltages on any fast adjustable instances in the simulation. Basically, all project-wide settings are specified in an IOB file, so running a SIMION simulation actually means running the IOB file. Figure 2.9 sums up the pieces that comprise an IOB file.

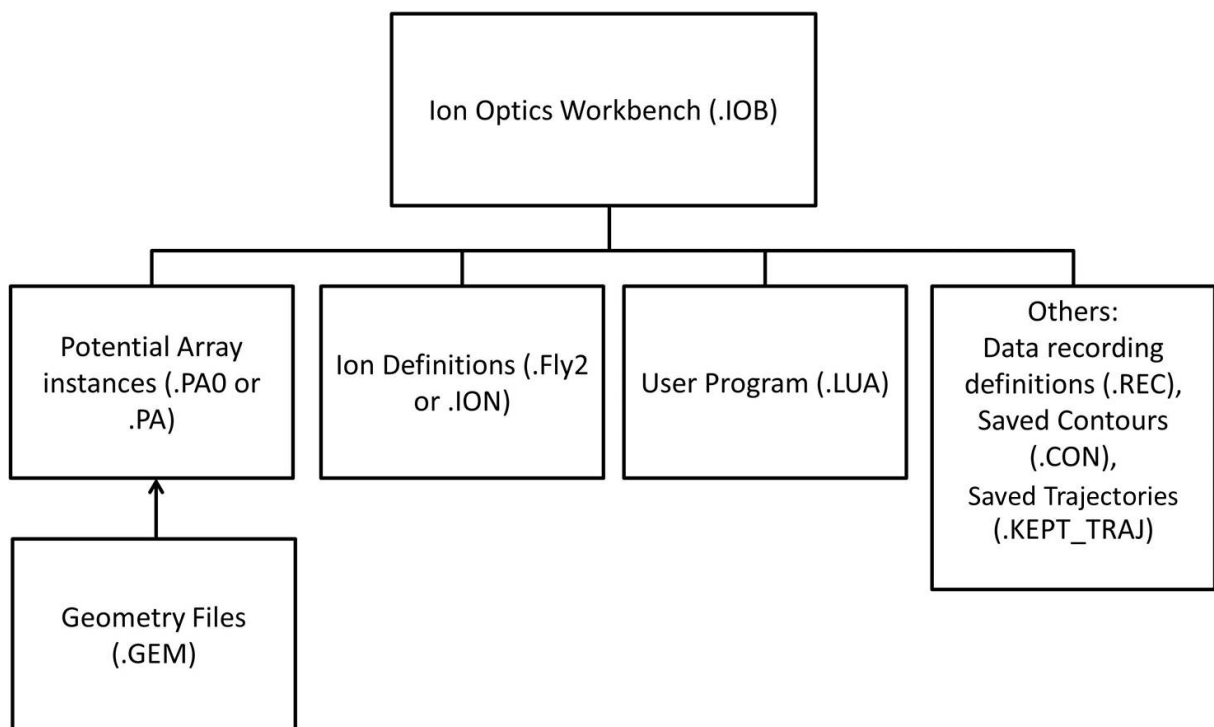


Figure 2.9: Components of a SIMION ion optics workbench

So far a few indispensable steps/components of a basic SIMION simulation have been introduced: (1) geometry files which define the geometry of any desired electrodes in ion

optics; (2) potential arrays, which are generated by refining the geometry files, and are responsible for constructing electric potential surfaces inside the simulated ion optics; (3) the ion optics workbench, which describes the entire simulation. As mentioned earlier, besides offering a fictitious space where we load in all parts of the simulated instrument, an IOB file also contains other useful files, such as ion definitions and user programs, which work together in a SIMION simulation project. These will be detailed in the following subsections.

2.2.4 Defining An Ion

Before flying a charged particle in SIMION, one has to define each particle using a collection of parameters. The ion's mass is defined in atomic mass unit (amu) and its charge is defined in elementary charge unit (e). The repeller determines the ion's starting location. Based on an assumption that the topography of the repeller (and sample) surface is flat, all simulated ions are created from a single plane. In our experiment, samples are prepared using an electrospray device (to be introduced later in Chapter 3), which forms a very homogeneous surface. The variance in thickness of samples is less than 50 nm [106].

To define the creation time of ions correctly, we need to look into a quantitative model which depicts the ionisation process of UV-MALDI. This model was proposed by R. Knochenmuss and coworkers in ref. [107]. A two step frame was described in the model which includes a primary and a secondary ionisation process. A key microscopic mechanism called “energy pooling” occurs in the primary ionisation process, which illustrates how the electronic excitation energy is redistributed between two neighbouring excited molecules. Basically, through a pooling process photon energy gets concentrated on one matrix molecule and eventually ionises it. In the secondary ionisation process, the density changes slowly enough for many collisions to take place. Consequently, the primary matrix ions react with the neutral analytes as follows:



where M stands for matrix and A for analyte. So analyte ions are produced in this process. The plume of laser-desorbed material is rather dense and lasts long enough in typical MALDI experiments. So it can be studied by conventional kinetic and thermodynamic methods. For example, rate equations were solved numerically and modeled in Figure 2.10. 2,5-dihydroxybenzoic acid (DHB) was used as the matrix molecule used in this example. The laser wavelength was 355 nm and the laser fluence was 24 mJ/cm^2 . The matrix-to-analyte reaction exoergicity was 150 kJ/mol , typical for proton transfer from $(\text{DHB})\text{H}^+$ to small peptides. The concentration of analyte (1,000 Da) was 0.015 mol/L .

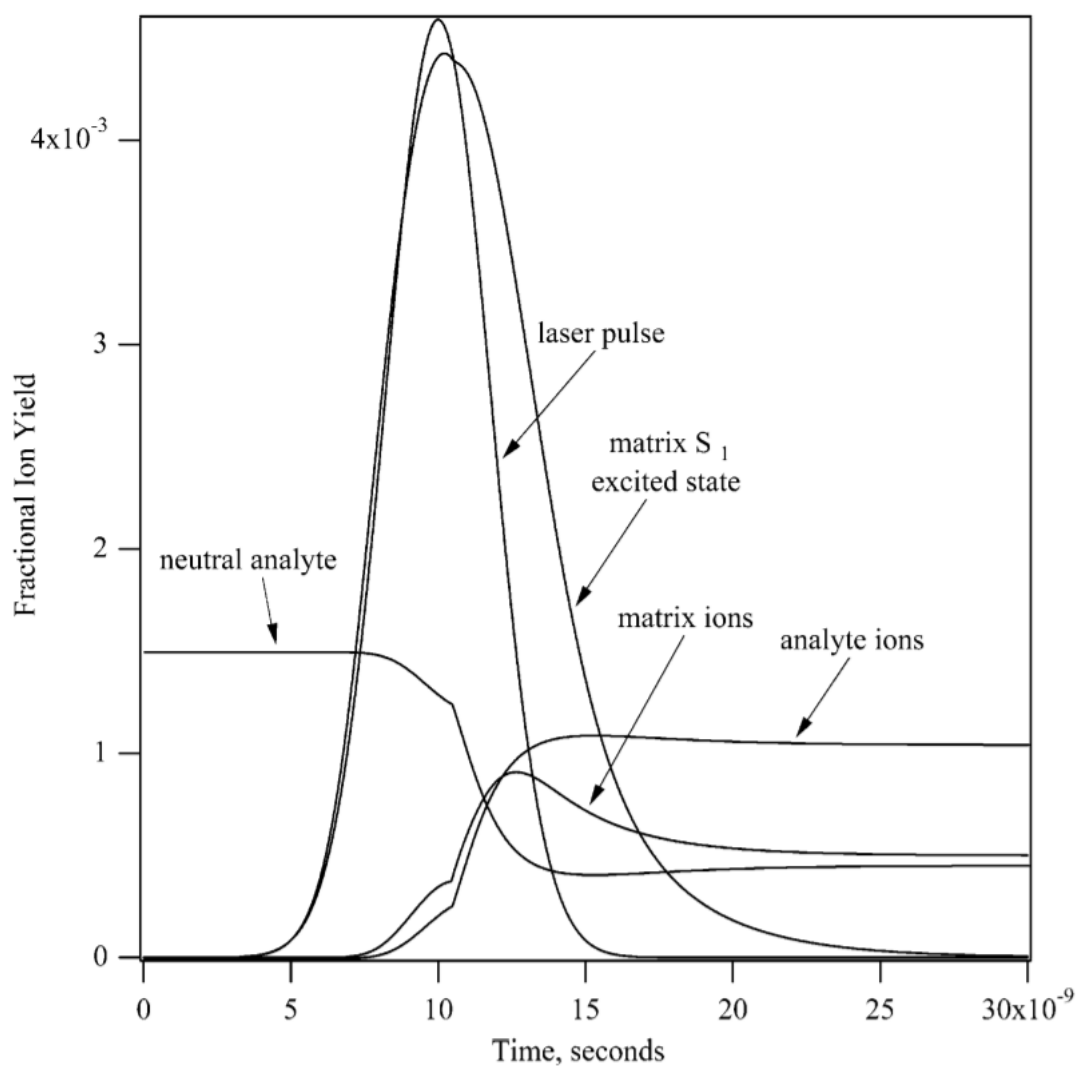


Figure 2.10: The time-dependent fractional ion yield of MALDI sample in the primary-secondary ionisation model. Reprinted from [107]

The number of matrix molecules in S_1 excited states rapidly increases during the laser pulse. In the primary ionisation process, matrix ions are yielded following the energy pooling among abundant excited matrix molecules. In the secondary ionisation process, reactions between matrix ions and neutral analytes give rise to analyte ions while decreasing the number of the matrix ions. It should be noted that secondary ionisation processes begin as soon as primary ions are created, so the two steps occur at the same time. As the plume expands and becomes dilute, the bulk of the reaction stops within 10 ns. Only the time-dependence of analyte ion yield actually matters in our SIMION simulation. As shown in Figure 2.10, the primary and secondary ionisation processes begin to accelerate at ≈ 11 ns as the sample starts to sublime when its temperature rises to 450 K. Therefore, the analyte ion yield follows a piecewise function over time. However, for simplicity in the simulation, a continuous normal cumulative distribution function was used as an approximation,

$$\text{yield}(x) = \frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^x e^{-\frac{(t-\mu)^2}{2\sigma^2}} dt. \quad (2.51)$$

This fits the yield curve reasonably well. Thus, the analyte ion yield rate or Time of Birth (TOB) of the analyte ions follows a normal distribution with mean TOB (μ) equal to 11 ns and standard deviation (σ) equal to 3 ns.

In the experiments described in this thesis, ions generated by laser ablation always possess a spread of non-zero initial speed along the ToF axis, which is responsible for a limitation of linear ToF analysis (more details on this issue will be discussed in Chapter 4, where the PEDDA method along with its two variants will be introduced). There is also an angular distribution of the initial velocities of the ions as they leave the sample surface. This is another critical parameter in simulation: since angular spread also contributes to the deterioration of mass resolution (for ions of identical m/z and same initial kinetic energy, if their starting directions are different, their speed along the ToF axis will be as well, resulting in a difference of ToF). More importantly, it is the angular distribution

that causes the blur of ion images recorded by the detector. As discussed earlier in this chapter, owing to the lensing effect of inhomogeneous extraction electric fields, our spatial imaging instrument is able to minimise this blur (or in other words to spatially focus the ions on to the detector plane) and achieve a relatively good spatial resolution. Therefore, the efficacy of simulating our experiment in SIMION relies heavily on a solid estimate of the distribution of the ion's starting kinetic energy and ion's starting direction. As shown in Figure 2.11, two angles, namely azimuth (Az) and elevation (El), are used to define an ion's starting direction. The X , Y , and Z axes in Figure 2.11 are the same coordinates used to define the particles' starting location.

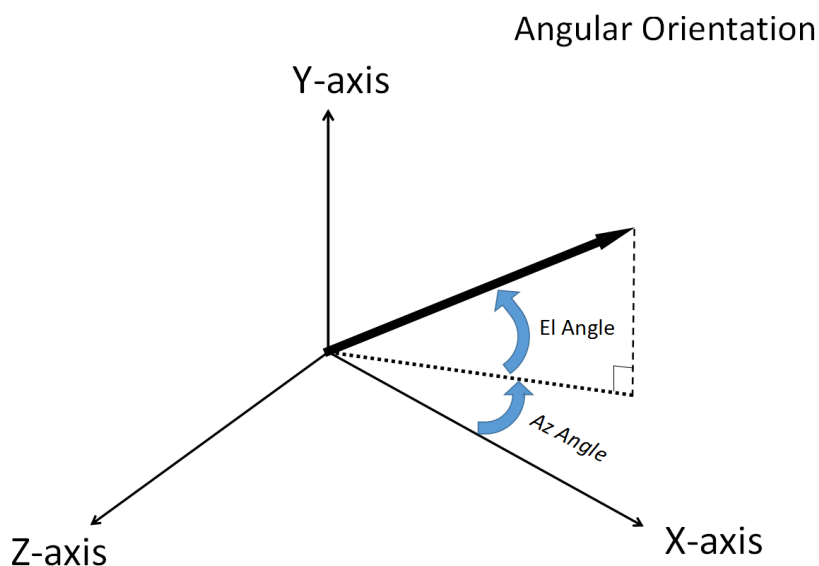


Figure 2.11: Illustration of azimuth (Az) and elevation (El) angles

Beavis and Chait [108] measured the velocity distribution of intact protonated polypeptide ions (in a mass range between 1,000 and 15,600 Da) emitted along the sample surface normal (*i.e.* the ToF axis) during MALDI. An mean axial velocity of approximately 750 m/s was determined by them and it is independent of the molecular mass. Verentchikov and coworkers [109] confirmed this lack of dependence of the axial velocity on the molecular mass over a broader range of polypeptide masses, but the mean axial velocity they obtained is slightly lower (approximately 500 m/s). So in MALDI experiments, the ini-

tial velocity v_0 of analyte ions (peptides and proteins) is typically between 300 and 800 m/s and approximately independent of analyte mass. Moreover, the initial ion velocity is neither affected by the ion's charge state and polarity, or the laser fluence within the practical fluence range. v_0 merely depends on the matrix choice and other experimental parameters that may affect crystallisation, such as solvents and additives. These variables may lead to a considerable increase or decrease of v_0 . Typical initial ion velocities of various analyte and matrix combinations are summarized in Table 2.1. v_0 also differs for samples in different crystallisation states. For example a coarsely crystalline droplet rim and a microcrystalline inner part. As a result, the initial velocity can show a considerable spread ($v_0 \pm 0.5v_0$).

analyte	matrix	ion	m/z	v_0
bovine insulin	SINA	$[M + H]^+$	5735	332 ± 29
bovine insulin	α CHCA	$[M + H]^+$	5735	291 ± 51
bovine insulin	α CHCA	$[M + 2H]^{2+}$	2868	251 ± 75
bovine insulin	3-HPA	$[M + H]^+$	5735	620 ± 87
DNA-24mer	3-HPA	$[M + nH]^{n+}$ ($n = 1, 2$)	7374/3687	619 ± 16
bovine insulin	THAP	$[M + H]^+$	5735	306 ± 32
bovine insulin	THAP + THAC	$[M + H]^+$	5735	201 ± 19
bovine insulin	2,5-DHB	$[M - H]^-$	5733	535 ± 48
bovine insulin	2,5-DHB	$[M + H]^+$	5735	543 ± 40
bovine insulin	2,5-DHB + fructose	$[M + H]^+$	5735	659 ± 49
carbonic anhydrase	2,5-DHB	$[M + H]^+$	29025	599 ± 119
melittin	2,5-DHB	$[M + H]^+$	2848	530 ± 52
maltoheptaose glycosylamine	2,5-DHB	$[M + H]^+$	1154	515 ± 66
maltotetraose	2,5-DHB	$[M + Na]^+$	689	187 ± 97
human-milk oligosaccharide 1	2,5-DHB	$[M + Na]^+$	730	340 ± 10
human-milk oligosaccharide 2	2,5-DHB	$[M + Na]^+$	1242	461 ± 04
human-milk oligosaccharide 3	2,5-DHB	$[M + Na]^+$	2776	545 ± 29

Table 2.1: Initial ion velocities (v_0) of some analyte ions using different matrices [110].

In terms of angular distribution, the radial initial velocity is not comparable to the axial initial velocity, creating a strongly forward peaked MALDI plume. Therefore, the initial angular distributions of ions are strongly forward peaked. Zhang and Chait determined the radial velocity distributions of both analytes and matrix ions with a laser power just greater than the threshold for ion production [109]. The angular distribution follows a form of $\cos^n \theta$, where n was dependent on the ion mass. The angular distribution for protonated dynorphin 1-13 can be fitted well by a $\cos^{28} \theta$ distribution, while that for the protonated CHCA dimer is best fitted by a $\cos^{12} \theta$ distribution. Therefore they concluded that heavy peptide species have a considerably stronger forward orientation than those

light matrix ions. Fly2 files are used to define particles for SIMION simulations, but there is no option for a $\cos^n \theta$ distribution of angles. Instead, a Gaussian function is used to approximate this.

Since there is no consensus on defining ions' starting direction when simulating MALDI process, the average initial speed was set at 500 m/s with a FWHM of 500 m/s, and the average direction was set normal to the sample surface (Az and El angles are 0°) with a FWHM of 60° (in both the Az and El angle distributions). These values are estimated from existing studies [108, 109, 111, 112] and proved a good approximation of reality in previous work [94].

2.2.5 User Programs in SIMION

A user program plays an extremely powerful role in SIMION, which considerably extends the scope of “simulatable” situations and automates essential tasks. Although various programming languages, including Lua, C/C++, PRG, and others, can be used to code the user program, LUA was chosen for its efficiency, flexibility, and simplicity. Here are a few examples of tasks that user programming interface does in the simulation:

- (1) Initializes variables and defining useful functions.
- (2) Initializes the system state at the start of a simulation run, such as electrode voltages.
- (3) Applies time-dependent voltages to electrodes. For example, in the original PEDTA technique (to be discussed in Chapter 4), voltage on the extractor steps up after certain delay time when ions are flying.
- (4) Enables “ion jumping” tricks that estimate the lensing effect of a grid on ion trajectories. A large repeating grid of wires is represented by a small section of it by jumping ions into and out of that small representative section (more details in Chapter 5).
- (5) Outputs data and performs calculations. The user program can choose what data to record after a simulated experiment and do a series of calculations based on these data

to present informative results such as magnification, spatial resolution, mass resolution, and so on.

2.2.6 Interpreting Simulation Results

Once the entire experiment has been modeled, SIMION “flies” simulated ions through all the ion optics until they impact the simulated detector plane. In the user program, with the help of `segment.terminate()` function, two useful types of data can be recorded: when and where an ion hits the detector (*i.e.* Time of flight (ToF) and (X,Y,Z) position of an ion when its flight ends). From the recorded ToF and position data, various information can be extracted to characterise the performance of the instrument, including its field of view, hit/miss ratio, magnification, and most importantly mass and spatial resolutions.

The initial position of the ions are defined as a sequence of equally spaced discrete point sources along the Y axis, with the first point source located at the centre of the repeller (see Figure 2.12(a)). From each ion source, 500 particles are generated with a distribution of starting speed and direction defined in subsection 2.2.4. The one dimensional sequence of ion sources is sufficient to represent the two dimensional sample surface thanks to the cylindrical symmetry of the ion optics.

Once the flight time has been collected for each ion, we are able to calculate its mean $\bar{t}$ as well as the standard deviation σ_{ToF} for the entire ion package. The distribution of flight time is shown in Figure 2.12(c). The mass resolution of our simulated instrument is defined as

$$m/\Delta m \approx \frac{\bar{t}}{2\Delta t} = \frac{\bar{t}}{2 \cdot 2\sqrt{2\ln 2}\sigma_{ToF}} \approx \frac{\bar{t}}{4.71\sigma_{ToF}}, \quad (2.52)$$

where Δt and σ_{ToF} are the full width half maximum (FWHM) and the standard deviation of the arrival time distribution, respectively. If the arrival time is assumed to be a normal distribution, the ratio between FWHM and standard deviation is a constant $2\sqrt{2\ln 2}$. Here the ToF resolution is used to approximate the mass resolution. However, Equation (2.52)

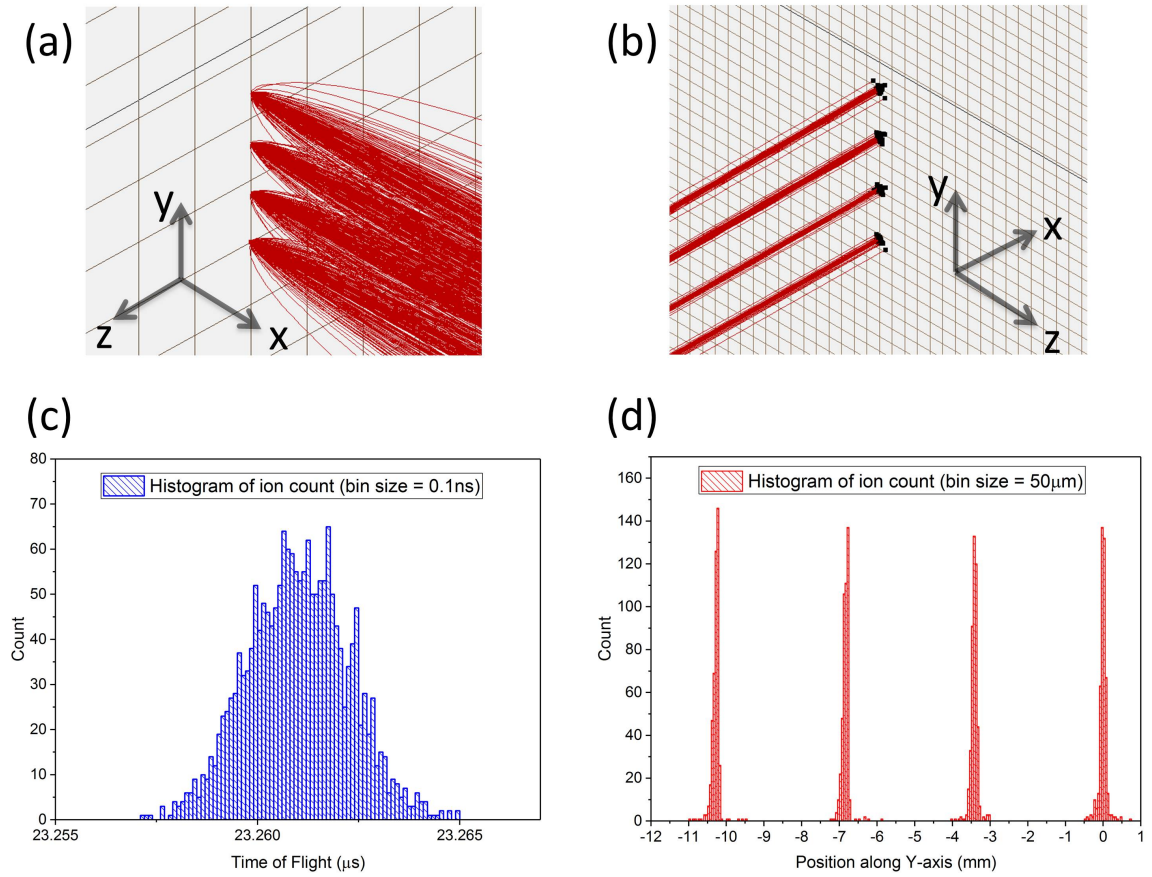


Figure 2.12: SIMION data: (a) a progression of four point ion sources with 500 particles originating from each, (b) ions are projected onto the detector plane to form four separate splats, (c) histogram plot of ion count as a function of total time of flight, (d) histogram plot of ion count as a function of final position on the detector plane along the Y-axis.

is valid only when the response time of the detector assembly is negligible compared with Δt , which must otherwise be taken into account when calculating mass resolution.

As will be described in more details in Chapter 3, the ToF signals collected in these experiments are usually photons emitted from a scintillator. Therefore the fluorescence lifetime of the scintillator contributes largely to the overall detector response time. For example, the rise time (from 10% to 100% of peak intensity) and the fall time (from 100% to 10% of peak intensity) of the Exalite 404 scintillator screen are 3.5 ± 0.2 ns and 7.7 ± 0.3 ns respectively, which are comparable to the ≈ 5 ns temporal spread of ions arriving at the detector as indicated by the ion trajectory simulations under the operating conditions of the spectrometer. Therefore the total distribution of observed

signal has to be the result of the convolution integral of the arrival time distribution and the response time distribution. If both of the aforementioned distributions are assumed to be close to Gaussian-like functions with standard deviations σ_{ToF} and σ_{det} respectively, the convolution of them will be another Gaussian function whose standard deviation σ_{tot} has the following form:

$$\sigma_{tot} = \sqrt{\sigma_{ToF}^2 + \sigma_{det}^2}, \quad (2.53)$$

and as a result the mass resolution after taking the detector response time into consideration is:

$$m/\Delta m \approx \frac{\bar{t}}{2\Delta t_{tot}} \approx \frac{\bar{t}}{4.71\sigma_{tot}} = \frac{\bar{t}}{4.71\sqrt{\sigma_{ToF}^2 + \sigma_{det}^2}}, \quad (2.54)$$

where Δt_{tot} is the total FWHM of temporal distribution of the observed ToF signal.

Since

$$\lim_{\sigma_{ToF} \rightarrow 0} \Delta t_{tot} = 2\sqrt{2\ln 2}\sigma_{det} = 2.355\sigma_{det}, \quad (2.55)$$

the response time of the detector assembly puts a practical limit on the highest mass resolution that an ideal ToF mass spectrometer can possibly reach. Also, it should be noted that the mass resolution obtained here is valid for one single mass or mass-to-charge only, as will be shown later in Chapter 4, the mass resolution is highly mass-dependent.

Just like a conventional optical microscope, magnification M of our system refers to the degree to which the object observed is enlarged. As shown in Figure 2.12, an image consisting of four point ion sources is projected onto the detector plane by the ion optics. The magnification for the second to fourth “splats” is defined as:

$$M_i = \frac{Y_i - Y_1}{(i-1)d} \quad (i=2,3,4), \quad (2.56)$$

where d is the distance between two adjacent point sources, Y_i is the location of the i^{th} splat centre. A splat centre is defined as the mean y position on the detector of all ions coming from an same point source. The overall magnification $\overline{M}$ is simply the average of

all M_i .

Figure 2.12(d) shows the spatial spreads of simulated ions originating from four discrete point sources along the y-axis of the detector plane. If we assume the spatial spreads are normal distributions, the standard deviation σ_y^i of the distance with respect to each ion source's own splat centre can be used to estimate the FWHM of distribution at the corresponding point on the detector surface

$$\Delta R_{50\%}^i = 2\sqrt{2\ln 2}\sigma_y^i \approx 2.354\sigma_y^i \quad (i=1,2,3,4). \quad (2.57)$$

The obtained FWHM can then be used, along with the previously calculated magnification, to compute the spatial resolution over the sample surface,

$$\Delta r_{50\%}^i = \frac{\Delta R_{50\%}^i}{M_i} \quad (i=1,2,3,4), \quad (2.58)$$

where M_1 can be estimated by either taking the value of M_2 or $\overline{M}$. Using $\Delta r_{50\%}^i$ as the spatial resolution may seem a very natural thing to do, but in practice it is impossible to perfectly materialise discrete point ion sources like those simulated in SIMION. As a result, dye samples of a repeating, well defined square pattern are often used to experimentally determine the spatial resolution obtainable with an instrument. And the distance from 20% to 80% of the maximum intensity of an edge of such square pattern is commonly seen as a good measure of spatial resolution (there are more details in Chapter 4). Since the size of the square pattern (usually a few hundreds of microns) is often much larger than the spatial resolution (less than 50 μm) of the instrument, the rising edge of a square profile will be a product of the underlying shape convolved with a Gaussian response function. E. Halford numerically calculated the relationship between the standard deviation σ_y^i and the 20% to 80% distance $\Delta R_{20-80\%}^i$ in his thesis [106],

$$\Delta R_{20-80\%}^i \approx 1.683\sigma_y^i \quad (2.59)$$

$$\approx 0.715\Delta R_{50\%}^i \quad (i=1,2,3,4). \quad (2.60)$$

Equation 2.59 is only valid under the condition that the size of each square pattern is no less than ten times larger compared with the spatial resolution. Both the magnification and spatial resolution are to some extent dependent on the final positions of the ions on the detector surface. The standard deviations of the magnification and spatial resolution can be seen as a good measure of the degree of this aberration, which is the curve focusing plane aberration discussed in Chapter 1.

2.2.7 Summary

In this chapter, two different approaches for modeling an ion optical system *in silico* were introduced. The first is the transfer matrix method, which characterises the movement of a charged particle by multiplying a series of transfer matrices with its initial state vector $[r, r']$. Here r is the particle's distance from the optical axis and r' is the slope of its trajectory. Each transfer matrix represents a specific region of the system, such as a uniform electric field between flat electrodes, field-free space, or an aperture that separates two electric fields of distinct strengths. Transfer matrices have been derived from Newton's laws and basic electromagnetic dynamics. In particular, the deflecting effect of an aperture follows exactly the same formula that describes the refraction of an optical lens. The lensing effect of apertures explains the imaging capability of ion optical systems. A typical three-electrode spatial imaging ion optical system as well as the five-electrode system used here were modeled with the transfer matrix method, which is good at dealing with complex optics assemblies and offers an intuitive way to understand how each component functions. However, based on the assumption of ideal lenses, transfer matrix method cannot estimate accurately the spatial resolution of an instrument. Moreover, due to the fact that various approximations are involved when deriving transfer matrices, it is also less accurate when predicting the potentials that must be applied on each electrode. In addition, only cylindrically symmetric system can be modeled by transfer matrix method because it can only deal with 2-dimensional problem.

Another method used to model our experiment is SIMION. Given a configuration of electrodes with voltages and particle initial conditions, SIMION is able to simulate electric fields and the motion of charged particles inside those fields. Various building blocks of a SIMION simulation workbench have been detailed. Potential arrays define electrode geometries and potentials at each point in space. Ion definition files characterise important properties of an ion such as its mass to charge ratio, starting location, initial velocity, and so on. User programs provide considerable convenience through automating many tasks during simulations, including the adjustment of voltages and analysis of the results. Since highly efficient and rigorous numerical computation methods are used to simulate electric fields and ions' motion inside them, SIMION is far more accurate and powerful compared with the transfer matrix method. Therefore, most of the design work described in this thesis is performed with SIMION.

Chapter 3

Experiment Methods

3.1 Introduction

The modeling and simulations described in chapter 2 will assist in the development of new ideas to improve the performance of microscope MSI. Validating these ideas requires experimental implementation. Each section of this chapter will cover a key aspect of the mass spectrometer used to achieve this, including its ion optical system, ion source, detector, sample preparation, clock synchronisation, data acquisition (DAQ) system, and time-of-flight (ToF) to m/z calibration.

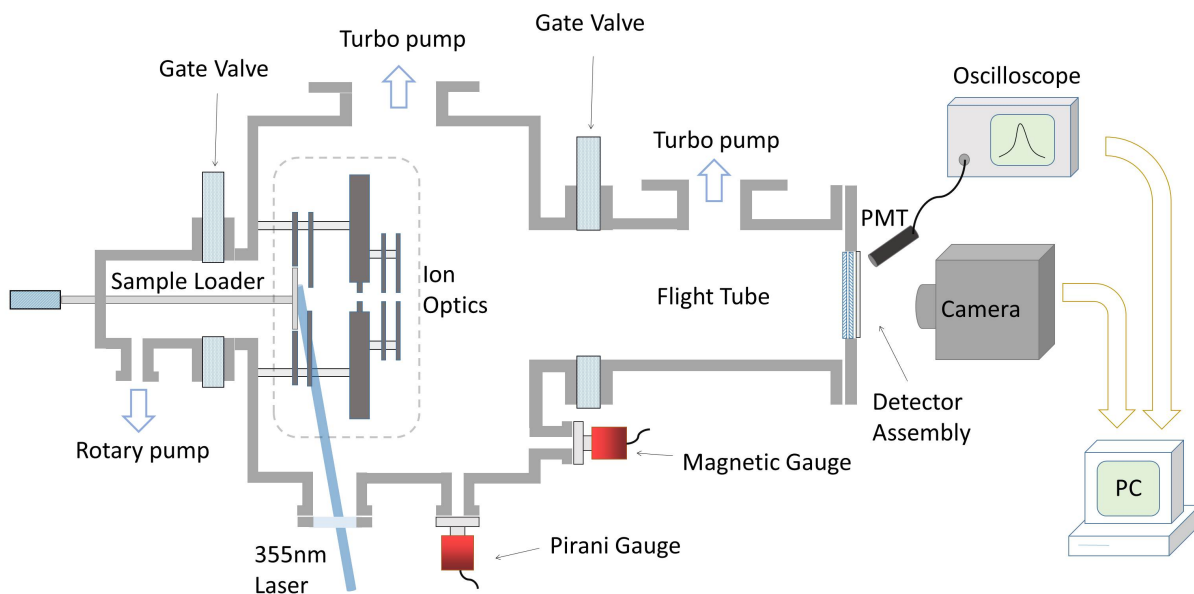


Figure 3.1: Schematic diagram of the microscope mode MSI system. The instrument was developed by B. Winter [113].

The home-built microscope MSI instrument is illustrated in Figure 3.1. It consists

of three vacuum chambers: a sample loading chamber, an ion source chamber, and a flight tube. Through the loading chamber, samples are transferred into the ion source chamber, where they are ionised by a laser beam and spatially mapped by the ion optics onto a detector assembly at the end of the flight tube. Then ion signals are recorded by a photomultiplier and a camera before being stored in a computer.

The ion source chamber and the flight tube chamber are connected through a gate valve. By closing this gate valve, the flight tube chamber that contains the detector assembly can be isolated and kept under vacuum while the ion optics chamber is open. It is necessary to store the microchannel plates (MCPs) of the detector assembly in a vacuum environment to avoid trapping gas molecules in their pores and hence minimise the risk of damaging them due to electrical breakdown. The ion source chamber is pumped down by a 510 l/s turbo pump (Pfeiffer Vacuum TMH-521P) and the flight tube chamber is evacuated with a 70 l/s Pfeiffer Vacuum TMU-071P pump, both of which are backed by a Leybold Trivac D40V rotary pump. The pressure inside the instrument is monitored by two different types of vacuum gauges, which are a Pirani gauge and an Active Inverted Magnetron gauge. The Pirani gauge is a type of thermal conductivity gauge, offering pressure measurements from atmosphere to 10^{-3} mbar. The active inverted magnetron gauge measures the number density of neutral gas phase molecules by first ionising them and then collecting their product ions. Its pressure range is typically 10^{-2} to 10^{-9} mbar. Experiments are typically operated at a pressure of 10^{-7} mbar.

3.2 Ion optics

The ion optical system is located within the ion source chamber and consists of five concentric electrodes (see Figure 3.2). The first electrode is the repeller, which is a circular metal plate with a high positive electric potential that accelerates cations along the ToF axis. The outer diameter of the repeller is 148 mm (as shown in Figure 3.3(a), the complete repeller is formed of a sample holder plate and a complementary metal ring).

the rotary pump.

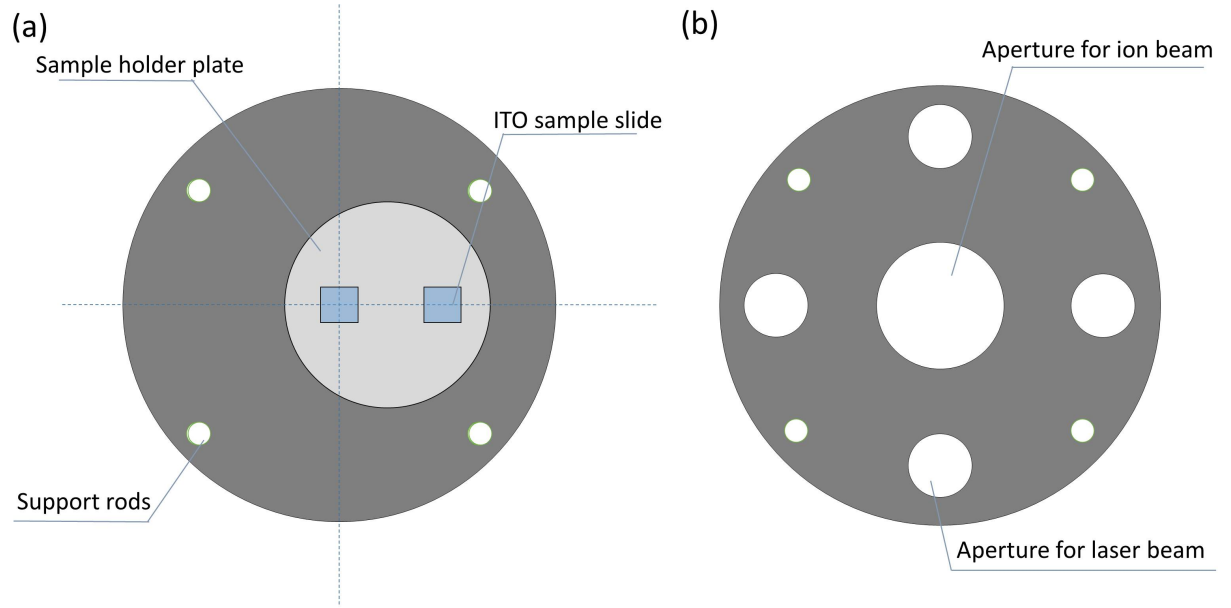


Figure 3.3: (a) The repeller electrode and (b) the extractor electrode.

An extractor electrode is located 14.5 mm away from the repeller. Its outer diameter is the same as the repeller with a thickness of 0.9 mm. Four holes are symmetrically distributed around a central hole on the extractor (as shown Figure 3.3(b)). The central hole is for ion transfer, while one of the four symmetric holes allows the laser beam to pass through the plate and illuminate the sample on the repeller. The other three maintain the symmetry of the electric field. The diameter of the central extractor's aperture is 19.5 mm and the diameter of the four symmetric holes is 18 mm.

The first grounded electrode of a three-electrode einzel lens assembly is 39.5 mm away from the extractor. It is a 10 mm thick plate with 180 mm diameter and a removable 2 mm diameter aperture at its center. The central aperture coincides with the velocity-map imaging plane of the ion optics. It therefore acts as a contrast diaphragm that blocks ions of large initial transverse velocities. This is an efficient way to reduce chromatic aberrations in the recorded ion images. According to SIMION 8.0, about 98% of all the ions generated on the sample slide can pass through the 2 mm aperture and eventually make their way to the detector. The distance between the first and second electrodes of the einzel lens assembly is the same as that between the second and third, which are

both 7.9 mm. Two identical electrodes are used for the second and third electrodes of the einzel lens assembly respectively, with an outer diameter of 100 mm and a central aperture diameter of 5 mm.

The repeller, extractor, and the first grounded plate are held in position by four ceramic (Macor) isolated stainless steel rods mounted to the chamber wall and the other two electrodes of the einzel lens are fixed by four rods connected to the first grounded plate (as shown in Figure 3.2).

3.3 Laser

All experiments described in this thesis used a Nd:YAG laser (Continuum Powerlite 8010) to produce ions. Nd:YAG crystals are widely used active mediums for solid-state lasers. A small fraction of yttrium ions (1%) in the Yttrium Aluminum Garnet (YAG) crystal are replaced with triply ionised neodymium (Nd) which serves as a dopant. As shown in Figure 3.4, Nd:YAG lasers are four-level systems. In our case, a flash lamp pumps many ground-state electrons to an excited state, leading to population inversion where stimulated photon emission is greater than photon absorption. Light amplification occurs by reflecting the emitted photons back and forth through the active medium in an optical cavity, creating coherent 1064 nm light.

Q-switching is employed to obtain energetic 3–7 ns laser pulses of 1064 nm light, which have much higher peak powers than continuous wave lasers. Producing Q-switched pulses involves three steps. In the first step, the optical cavity losses are kept high (which corresponds to a low Q factor). As lasing cannot occur under this condition, the energy fed into the gain medium builds up there. Then, the optical cavity switches to a high Q factor (losses are sharply reduced), so that lasing begins. Finally, lasing amplifies light in the gain medium until its stored energy depletes, leading to an intense pulse.

The fundamental wavelength generated by the Nd:YAG medium is frequency-doubled (532 nm) and tripled (355 nm) using a nonlinear Potassium Dihydrogen Phosphate (KDP)

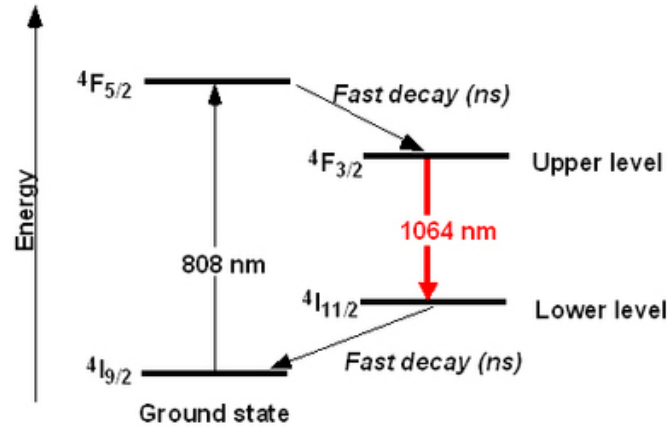


Figure 3.4: Simplified energy level diagram for a Nd:YAG laser. Since the absorption wavelength (808 nm) is different from the emission wavelength (1064 nm), losses due to reabsorption of the laser radiation are avoided.

crystal. In the experiments reported here, the third harmonic was used due to its efficiency in desorbing and ionising neutral molecules. The laser fires at a repetition rate of 10 Hz, which determines the experimental duty cycle, and its pulse width is between 3–7 ns. Its power is controlled by varying the delay time between the triggers for the flash lamp and the Q-switch (as shown in Figure 3.5). The maximum laser pulse energy is 350 mJ/pulse, with 1 to 10 mJ/pulse (measured before entering the vacuum chamber) used in the experiments described here. The optimal laser power, which depends on the sample under study, should be sufficient to surpass the sample ionisation threshold and to provide ample ion signals. However, the laser power should not be too high, as too many ions would be created simultaneously, which may lead to strong space charge effects that would distort the ion trajectories. After exiting the laser head, the laser beam is split in two by a 20:80 beam splitter. The more intense beam is directed into another instrument and the less intense one is reflected by mirrors and prisms before entering the ion source chamber through a fused silica window. As shown in Figure 3.1, the laser beam illuminates the sample plate at a small incidence angle ($\approx 20^\circ$) through a hole in the extractor electrode.

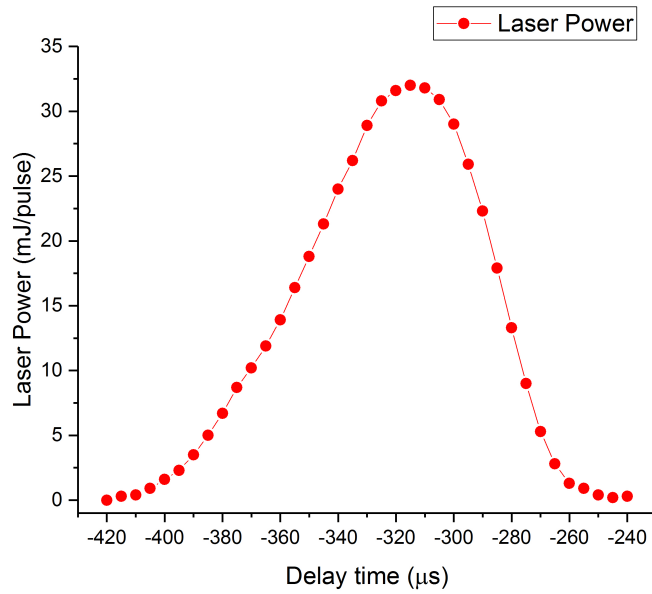


Figure 3.5: Laser power before entering the vacuum chamber as a function of the delay time between flash lamp and Q-switch ($t_{flash} - t_Q$). In practice, 1–10 mJ/pulse are used to desorb and ionise samples, which corresponds to a delay time around -410 to -370 μs .

3.4 Detector assembly

At the end of the field free drift path is a detector assembly, where incident ions are detected and converted into a usable signal. For microscope MSI, the detector assembly has to fulfill a few requirements simultaneously. For example, the detector should be able to generate an electronic signal that is proportional to the number of ions impacting on it, the signal has to be registered with a very high time precision (typically on the order of nanoseconds), and the 2D spatial distribution of ion impacts must be recorded. A detector assembly combining microchannel plates, a scintillating screen, a photomultiplier, and a camera, has long been used for performing such a task [19] (the detector assembly is shown on the right part of Figure 3.1). In this section, the different components of the detector assembly will be detailed.

3.4.1 Fast scintillator

A phosphor screen emits photons when impacted by electrons with high kinetic energies. Such impact-induced fluorescence can be illustrated by a Jablonski diagram (Figure 3.6(a)). In the Jablonski diagram, each horizontal line corresponds to an energy level of a specific phosphor molecule. Each bold horizontal line represents the lower limit of an electronic energy state and other horizontal lines represent various vibronic energy states of this electronic energy state. At room temperature, the majority of electrons are occupying the ground electronic energy state according to a Boltzmann distribution. In the first step of the fluorescence process, electrons at lower energy levels are excited to higher energy levels after absorbing the energy from an incident electron beam. This transition occurs within a few femtoseconds. Straight arrows that point up are used to describe this excitation process. In the second step, molecules dissipate excess energy through two mechanisms, “vibrational relaxation” and “internal conversion”. Vibrational relaxation non-radiatively transfers energy from excited vibrational modes to kinetic modes of the same molecule or the surroundings, which means this energy transfer can be either intramolecular or intermolecular. In Figure 3.6(b), vibrational relaxation is indicated by the blue wavy arrow pointing downwards, which only occurs between vibrational states of the same electronic level. However, at higher excited states, the manifold of vibrational levels strongly overlaps with those of other electronic states. According to the Franck-Condon Principle, transitions between two vibrational levels of different electronic states therefore becomes possible. If the excess energy caused by external perturbation is dissipated through this kind of transition, the process is called “internal conversion”. It is represented in the Jablonski diagram as a green wavy arrow. Internal conversion is also a non-radioactive process and its underlying mechanism is identical to vibrational relaxation. Both vibrational relaxation and internal conversion are very fast, occurring in 10^{-14} to 10^{-11} s. After the non-radiative dissipation of energy, most excited electrons transition to the ground vibrational level of the first excited electronic state. The last step of the

fluorescence process is to emit a photon. This is indicated in Figure 3.6(c) as a straight line going down from the ground vibrational level of the first excited electronic state to a vibrational level of the ground electronic state. Unlike vibrational relaxation or internal conversion, fluorescence is a relatively slow process (between 10^{-9} and 10^{-7} seconds). As shown in the Jablonski diagram, a large number of vibrational excited states exist in the manifold of the ground state, which results in a relatively broad range of wavelengths of photons emitted in the final transition.

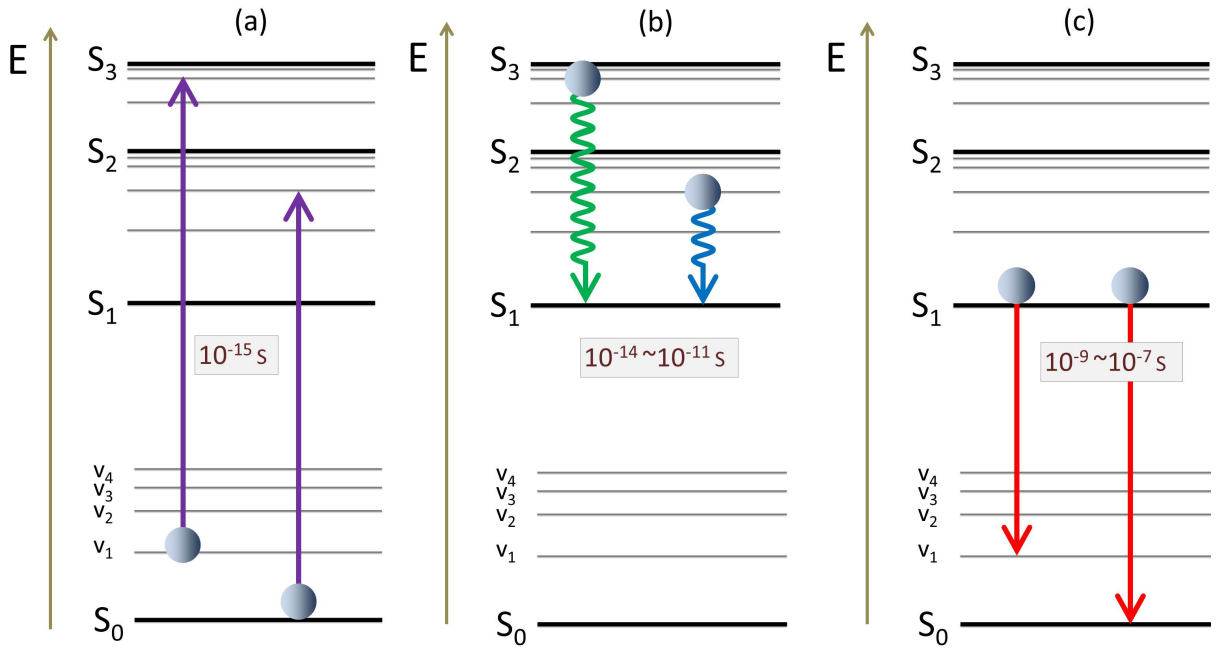


Figure 3.6: Jablonski diagram of a fluorophore: (a) electron-induced excitation, (b) energy dissipation through vibrational relaxation and internal conversion, (c) photon emission.

Cathode ray tube (CRT) displays used in early televisions and oscilloscopes are common examples of phosphor screens. These phosphors were standardised and named by the letter “P” followed by a number. For example P43, P46, and P47 are widely used for image intensification. As shown in Figure 3.7, the back surface of the phosphor screen is coated with a thin layer of aluminum. Photons which otherwise would not enter a sensor are reflected by the aluminum layer towards the proper direction, maximizing the total visible light output.

Choosing the right scintillating material is extremely important for a microscope MSI

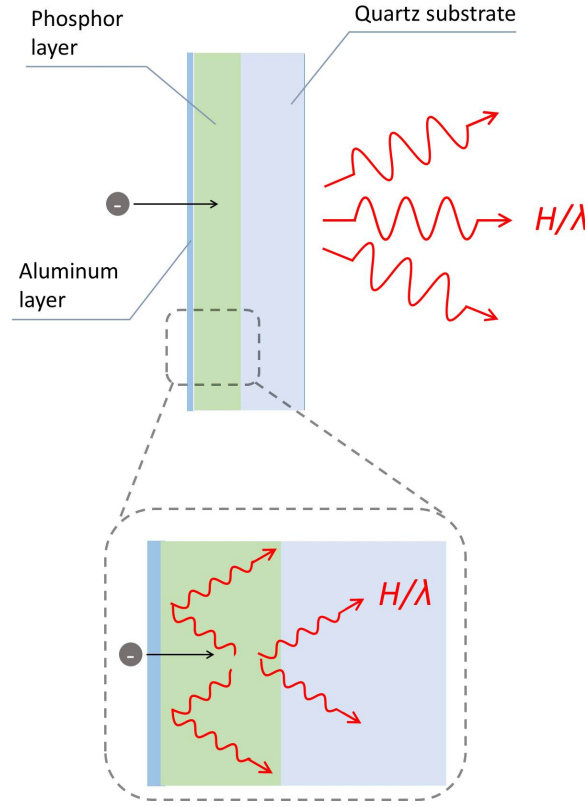


Figure 3.7: The structure of a typical phosphor screen, where electron impacts are converted into light signals.

system. There are many parameters that need to be taken into consideration for a candidate material, such as fluorescence lifetime, brightness, the emission spectrum, the spatial resolution, and so on. Among these parameters fluorescence lifetime is worth an in-depth investigation. Fluorescence lifetime is defined as the time a fluorophore spends in its excited state before decaying to its ground state through spontaneous emission (the process is shown in Figure 3.6(c)). Lifetimes span from picoseconds to hundreds of nanoseconds. If a large number of fluorophores is excited, which is often the case for ion microscopy, the lifetime τ is defined as the time it takes for the number of excited fluorophores to drop to $1/e$ of the original population. However, in practice τ is determined by the time required for the fluorescence intensity to decline to $1/e$ of its initial value, as expressed by:

$$I = I_0 e^{-t/\tau} \quad (3.1)$$

where I represents the emission intensity at time t and I_0 is the initial intensity.

A long τ may severely limit the mass resolving power of a ToF-MS, as mass resolution is inversely proportional to the FWHM Δt of a ToF peak. According to SIMION, the PEDTA technique can reduce the FWHM of the arrival time spread of a m/z ratio to less than 10 ns, corresponding to a mass resolution of more than 1,000 $m/\Delta m$. However, if a common scintillator were used, such as P47 which has a fluorescence lifetime around 60 ns, the total FWHM of a ToF peak after convolving its arrival time spread with the fluorescence lifetime would be much wider than 10 ns, which corresponds to a mass resolution of around 200 $m/\Delta m$. In order to maximize the mass resolution of a ToF-MS, a fast scintillator screen with a short fluorescence lifetime is therefore necessary.

A selection of materials were studied by B.Winter and coauthors in a search for such a fast scintillator [114]. The fluorescence times of the candidates are summarised in Table 3.1. Two methods, sedimentation and sublimation, were employed to coat these scintillating materials over ITO substrates to form thin and homogeneous layers. The sedimentation method was found more suitable for preparing inorganic phosphors such as P47, while sublimation worked better for organic dyes such as Exalite 404. The sublimation method was used to prepare the scintillator for the experiments included in this thesis, and is detailed later in this section. The fluorescence times were determined using electron ToF spectra. In the ion source chamber of a standard velocity-map imaging spectrometer, electron pulses were generated by the laser-induced ionisation of gas phase methyl iodide molecules, and propelled towards the scintillator screen by negatively charged ion optics. The electron impact excitation of the scintillating materials lead to the emission of fluorescence light, the intensity of which was recorded as a function of time using a photomultiplier. As the temporal spread of each electron pulse was sufficiently narrow, the electron ToF spectrum were seen as a good approximation for the fluorescence process. There are also other pathways of energy dissipation, such as intersystem crossing, external conversion, and quenching, that compete with fluorescence as the fluorophore retreats to its ground state. The τ values listed in Table 3.1 are the upper limits for each scintillating material, because there are several other factors that may also contribute to

the broadening of an electron ToF peak. For example, the pulse width of the ionisation laser (≈ 10 ns), the temporal spread of an electron pulse (≈ 3 ns), and the response time of the photomultiplier (< 3 ns). So the actual τ , as well as the rise and fall times (denoted by t_{rise} and t_{fall} respectively), are probably smaller than those in Table 3.1.

Coumpound	τ (ns)	t_{rise} (ns)	t_{fall} (ns)	Signal strength
P47	69.3 ± 0.8	6.7 ± 0.2	13.0 ± 1.3	strong
Exalite 384	4.7 ± 0.5	4.2 ± 0.8	9.3 ± 1.5	strong
Exalite 389	3.7 ± 0.5	3.5 ± 0.6	7.7 ± 1.0	strong
Exalite 398	9.8 ± 0.4	5.1 ± 1.1	26.1 ± 1.9	strong
Exalite 404	3.7 ± 0.2	3.5 ± 0.2	7.7 ± 0.3	strong

Table 3.1: Comparing the lifetime of different phosphor molecules. The values listed for Exalite 384, 389, 398, and 404 are only upper estimates due to the precision limitations of the measurement device [114].

Due to their relatively short fluorescence lifetime, Exalite 389 and Exalite 404 were investigated further [114]. Their emission wavelength ranges were determined and compared to the industry standard P47. Their electron-impact-induced emission spectra were recorded using a monochromator. In spite of some minor differences in their peak wavelengths, the emission ranges of Exalite 389, Exalite 404, and P47 were basically the same (350 – 550 nm) and were compatible with most imaging sensors and photonmultipliers. Their conversion efficiencies were also determined to investigate the electron-to-photon sensitivity of each candidate. Due to the difficulty and complexity in measuring an absolute value of photon-electron conversion efficiency, a relative scintillation efficiency was obtained instead. The experimental setup was very similar to that used to measure the fluorescence lifetime constants, but the polarity of the ion optics potentials was inverted and I^+ ions were velocity mapped onto the detector assembly. Moreover, the scintillator screen was segmented into four quadrants, two of them were P47 phosphor screens and the other two were candidate materials. The potential applied on each quadrant controlled the impact energy of incident electrons emitted from the back face of MCP (more details in subsection 3.4.4), therefore the brightness of a scintillator screen would increase with

increasing potential. Hence, if incident ion beams are identical on the two screens, the relative brightness is equivalent to the relative conversion efficiency. Using one P47 quadrant held at 2.5 kV as a reference, the relative brightness of each candidate was determined as a function of scintillator potential. The conclusion was that Exalite 404 outperformed the P47 phosphor and Exalite 389 in terms of sensitivity over the entire scintillator potential range. So Exalite 404 (see Figure 3.8) was chosen as the fast scintillating material to fabricate a home-built detector assembly. Moreover, further study showed that the stability of the Exalite screen was also impressive: after a 47-hour continuous electron strike, there was no sign of signal degradation.

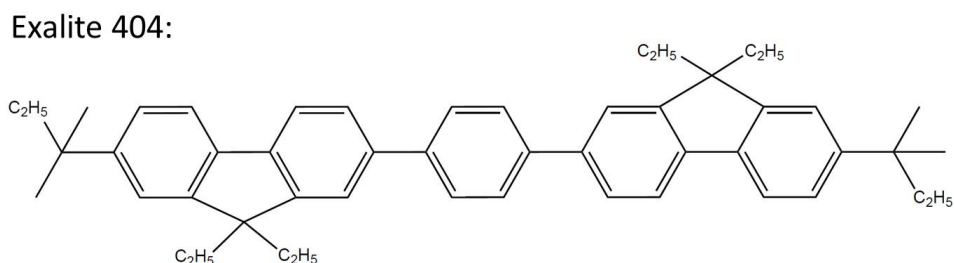


Figure 3.8: Molecular structure of Exalite 404.

3.4.2 Scintillator preparation

If a substance is heated to a certain temperature at a pressure lower than its triple point, it will sublime straight from the solid phase to the gas phase. When the vapour reaches a cooler surface, it returns to the solid phase, through an inverse process called deposition or desublimation, as a uniform layer over that surface. Sublimation has proved a highly efficient and reproducible method to prepare scintillators of superb quality. Through the sublimation and desublimation process, luminescent particles are homogeneously deposited on the substrate, resulting in a consistent fluorescence response over the entire screen.

The scintillator preparation device consists of an Ace vacuum sublimation apparatus (60 mm internal diameter), a vacuum pump, and a hot/stirring plate (Fisher Scientific) as shown in Figure 3.9. A substance container is connected to the cold finger of the

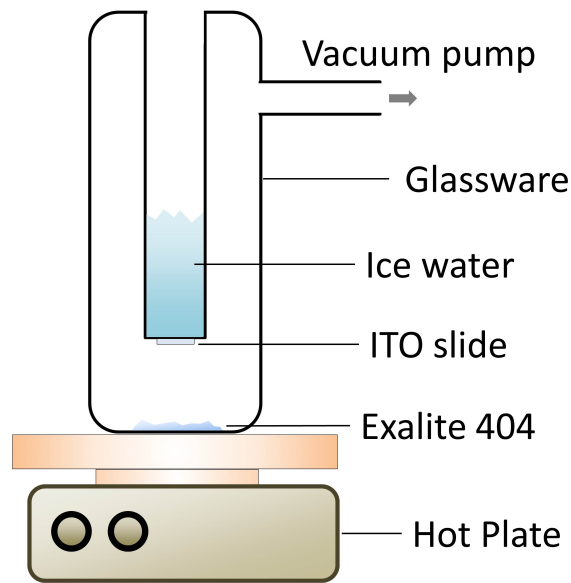


Figure 3.9: Scintillator preparation device. This device were developed by B. Winter [113].

sublimation apparatus through O-ring sealed flanges that are joined by a quick-release clamp. A small quantity of Exalite 404 (0.05 g to 0.15 g) is spread evenly over the bottom of the substance container, facilitating a faster and more thorough sublimation. The substance container is evacuated down to 10 mbar to furnish a sufficiently low pressure for sublimation to occur. The bottom face of the cold finger is made of copper, which offers a higher thermal conductivity than glassware. A clean ITO-coated glass slide is fixed onto the metal bottom with Kapton tape. Electrons that initiate the fluorescence need to be drained off from the scintillating material layer, otherwise they may accumulate on its surface over time and repel following electrons. So one has to ensure that it is the conductively coated side of the ITO slide that faces towards the Exalite dye sample. The distance between the ITO slide and the dye powder is about 1 – 2 cm. A mixture of water and ice is filled into the cold finger as a cooling agent that maintains a low temperature on the metal bottom as well as the ITO substrate. The hot plate is placed under the substance container to mildly heat up the Exalite 404 dye. The temperature is a critical parameter for a successful sublimation, if it is too low, the sublimation process does not start or is very slow; if it is too high, the dye powder melts, ruining the homogeneity of the

scintillating layer. For this setup, the target temperature of the hot plate is set to around 260 °C. Shortly after being heated, Exalite 404 begins to crystallise on the conductive surface of the ITO slide. At the end of the sublimation, the layer should be sufficiently thick to produce ample fluorescence but not too thick, otherwise parts of it may easily flake away.

3.4.3 Photomultiplier tube

Photomultiplier tubes (PMTs) are light sensors, that are widely used in mass spectrometers. PMTs record optical signals from scintillator screens, convert them into detectable currents, and output the current to external devices. They have extremely high photon-detection sensitivity over a broad range; from the ultraviolet all the way to the near-infrared region. PMTs may also have short response times (no more than 1 ns), which is particularly suited to ToF-MS where high temporal resolution is required. A PMT is essentially an evacuated glass tube, that makes use of the photo-electric effect to convert incident photons into current, and to amplify this current by a factor of 10^8 . The vacuum inside a PMT can be kept as low as 10^{-8} to 10^{-7} mbar. A schematic view of a PMT is shown in Figure 3.10. Light enters the PMT through its input window and illuminates a photocathode, which is usually coated onto the inside of the input window. The photocathode is a layer of photosensitive semiconductor material, capable of emitting photoelectrons into the vacuum tube after being struck by incident photons. A negative voltage (about -1 kV in these experiments) is applied on the photocathode to accelerate the generated photoelectrons away from its surface. These photoelectrons are then focused and guided by a focusing electrode towards the first dynode. This dynode is capable of multiplying electrons through secondary emission, a process where a single impinging electron turns into a cloud of electrons with lower kinetic energies. As shown in Figure 3.10, there is a series of discrete dynodes in a PMT, which, including the aforementioned photocathode and an anode at the end of the tube, are connected through a resistor chain. This resistor chain is used as a voltage divider circuit that creates a potential gradient in

the PMT. Each dynode is held at a more positive voltage relative to its preceding one. So, those secondary electrons generated by the first dynode are guided to bombard the second dynode after being accelerated by the inter-dynode potential difference. Again, more electrons are generated from the second dynode in another round of secondary emission. This cascade process continues as the secondary electrons go down the tube and the number of electrons is amplified rapidly. The final output current depends on two factors, which are the number of dynodes in a PMT and the accelerating potential difference between them. The number of dynodes determines how many rounds of amplification the electrons will go through. The accelerating potential difference determines the kinetic energy gain of electrons after each round of secondary emission and hence how many more secondary electrons will be produced in the next round. At the end of the PMT is an anode which is an electrode held very close to ground potential. The anode collects the final flow of secondary electrons amplified by the multi-stage dynodes and outputs the a detectable current to an oscilloscope. In addition, as shown in Figure 3.10, there are capacitors in parallel with the last few dynodes that supply charges to help maintain the potential on the dynodes as electron avalanches propagate through the PMT.

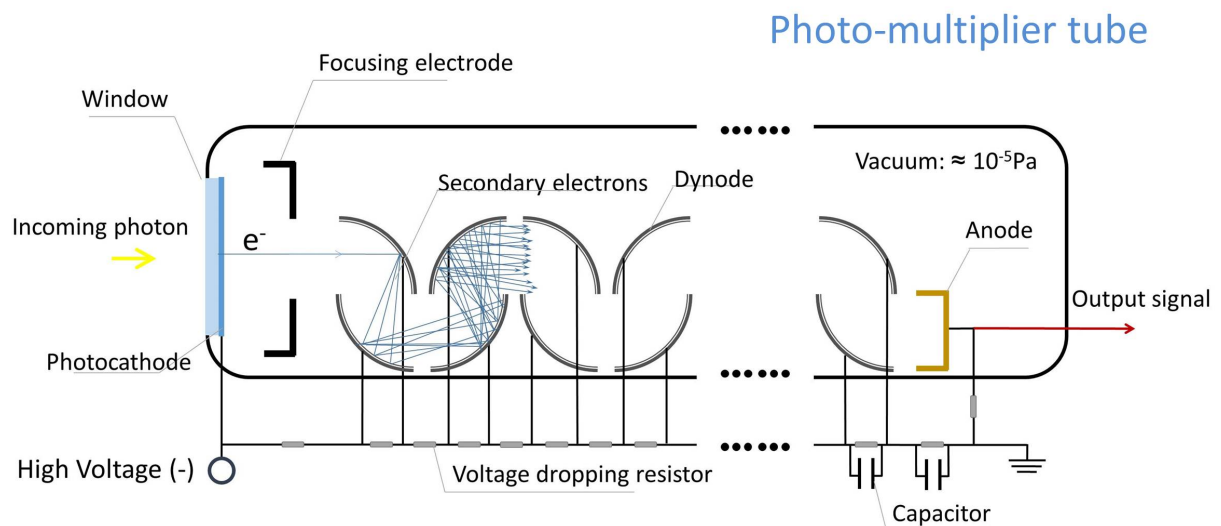


Figure 3.10: A schematic view of a photonmultiplier tube (PMT).

3.4.4 Microchannel plates

Microchannel plates (MCPs) are used to significantly amplify detected ion signals. An MCP is a circular glass slab with a regular array of parallel cylindrical open pores (microchannels) (see Figure 3.11). MCPs are suited to position sensitive detection because

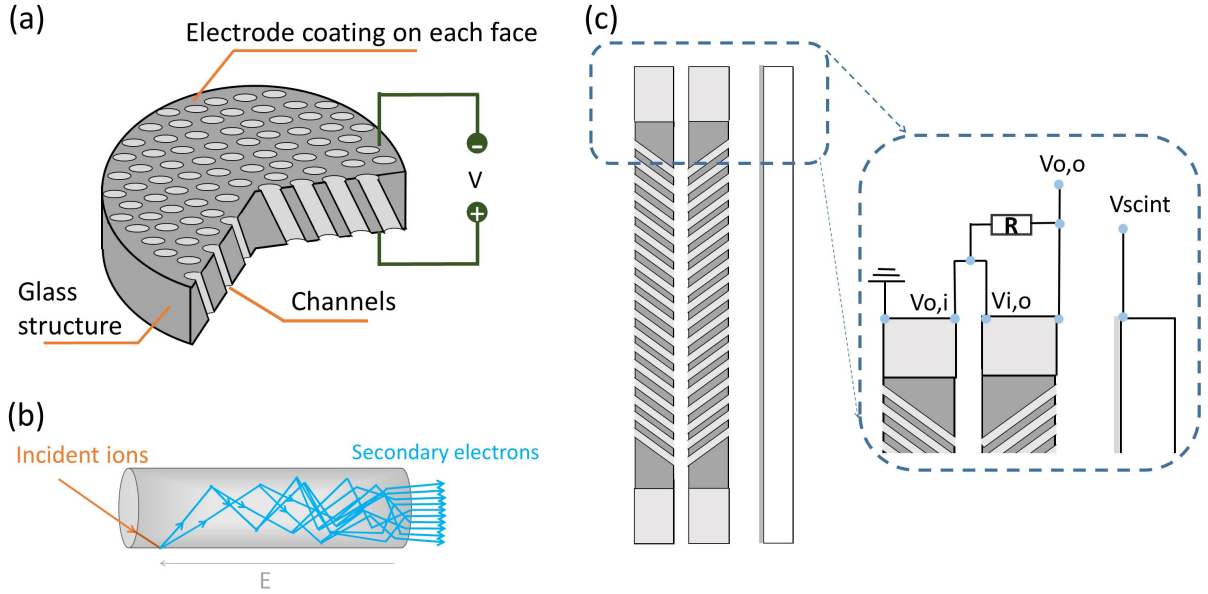


Figure 3.11: Microchannel plate (MCP) assembly: (a) schematic, (b) principle of ion signal multiplication, (c) the structure of a 2D position sensitive detector assembly.

they have a relatively large surface area, and, more importantly, the individual channels guarantee that the spatial patterns of electrons emitted at the rear of the array preserve the image of the incident particles on the front face. The outer diameter of the MCPs (B1425, Photek, UK) used in our system is 32.8 mm with a thickness of 0.42 mm. It should be noted that only a smaller (26.5 mm diameter in our case) concentric circular area performs signal amplification, which is called the “active area”. The active area is very densely populated with micro-channels, the centre-to-centre spacing between two adjacent pores is only $12.2 \mu\text{m}$ and the diameter of a pore itself is $10 \mu\text{m}$. The inner wall of each individual channel is covered with a semiconductor, which facilitates the secondary emission process. A metallic coating (chrome) is deposited on both the front and the rear faces of the MCP. A large potential difference is applied to the two conductive

faces ($U_{rear} \gg U_{front}$) to form a strong electric field in each channel. Therefore every single channel can be seen as a miniature continuous dynode electron multiplier. Once an ion enters one of the tubes, it first experiences a rapid acceleration in the aforementioned strong electric field, and then strikes the channel wall because the channel axes are biased at an angle (12°) to the surface normal of the plate (see 3.11(b)). This impact triggers a cascade of secondary electron emissions that is repeated many times as electrons propagate down the channel. At the exit orifice, the output signal can be several orders of magnitude more intense than the input signal. The amplification factor depends on the electric field strength in the tube: when the front face is grounded and the rear face is held at the maximum voltage (1 kV), the maximum gain of the MCPs used here is 10^4 , MCPs have relatively fast response times because the paths of secondary electrons inside a channel are usually very short. As a result, MCPs are perfect for ToF analysers, which require precise arrival times and narrow peak widths. As shown in Figure 3.11(c), our detector assembly consists of two MCPs turned 180° along their axes with respect to each other, forming a chevron configuration. Using a very simple circuit where two resistors are connected in series, high voltage ($V_{o,o}$) from the power supply can be evenly divided among two MCPs: the rear face of the second MCP is held at $V_{o,o}$, the front face of the second MCP and the rear face of the first MCP are held at $V_{o,o}/2$, and the front face of the first MCP is grounded. The advantage of stacked MCPs is of course a higher gain, as the electron avalanche emitted from the first MCP will get further intensified by the second one. Positive ion feedback is an annoying phenomenon that limits the length of a microchannel and hence its gain. As the electron cascade gets bigger and faster on its way through a microchannel, atoms from the rest gas or those adsorbed on the inner channel wall may be ionised by electron impacts. The ions generated in this process are called feedback ions. Positive feedback ions are accelerated by the electric field towards the input side of the MCP. If no counter-measures are taken, feedback ions can achieve enough energy to release electrons as they hit the channel wall. So a second cascade will be initiated. These false after-pulses not only disturb the measurement, they may even

lead to a permanent glow discharge and damage the MCP. The chevron (V-like) shape can help to trap feedback positive ions at the interface of the two plates. It should be noted that MCPs must operate under high vacuum circumstance and strictly below their maximum voltage, otherwise they can be easily damaged due to electrical breakdown.

3.4.5 CCD camera

Charge-coupled device (CCD) cameras play important roles in microscope MSI, where they record two dimensional optical images emitted by scintillator screens. The fundamental building blocks of CCD sensors are light sensitive “pixels”. Each CCD sensor consists of an array of pixels, the intensified CCD camera (Photonic Science) used in our experiments has 768×576 pixels. Photons incident on each pixel proportionally generate electrons that can be stored, transferred, read by electronics, and reconstructed into a digital map of the light patterns falling on the camera.

A pixel is constructed by etching an integrated circuit onto a semiconductor surface, as shown in Figure 3.12. After incident photons strike the silicon surface, free electrons are generated as a result of the photoelectric effect. These electrons are then stored by a capacitor within the pixel. There is also a series of gate electrodes on the pixel surface, which are capable of attracting electrons if positively charged. By switching those gates on and off, electrons can be transported both within a pixel and across neighboring pixels. To read out a signal, electrons migrate in two directions on a CCD sensor: a parallel shift from left to right until electrons have reached the register column, which is on the edge of a CCD sensor, and a serial shift from top to bottom along the register column until electrons arrive at the output unit. So an electron packet can be moved from the pixel they originate from to the measurement electronics at the serial output, where a voltage created by the packet is measured. This is how the light signal collected by each pixels during one exposure is quantified, the final “image” one gets is simply a two-dimensional plot of the electronic signal.

At its peak absorption wavelength, CCDs have a high light sensitivity and a very large

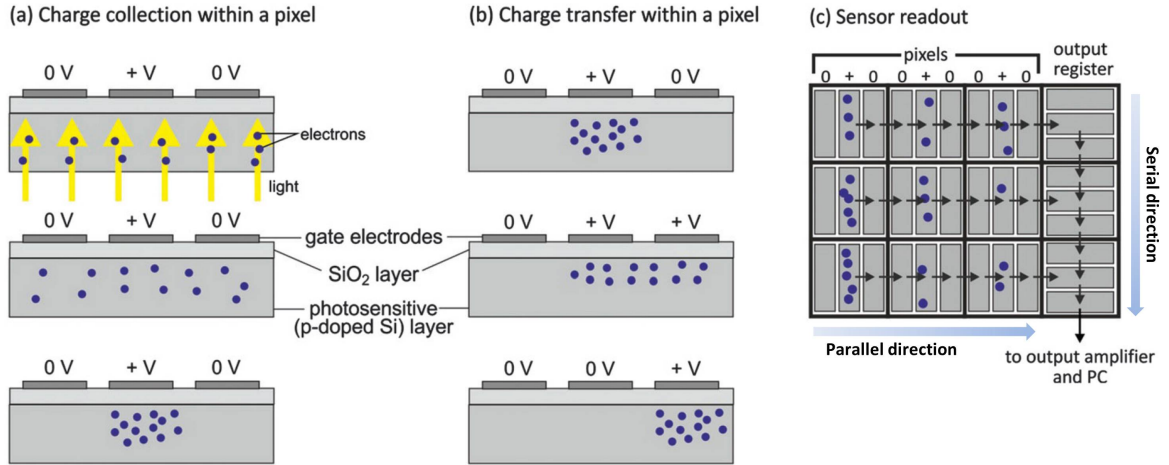


Figure 3.12: A schematic view of a CCD camera sensor, which illustrates how it operates: (a) electron collection within a pixel; (b) electron migration within a pixel; (c) electron migration among pixels before being read out. Adapted from ref. [63].

dynamic range. However, one major downside of CCD cameras is their slow readout speed which considerably limits the applicability of a conventional CCD sensor in the area of microscope MSI. The standard frame rates of a CCD camera are generally less than one hundred frames per second (*i.e.* a worse than 10 ms temporal resolution). This is far too slow compared to the microseconds timescale of the time-of-flight difference between adjacent ion peaks. To overcome this hurdle, one has to gate the CCD camera to a narrow time window of a few hundred nanoseconds. By overlapping the time window with a certain ToF peak, the CCD camera records positional information of that single ion species. The gating occurs in the image intensifier tube of the camera, which is an evacuated cylinder comprising a photocathode, MCP, and a phosphor screen (a very similar layout to the detector assembly described above). The output of the image intensifier is then transmitted to the CCD sensor through a fiber optic coupler.

In order to cover the entire Exalite screen, the lens of our CCD camera has to be located about 10 to 30 cm (depending on the specific focal length of the lens) away from the screen. The body of the CCD camera is attached onto a frame fixed by three threaded stainless steel rods which are screwed into the vacuum flange of the detector housing. To

refrain any ambient light from entering the camera, black curtains are used to tightly close off the space between the camera and the detector screen. Ambient light has to be cautiously avoided because it may interfere with flashes coming from actual incident ions or increase the possibility of wear and damage to the intensifier of the camera.

3.5 Sample preparation

Dye molecules were employed to characterise the performance of our microscope MSI system. In order to test the spatial mapping capability, a thin, uniform layer of dye molecules with a well-defined pattern was created using a home-built electrospray device and metal mesh.

Electrospraying is liquid nebulisation by electrical forces [115], and has traditionally been used as a deposition technique to prepare thin, uniform samples. For example, researchers employed electrospraying to prepare thin films for nuclear-physics apparatus [116,117], solar cells [118,119], fuel cells [120,121], Lithium batteries [122,123], and micro- and nanoelectronic devices [124,125].

In the field of MALDI-MS, the feasibility of preparing samples by electrospray deposition has been demonstrated. Hensel and coworkers [126] reported that electrospray deposition could produce significantly more homogeneous sample surface than the traditional air drying method, which allowed MALDI ToF-MS to be used as a quantitative analytical tool. Both the within-sample spot and between-sample spot variability were reduced by electrospray sample preparation, which led to a higher uniformity for the acquired MALDI mass spectra. This conclusion was also confirmed by Axelsson in ref. [127]. Matrix crystals produced by electrospray are on the order of micrometers or even sub-micrometers, resulting in a fine and homogeneous layer.

A schematic diagram of the home-built electrospray deposition device is shown in Figure 3.13. Under atmospheric pressure, a solution of analytes flows through a metal capillary tube held at high potential (4 to 5 kV). The diameter of the capillary is typically

less than 1 mm. A conductive sample slide is laid upon a large planar counter electrode that is 1 – 3 cm under the bottom tip of the capillary tube. An electrospraying process can be divided into three major stages, which are (1) droplet formation, (2) droplet nebulisation, and (3) gaseous ion formation [115]. The three stages are illustrated in Figure 3.14.

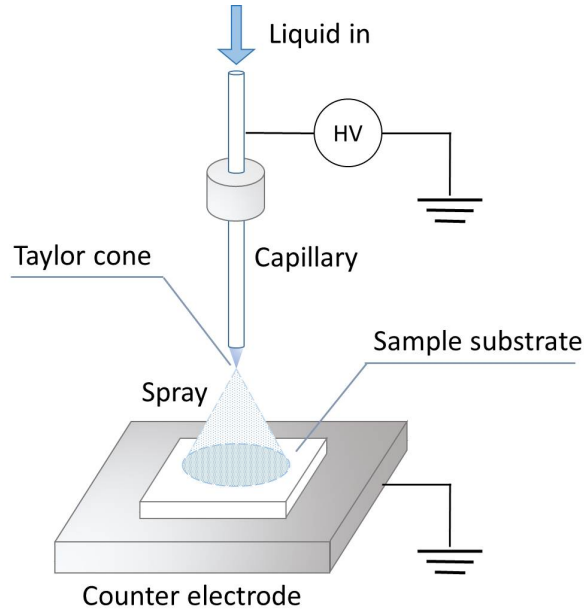
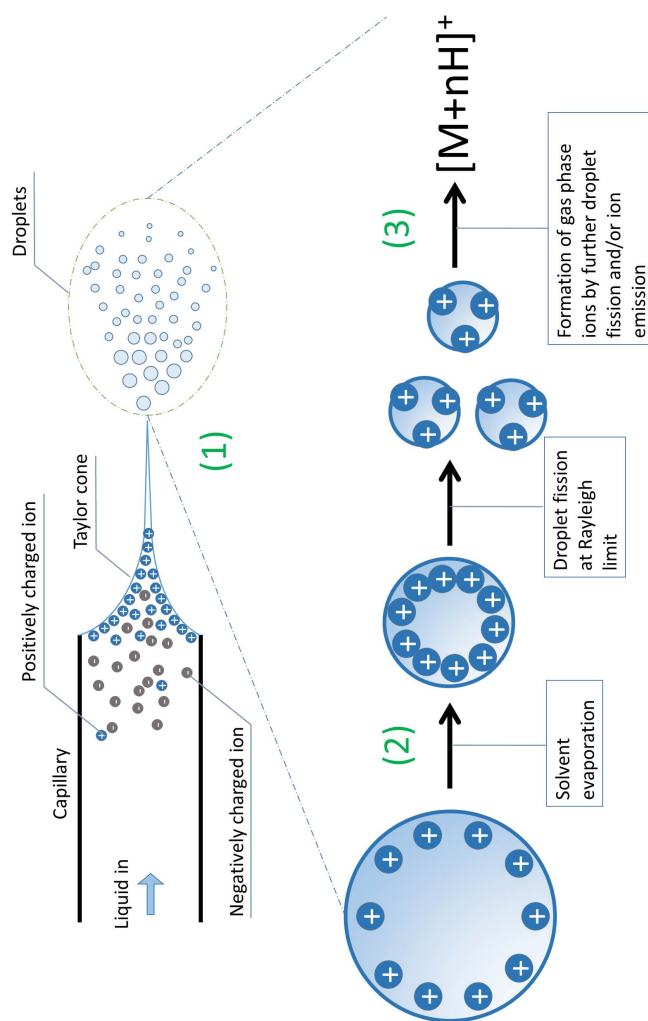


Figure 3.13: A schematic diagram of the home-built electrosprayer. This apparatus was developed by B.Winter [113].

In stage (1), the electric field strength at the capillary tip can be determined by

$$E_c = 2V_c \left[r_c \ln \frac{4d}{r_c} \right]^{-1}, \quad (3.2)$$

where r_c is the radius of the capillary and d the distance between the planar counter electrode and the capillary tip [115]. V_c corresponds to the high voltage applied on the capillary, it is assumed positive for convenience of discussion. As shown in Equation (3.2), the field strength E_c is in proportion to V_c and approximately inversely proportional to the capillary radius r_c . It also decreases slowly with a logarithmic dependence on the distance d . Under typical electrospray conditions, where r_c is very small and V_c is very large, E_c is of a magnitude of 10^6 V/m.



c

Figure 3.14: Diagram of electrospray sample deposition: (1) Under high voltage, the Taylor Cone emanates a mist of liquid drops. (2) The solvent gradually evaporates away from the droplets, leaving them more and more charged until the charge exceeds the Rayleigh limit and the droplets explosively decompose. (3) Ions of sample molecules are formed.

At the capillary orifice the imposed field also partially penetrates into the solution. As a result, positive and negative charges are redistributed: some cations in the solution drift towards the liquid surface while some anions drift away from it. Therefore an internal electric field is formed inside the solution, which essentially cancels out the imposed field. There are two competing forces on the positive charges: they are drawn away from the liquid by the strong electric field and held back by the surface tension. As a result, if one examines the nascent drop forming at the orifice while raising V_c , at low voltage the drop appears as a hemisphere, then stretches out until a liquid cone forms with convex

sides and a rounded tip. This is called the “Taylor cone” after the British physicist who theoretically described this phenomenon in 1964 [128]. As the voltage keeps rising, the effect of the electric field on the liquid becomes more prominent and the Taylor cone becomes more and more unstable. Once a certain threshold voltage has been reached, a liquid filament with a diameter of a few microns, whose surface is loaded with cations, is emanated from the cone tip. The threshold voltage for a given solvent surface tension λ [115] is

$$V_{on} \approx 2 \times 10^5 \sqrt{\lambda r_c} \ln \frac{4d}{r_c}. \quad (3.3)$$

Therefore, when the capillary geometry is fixed, the magnitude of the onset voltage for electrospray deposition is dictated by the distance between the capillary orifice and the counter electrode. For stable operation, the voltage is usually raised one or two hundred Volts above V_{on} . However, if the applied voltage is too high, it may reach a multispray condition where the major central cone disappears and droplets are emitted from multiple tiny cones. In practice, multispraying should be avoided because it is less stable and the shape of the deposited sample becomes rather irregular.

At some distance downstream, the liquid filament decomposes into a mist of separate droplets. Each droplet possesses an excess of positive electrolyte ions on their surface because the negative counterions remain in the liquid inside the capillary. The droplets begin to shrink due to the evaporation of solvent molecules. The initial charge and size of a droplet depend on the liquid flow rate and V_c . Generally, its radius is about $1.5 \mu m$ and its charge is on the order of 10^{-14} C, which corresponds to around 50,000 singly charged ions. As the radius of a droplet reduces along with its surface area, ions on the surface are getting closer and closer to each other, leading to stronger and stronger Coulomb repulsion among them. A “Coulomb explosion” (also known as “fission”) occurs when the Rayleigh limit is reached. At the Rayleigh limit [129], the ions repel each other strongly enough to overcome the surface tension that holds the droplet together. The charge of Rayleigh

limit can be estimated by the following equation:

$$Q_R = 8\pi\sqrt{\lambda\epsilon_0 r^3}, \quad (3.4)$$

where λ is the liquid surface tension on a droplet, ϵ_0 is the electric permittivity in vacuum, and r is the droplet radius. From a first generation droplet, about 20 smaller offspring droplets are released, which have a diameter of about 0.1 μm .

There are two different mechanisms proposed to explain the formation of unsolvated gas-phase ions in stage (3). Dole [130] and Rollgen [131] proposed the “single ion in droplet theory” (SIDT) model: continuous solvent evaporation and fission may eventually lead to the formation of extremely small droplets ($r = 1 \text{ nm}$), which carries only one single ion. Non-solvated gas-phase ions may be derived after solvent is completely evaporated. In the other model, proposed by Iribarne and Thomson [132,133], ion evaporation (emission) occurs from small and highly charged droplets, and its driving force is the repulsion between a charged ion and all other charges of the droplet. Typically, once the radius of a droplet is reduced down to $r \approx 8 \text{ nm}$ with $N \approx 70$ singly charged ions inside, the droplet will stop fission but start to directly emit gas-phase ions. Once these ions of dye molecules are deposited over the sample substrate, they will be neutralised because the substrate is conductive and grounded.

The quality of a dye layer is determined by the size of particles or droplets that form the layer, as well as their monodispersity and their uniform distribution over the substrate. Small droplets of narrow size distributions are usually preferred because they can minimise the number and size of voids, flaws, and cracks. Moreover, to obtain an even layer thickness, the droplets have to be homogeneously dispersed over the surface. The electrospray method fulfills all these requirements. As discussed previously, electrospray allows the generation of fine droplets in the sub-micrometer range, with narrow size distribution. Electrostatic forces between the capillary tip and the substrate also enable a uniform dispersion of the droplets in the space. Moreover, the sample prepared by

electrospray deposition is highly reproducible and user independent thanks to its almost automated operation.

The sample prepared by electrospray deposition has a full circle shape since the charged droplets expands in space as a spherical cloud due to mutual Coulomb repulsion. Its size can be controlled by adjusting the distance d from the capillary tip to the sample plate. If the solution flow rate is fixed, by simply increasing (or decreasing) the spray time, the same thickness can be obtained over a larger (or smaller) area.

Our home-built electrospray apparatus has an injection needle (BD Microlance 3) as the metal capillary (the tip of the needle has to be deliberately filed to be flat). The outer diameter (r_c) of the needle is 0.81 mm and its length is approximately 30 mm. A voltage (V_c) of 4 – 6 kV is applied to the needle as V_c . Disposable indium tin oxide (ITO) coated glass slides (25×25×1.1mm, Sigma-Aldrich) are used as sample substrates. An xy -adjustable metal stage acts as the grounded counter electrode and the ITO slides are placed on top of that stage. The needle tip and the upper surface of the stage are separated by 1 – 5 cm (d). One side of the ITO slides is conductive and the other is insulated. The conductive side faces the needle and is connected to the grounded stage via a piece of metal in order to avoid charge accumulating on the plate, which would repel incoming droplets. A solution flow rate of 30 – 60 $\mu\text{L/h}$ is maintained using a syringe pump (Legato 100, KD Scientific Inc).

Two types of electroformed nickel meshes are used as stencils to create grid patterns (Goodfellows NI008720 with wire width = 67 μm and hole size = 500 μm and Industrial Netting BM0181-01 with hole size = 102 μm and wire width = 38.1 μm). Before spraying, a mesh is flushed against the conductive side of the ITO substrate with a droplet of solvent (methanol or acetone for instance), which also prevents the mesh from warping during spraying. Typically, a spray lasts between 5 to 20 minutes, which corresponds to 2.5 to 10 μL if the flow rate is set to 30 $\mu\text{L/h}$. The aforementioned electrospray procedures are illustrated in Figure 3.15 and a microscopic photo of a grid pattern of crystal violet is shown in Figure 3.16.

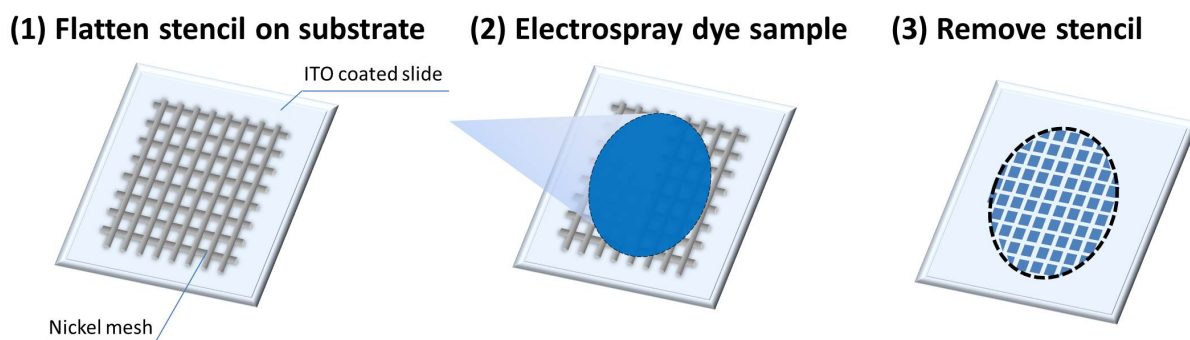


Figure 3.15: Using a stencil to create a grid pattern of dye.

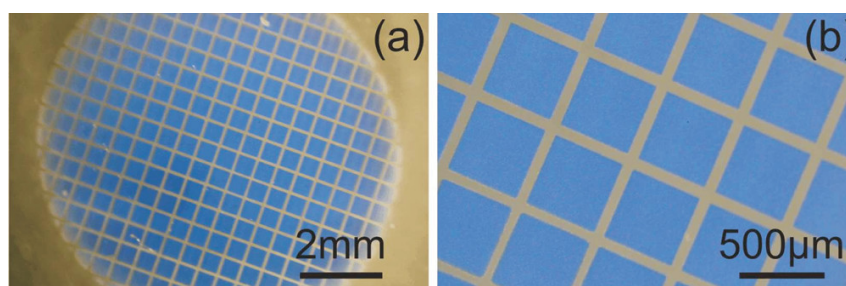


Figure 3.16: Optical microscope images of the grid pattern of crystal violet. Adapted from ref. [87].

To achieve an optimal electrospray deposition, trial-and-error is usually necessary. As shown in Equation (3.3), critical parameters, such as the high voltage applied to the capillary as well as the capillary tip to sample substrate distance, need to be tweaked carefully. Of course electrospraying also relies on the dye sample solution, its flow rate, concentration, and solvent.

3.6 Data acquisition and centroiding

The acquisition and processing of the spatial imaging data involves four steps: analogue-to-digital conversion, thresholding, centroiding, and accumulation. First, the analogue video signal output from the CCD camera is transmitted to a television and a computer respectively. The television is used to monitor real-time ion images while searching for optimal experimental conditions. The computer is where acquired ion images are actually processed and saved. However, the computer only handles digital data, whereas the

CCD camera produces analogue data. So, a frame grabber (Pleoras iPORT Analog-Pro External Frame Grabbers) is used to bridge the gap. A frame grabber is an electronic device which serves as an analogue-to-digital interface that captures individual, digital still frames (8-bit, 768×576 pixels) from an analog video signal. In the second step, a threshold is introduced to the digital image, which classifies any pixel intensity less than a certain (threshold) value as noise and reassigns it with a zero intensity. In comparison with real ion signal that has been amplified dramatically by the MCP assembly, low-level background noise is weaker, so thresholding can effectively filter them and improve the image quality. Choosing an appropriate discrimination value for thresholding is always a trade-off: if it is too high there is a risk of excluding actual ion signals with relatively low intensity levels while if it is too low unwanted noise may remain. In practice, a lower bound threshold is often set around 30 for an 8-bit pixel.

There is one problem with the detector assembly that may potentially impair the spatial resolving power of an imaging instrument, which is that a single incident ion can actually illuminate a group of pixels (rather than one single pixel) on the CCD sensor. During a conventional data acquisition process, captured ion images are directly summed up into a buffer for a certain number of frames before outputting a final accumulated image. The intensity distribution among the group of pixels lit up by a single incident ion approximates to a two-dimensional Gaussian distribution. Adding in the pixel intensity spread of each ion into the final output image is equivalent to applying a blurring filter to the obtained ion image.

The source of this blur is the electron amplification process in the detector assembly, where an incident ion turns into an electron packet after passing through a microchannel of the first MCP. Unfortunately this electron packet will spread in space before it reaches the second MCP. As a consequence, multiple electron avalanches will be triggered in neighboring pores. As the electron packet continues to expand during the flight from the second MCP to the scintillator, a small area rather than a single spot will be lit up on the scintillator, which eventually will be registered by a group of pixels on the CCD camera.

To solve this problem, a “centroiding” method is used to pin down a single position for one ion impact instead of accumulating the whole spread of illuminated pixels. This single position corresponds to one counted ion. It is worth noting that response inhomogeneities are also commonly seen over the surface of MCPs, scintillating screens, and CCD pixel-arrays, which means that identical incident ions may lead to different signal intensities depending on where they strike the detector. Moreover, the kinetic energies of different incoming ions are also slightly different, which further increases the ion to ion variability. With the centroiding method, an ion impact is always counted as one, regardless of its detected intensity profile, which effectively reduces fluctuation in signal level due to inhomogeneous response of detectors (the chance of two or more ions hitting the same spot is very small).

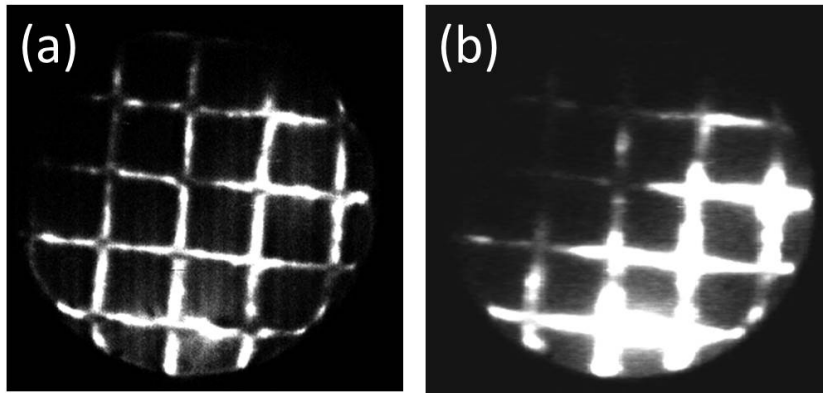


Figure 3.17: Ion images of a grid pattern of crystal violet: (a) with centroiding and (b) without centroiding. The one with centroiding is much clearer than the one without, showing that centroiding can significantly improve the spatial resolution of the microscope MSI system.

Centroiding is implemented by the Labview IMAQ “Find Circles function”, which is adept at detecting circles in binary images. Since the detector assembly is cylindrically symmetric, an area over the CCD pixel-array belonging to an ion event usually has a circular shape. Starting from an input image, “Find Circles” can find the radius and centre of any circular area even if it overlaps with multiple circular particles. In addition, a minimum circle radius value can be specified to further reduce any background noise from the image. The centre of a detected circle whose radius is above a threshold is regarded

as the actual coordinate (pixel) of a real ion event. This ion event is immediately summed into an accumulated image buffer by incrementing the intensity value at the corresponding pixel by one. This acquisition and centroiding process is repeated for about 10 000 laser shots before the final accumulated image is output. Each value stored at coordinate (x, y) indicates the total number of ions that have impinged that particular point during acquisition. Because centroiding is performed in real-time during each ToF cycle, the complete set of frame-by-frame centroided and raw ion images is not stored and therefore cannot be retrieved. A centroided ion image is compared with a raw image in Figure 3.17, it is obvious that the centroided image is significantly better resolved than the raw one. The data acquisition process is illustrated in Figure 3.18.

3.7 Delay generator

An eight-channel Digital Delay/Pulse Generator (Model 575, Berkeley Nucleonics Corporation) is used to synchronise all devices (the laser system, the voltage pulse box used in PEDDA, the oscilloscope, and the CCD camera gate) at a 10 Hz repetition rate. It provides precise delays and widths with 250 ps resolution for up to 1,000 seconds. As shown in Figure 3.19, the rising edge of the Q-switch (the moment a laser pulse is fired) is set as the T_0 of an acquisition cycle. The output power of the Nd:YAG laser is controlled by the delay time between the firing of the flash lamps and the Q-switch, which is typically set to be 360-410 μs . The laser power can be readily adjusted using this delay as mentioned previously and illustrated in Figure 3.5. The pulsed PEDDA voltage is created by a Behlke switch, which is triggered *ca.* 2.8 μs later than T_0 . The intensified CCD camera is gated on ToF peaks of interest through a special TTL signal generator device, which controls the delay time as well as the width of the camera gate. The time zero of this device synchronises with T_0 (denoted with a dashed rectangle in Figure 3.19). The width of a peak in the ToF spectrum is usually less than 20 ns, so in order to obtain an ion image of a particular peak, one simply moves the gate to overlap with it, and sets the gate width

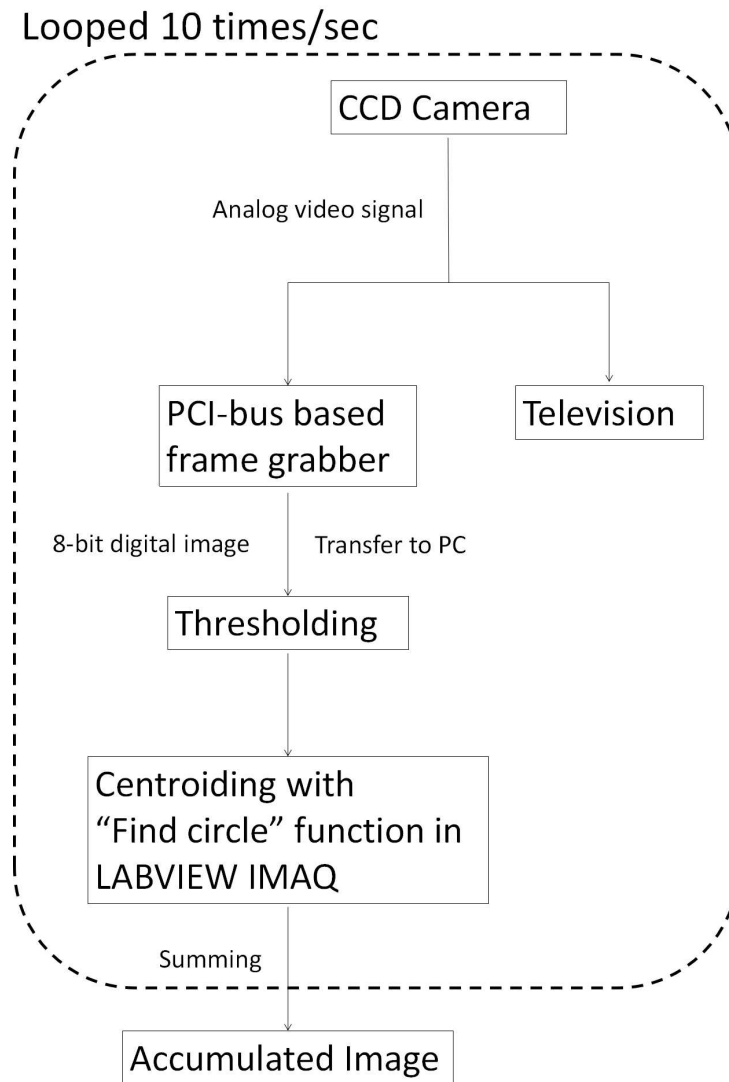


Figure 3.18: Schematic diagram of the image acquisition process. The Labview program that performs centroiding was developed by K. Amini [134].

slightly wider than that peak (roughly 50 ns).

3.8 Calibration

ToF-MS measures the arrival time of each ion, providing a ToF spectrum rather than a mass spectrum. This needs to be converted into a mass spectrum through calibration, a process that is necessary for good mass accuracy. The calibration equation is derived from the relationship between the kinetic energy (zU) and the flight time t in the field

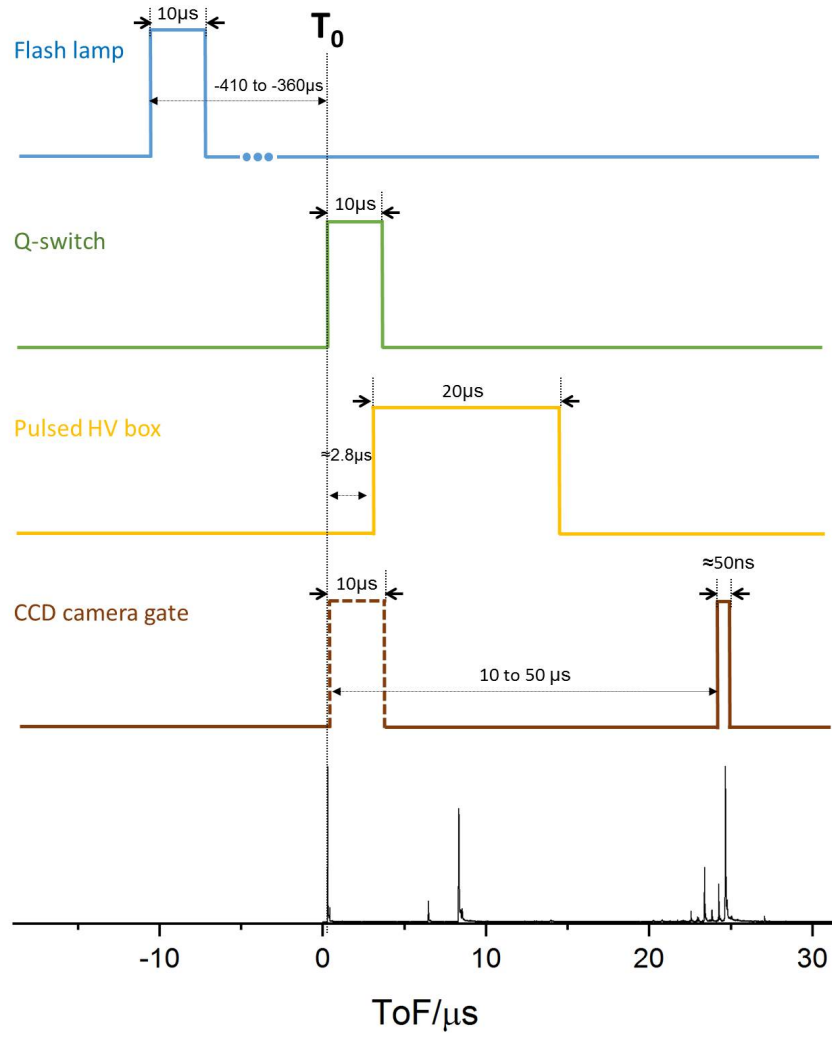


Figure 3.19: The respective delay times of the flash lamp, Q-switch, the PEDA voltage pulse, and the CCD camera gate. Shown at the bottom is an example of a ToF spectrum.

free drift region. If v denotes a particle's velocity along the ToF axis, then:

$$v = \sqrt{\frac{2zU}{m}}. \quad (3.5)$$

The particle's flight time t in the drift path d therefore has the following form:

$$t = \frac{d}{v} = \frac{d}{\sqrt{2U}} \cdot \sqrt{\frac{m}{z}}. \quad (3.6)$$

Consequently, t is linearly proportional to the square root of the mass to charge ratio. The conversion function between t and m/z can be fitted with a linear equation,

$$y = a + bx, \quad (3.7)$$

where y corresponds to flight time t , x corresponds to $\sqrt{m/z}$, the slope b characterises the linear dependence between t and $\sqrt{m/z}$, and an intercept a takes into account other factors that may shift the entire flight time, for instance the specific position of T_0 . In order to estimate the values of a and b , one needs to calibrate Equation (3.7) by a few ToF peaks with known corresponding m/z .

The ToF spectrum in Figure 3.20 (a) shows four major ToF peaks that correspond to different fragments of the parent ion of a reference compound crystal violet. The mass-to-charge ratios are already known for all of these fragments, thus Equation (3.7) can be fitted with a straight line as shown in Figure 3.20 (b), where the intercept $a = 1.83641 \times 10^{-6}$ and the slope $b = 1.17081 \times 10^{-6}$. Therefore, for an unknown ToF peak with a flight time t , its corresponding m/z can be estimated with the following calibration function:

$$\frac{m}{z} = \left(\frac{t - a}{b} \right)^2 \quad (3.8)$$

The accuracy of a calibration function is usually measured in the pseudo-unit “parts-per million” (ppm):

$$Accuracy = \frac{(m/z)_{Estimated} - (m/z)_{True}}{(m/z)_{True}} \times 10^6 \text{ (ppm)}. \quad (3.9)$$

where $(m/z)_{Estimated} - (m/z)_{True}$ calculates the residuals between the true m/z values of the ToF peaks and their respective estimated ones (the smaller this accuracy value, the better). Another dye, Rhodamine B, is used as an analyte to test the calibration equation. The ToF spectrum of Rhodamine B is recorded under the same conditions as the calibration experiments and one particular fragment ion $m/z = 398$ is chosen for comparison. The accuracy turns out not to be very ideal: the estimated m/z value

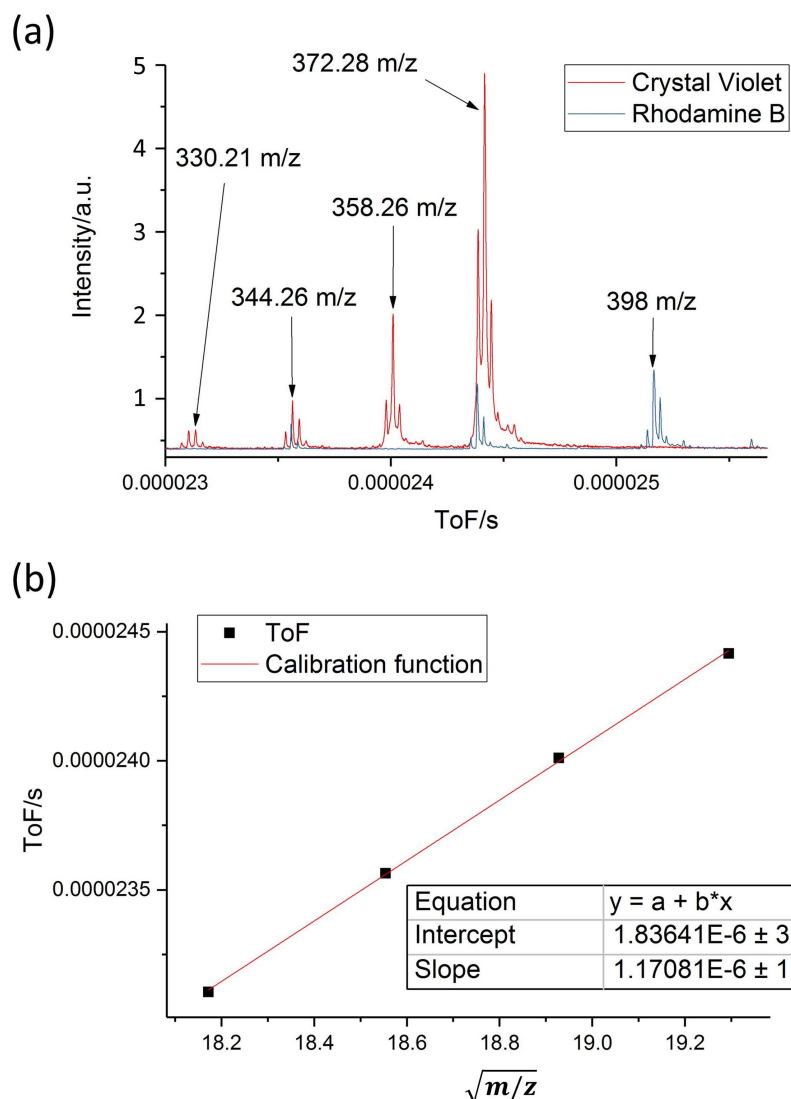


Figure 3.20: Converting a ToF spectrum to mass spectrum: (a) reference ToF peaks with known m/z values; (b) fitting the calibration function.

differs from the true value by -0.949 , which corresponds to an accuracy of -2384.2 ppm. Calibration results for the test ion and the four reference peaks are summarised in Table 3.2. There are a few possible sources of the errors in the determination of an accurate mass from the centroid of a ToF peak:

(1) The relationship between the flight time t and the square root of the mass-to-charge ratio $\sqrt{m/z}$ can not be fitted with a simple linear function. This can be confirmed by the large residuals of those four reference ions. Such nonlinearity is due to the fact that, during the acceleration process, the ions also spend time within the ion optics and

Table 3.2: Calibration results

Ion	$(m/z)_{True}$	$\sqrt{m/z}$	t/s	$(m/z)_{Estimated}$	Residual	Accuracy/ppm
1	372.28	19.295	2.442E-5	371.93	-0.34751	-933.47
2	358.26	18.928	2.401E-5	358.69	0.43285	1208.19
3	344.26	18.554	2.356E-5	344.41	0.14883	432.31
4	330.21	18.172	2.310E-5	329.98	-0.22982	-696.00
5	398	19.950	2.517E-5	397.05	-0.94893	-2384.24

do not just travel at a constant speed in the field free region, thus the relationship is more complex. Moreover, when PEDA techniques, especially the time-variable PEDA, are employed, the potential on the extractor becomes dynamic (it is raised up from a lower voltage to a higher one), which further complicates the situation. A way to deal with such issues is to use a quadratic or even higher order function to fit the underlying relationship.

(2) External calibrations almost always provide a worse mass accuracy than internal ones. In an external calibration, the spectrum of a known reference compound is recorded and used to produce the calibration function before an analyte is loaded and analysed by the MS system. Meanwhile in an internal calibration, the reference compound is introduced into the ion source together with the analyte. So ToF peaks from the reference compound and the analyte are in the same ToF spectrum. The internal method is usually preferable to the external one since the effects of any instrumental instability (such as temperature drift, voltage drift, and so on) are removed, and should be used whenever possible unless ions from the reference compound interfere with the analyte ions.

(3) Calibrant ions should cover as wide a mass range of analyte ions as possible, and generally speaking, the more known peaks are used in the calibration, the better the accuracy will be. This is because extrapolation of the calibration range is prone to inaccuracy. Calibrant ions densely distributed in a wide mass range is particularly crucial if a high order function is employed to fit the calibration equation.

(4) The mass peaks are not symmetric, which is a defect of the ToF-MS system. Each mass peak has a long tail due to the fluorescence lifetime of scintillators, and it is therefore

harder to determine the centroid of a peak.

3.9 Summary

The microscopic MSI instrument used in this thesis is a complex system. This chapter described the various modules involved in the system as well as their respective principles and functions. The first module described in this chapter is the ion optics setup and the overall layout of the MSI instrument. Then, the Nd:YAG laser system was introduced, which was used in experiments to ablate samples and turn them into detectable cations. Different components of the detector assembly were also discussed, especially the home-built fast scintillator. The approach to prepare a dye sample with a regular pattern using a home-built electrosprayer was also articulated. Steps involved in data acquisition and processing were detailed followed by a description of the synchronic control device. The last subsection was devoted to the calibration methods that transform a ToF spectrum into a mass spectrum, where possible sources of mass errors were also discussed.

Chapter 4

Pulsed Post-extraction Technique

The challenge in microscope MSI has shifted from multi-mass imaging to the simultaneous optimisation of mass and spatial resolution over a broad mass range. These are limited by the initial distribution of ion velocities at the sample surface. Mass resolution can be corrected using delayed extraction [135–138], but the spatial resolution will remain poor as nothing prevents ions from diffusing away from their initial time-of-flight axes. Aoki and coworkers resolved this by developing the post extraction differential acceleration (PEDA) method [139], which extracts ions from a surface immediately following ionisation to preserve their spatial resolution. Initial velocity distributions are then corrected by raising the potential of the extraction electrode after the ion packet has passed it. This causes slower ions of a particular m/z to accumulate more kinetic energy than quicker ions, creating a temporal focal point that is tuned to correspond with the detector plane. Using this approach, mass and spatial resolutions of $1450\ m/\Delta m$ and $4.0 - 5.5\ \mu\text{m}$ were simultaneously achieved. This chapter will introduce the idea of PEDA, then propose two variants that have broader mass ranges than the original.

4.1 PEDA

Figures 4.1(a) to (c) illustrate how the potential energy of a cation changes as it travels from the sample surface to the detector plane in the original PEDA technique. The initial electrical potential surface is shown in Figure 4.1(a), which is created by a three-electrode ion optical system with a potential of $V_E + V_A$ on the repeller, V_A on the extractor, and a

grounded electrode. The distance between the repeller and the extractor is L_1 and that between the extractor and the grounded electrode is L_2 . At $t = 0$, two cations, ion a and b , are generated at the repeller plane. They have the same mass-to-charge ratio but different initial velocities (the initial axial velocity of ion a ($v_0(a)$) is slower than that of ion b ($v_0(b)$)). Due to a positive potential difference between the repeller and extractor (V_E), both ions are accelerated along the ToF axis towards the detector. Ion b travels ahead of ion a and the distance between them gradually expands over time. At $t = \tau$ (Figure 4.1(b)), both ions are between the extractor and the grounded electrode, and the potential on the extractor is suddenly stepped up by ΔV_A . As a result, both ions gain potential energy from ΔV_A but the gain of ion a is larger than that of ion b : $\Delta V_A(a) > \Delta V_A(b)$. If τ and the magnitude of ΔV_A are chosen appropriately, the final speed of ion a ($v_f(a)$) will be larger than that of ion b ($v_f(b)$) in the field free drift region (Figure 4.1(c)). Hence after a certain flight path f , ion a will catch up with ion b at a temporal focal plane that should coincide with the detector. Consequently ions with the same m/z will arrive at the detector plane simultaneously no matter what their initial velocity is. This is the principle of PEDDA which is very similar to delay extraction (DE).

Figure 4.2 compares two ToF spectra of crystal violet ($m/z \approx 372$): the one on the left was collected without PEDDA and the one on the right with PEDDA. PEDDA's capability of narrowing the flight time dispersion of ions with the same m/z , but different initial velocities is illustrated here, the mass resolution on the left is about $180 m/\Delta m$, which increases to around $2400 m/\Delta m$ on the right. With PEDDA, adjacent ToF peaks that would otherwise be convolved become distinguishable and well-defined. PEDDA therefore allows for a better understanding of the sample composition, and can lead to or deeper insights into the structure of a particular molecule.

However, the mass resolving power of PEDDA is highly dependent on the mass of the analyte. Figure 4.14(a) shows this dependence for those analytes whose mass-to-charge ratios are in the vicinity of the optimal m/z ; in this case around $400 m/z$, PEDDA provides a reasonably good mass resolution (above $1000 m/\Delta m$) over a ≈ 65 Da range. However,

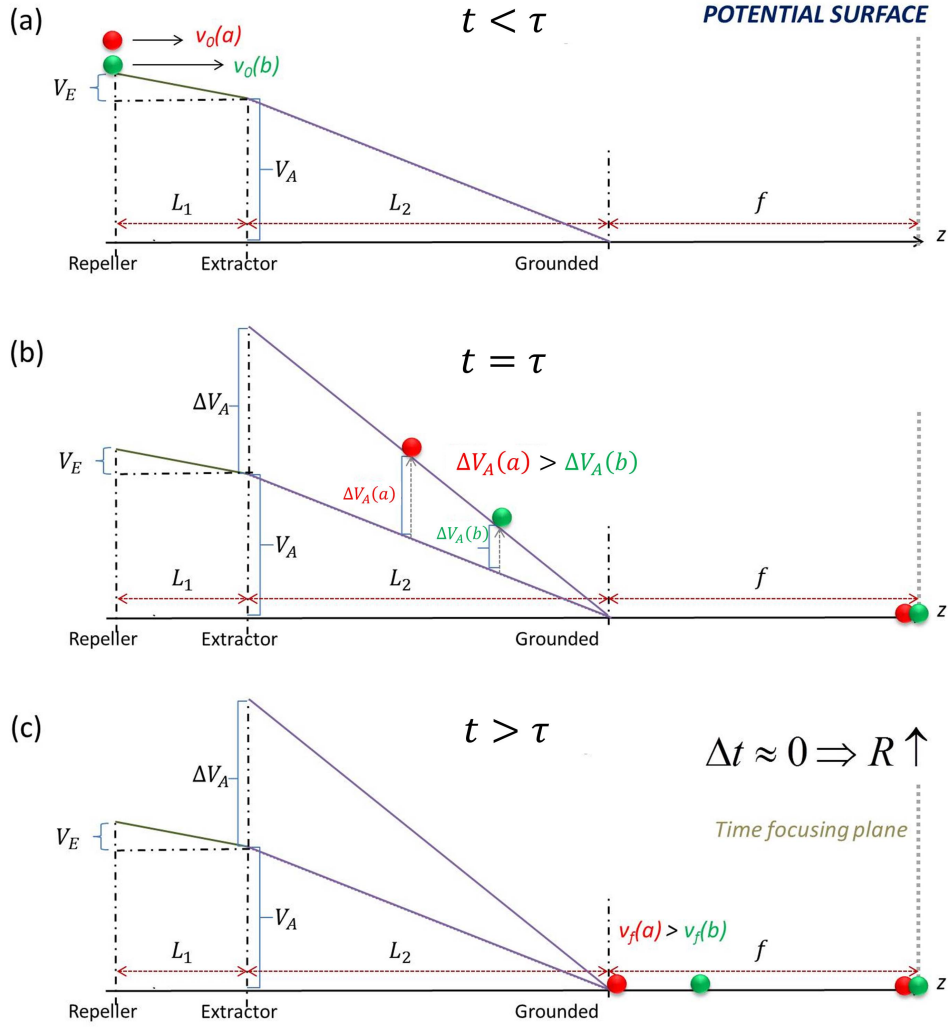


Figure 4.1: Illustration of the principle of PEDAs: (a) the initial velocity of ion a ($v_0(a)$) is smaller than ion b ($v_0(b)$), (b) when ions a and b are within the region between the extractor and grounded electrode, the potential on the extractor is pulsed up by ΔV_A at τ , which gives ion a a larger increase in potential energy ($\Delta V_A(a)$) than ion b ($\Delta V_A(b)$), (c) in the field free drift path, ion a will catch up with ion b at the time focusing plane due to its larger final velocity ($v_f(a) > v_f(b)$).

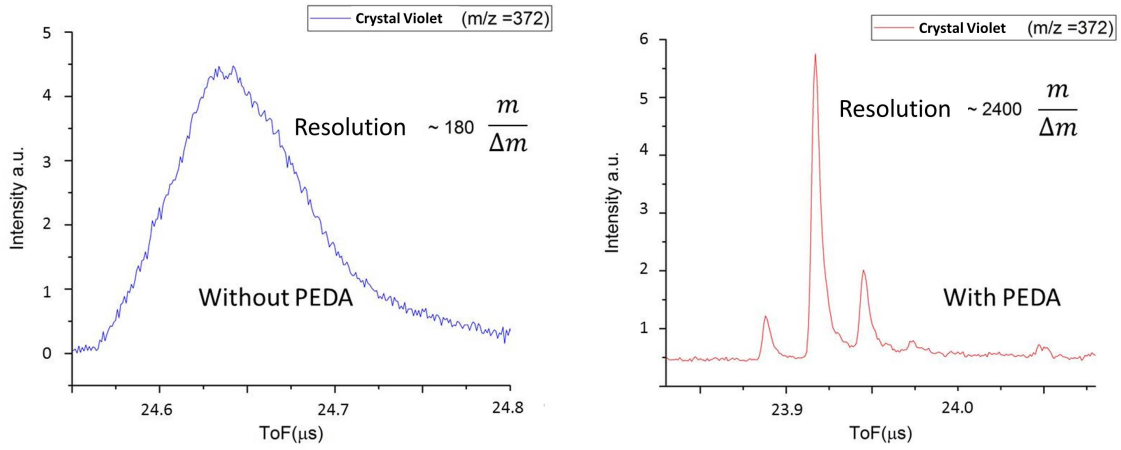


Figure 4.2: Comparing the time-of-flight resolution with and without PEDAs.

as m/z moves away from 400 m/z , PEDAs becomes less useful since the mass resolution drops rapidly.

PEDAs can also be applied over wide mass ranges using velocity corrected ion extraction [95]. For charged particles traveling through a uniform electric field formed by two electrodes with voltages of V_{in} and V_{out} respectively, it can be shown that the motion of two particles of different masses (m_1, m_2) can be replicated by tuning the acceleration potential by a scaling factor of x while maintaining the ratio between V_{in} and V_{out} :

$$\frac{V_{\text{in}} - V_{\text{out}}}{m_1} = \frac{xV_{\text{in}} - xV_{\text{out}}}{m_2}, \quad (4.1)$$

where x is determined by the ratio between m_1 and m_2 ($x = m_2/m_1$). Therefore, the optimal PEDAs window can be shifted to different m/z ranges, without changing the image magnification or the mass and spatial resolutions, by adjusting the applied extraction field accordingly. However, suppose there is a biological sample which contains thousands of analytes covering a wide mass range; if one wants to analyse the entire mass range with the PEDAs instrument using the velocity corrected ion extraction method, they would have to repeatedly adjust the voltages and calibrate for each mass window. This would be very tedious and impractical. Ideally, the mass range of PEDAs should be broadened without needing to tune the MSI instrument.

4.2 Double-field PEDA

This section describes an improved PEDA variant that uses an additional electrode to broaden the resolved mass window. The following subsections introduce this “double-field” PEDA concept, and experimentally compare it with the single-field method.

4.2.1 Theory and simulations

As shown in Figure 4.3, lighter ions in the original single-field PEDA have earlier temporal focusing points than heavier ions. As the field free drift length is fixed, ion optical

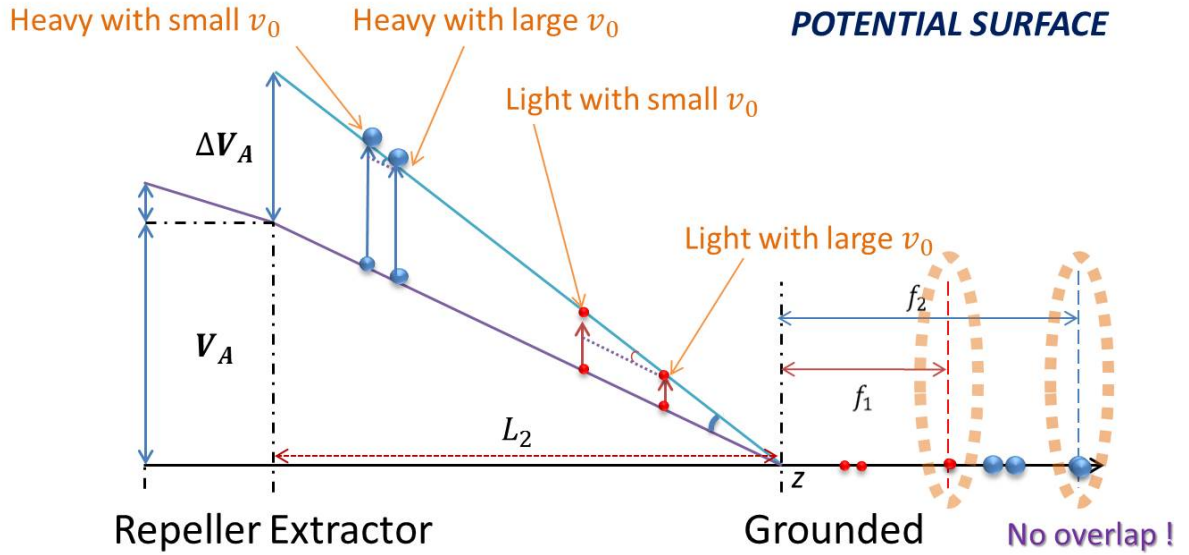


Figure 4.3: Lighter ions have an earlier time focusing plane than heavier ions. If the detector coincides with the temporal focusing point of the light ions, the heavy ions will not be focused simultaneously since they need a longer temporal focal length (f_2) than the light ions (f_1). As a result the instrument will provide a poor time-of-flight resolution for these heavy ions.

voltages must therefore be tuned to focus a particular m/z , and the effective mass range is consequently centred around this focal point. If one looks at this problem from another perspective, for a fixed focus length and delay time, the optimal pulsed electric field ΔE ($\Delta E = \Delta V_A/L_2$) is mass dependent. As illustrated in Figure 4.4, the optimal ΔE increases nonlinearly with larger m/z , or in other words, heavier ions need a harder “kick”

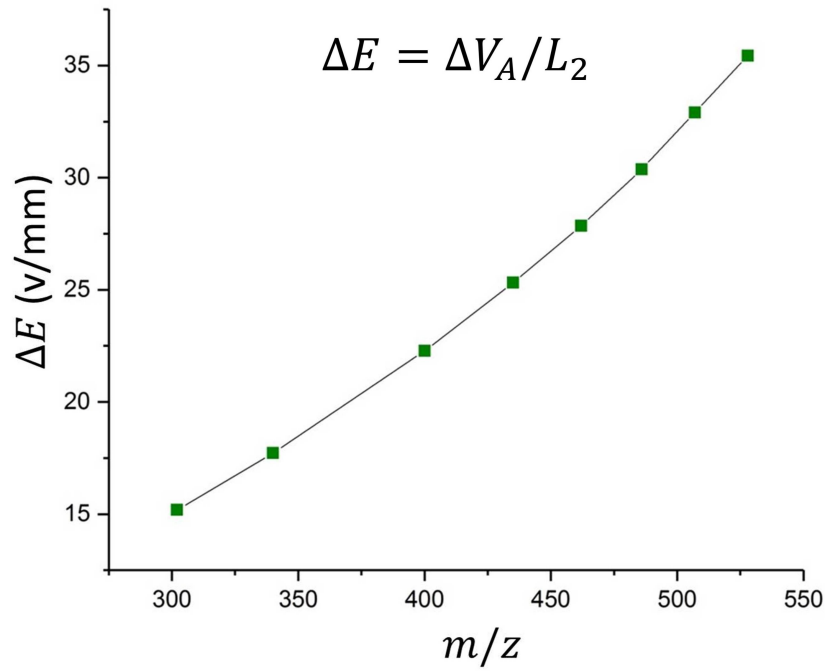


Figure 4.4: The optimum strength of the pulsed acceleration field (ΔE) as a function of m/z .

than lighter ions.

The temporal foci of two m/z can be made to overlap by introducing a second pulsed electrode to the PEDA acceleration region. This double-field PEDA method is illustrated in Figure 4.5, where the additional electrode is inserted between the extractor and einzel lens entrance. The raised potentials of the two pulsed electrodes are used to create a cusp in the potential surface that divides the acceleration region in two. When the pulse applied to the new plate is less than half of that applied to the extractor, heavier ions in the ‘early’ acceleration region will accelerate faster than lighter ions in the ‘late’ region. By adjusting the potential energy gradient on each side of the added electrode, ions with the same m/z ratio in different segments of the acceleration region can be simultaneously focused, thereby broadening the achievable mass range.

Following Aoki and coworkers, the extraction region in Figure 4.5 is defined with length L_1 and potential difference V_E . An ion with mass m , charge q , and initial velocity v_0 extracted from the charged surface at $x = 0$ will therefore accelerate to v_1 when it

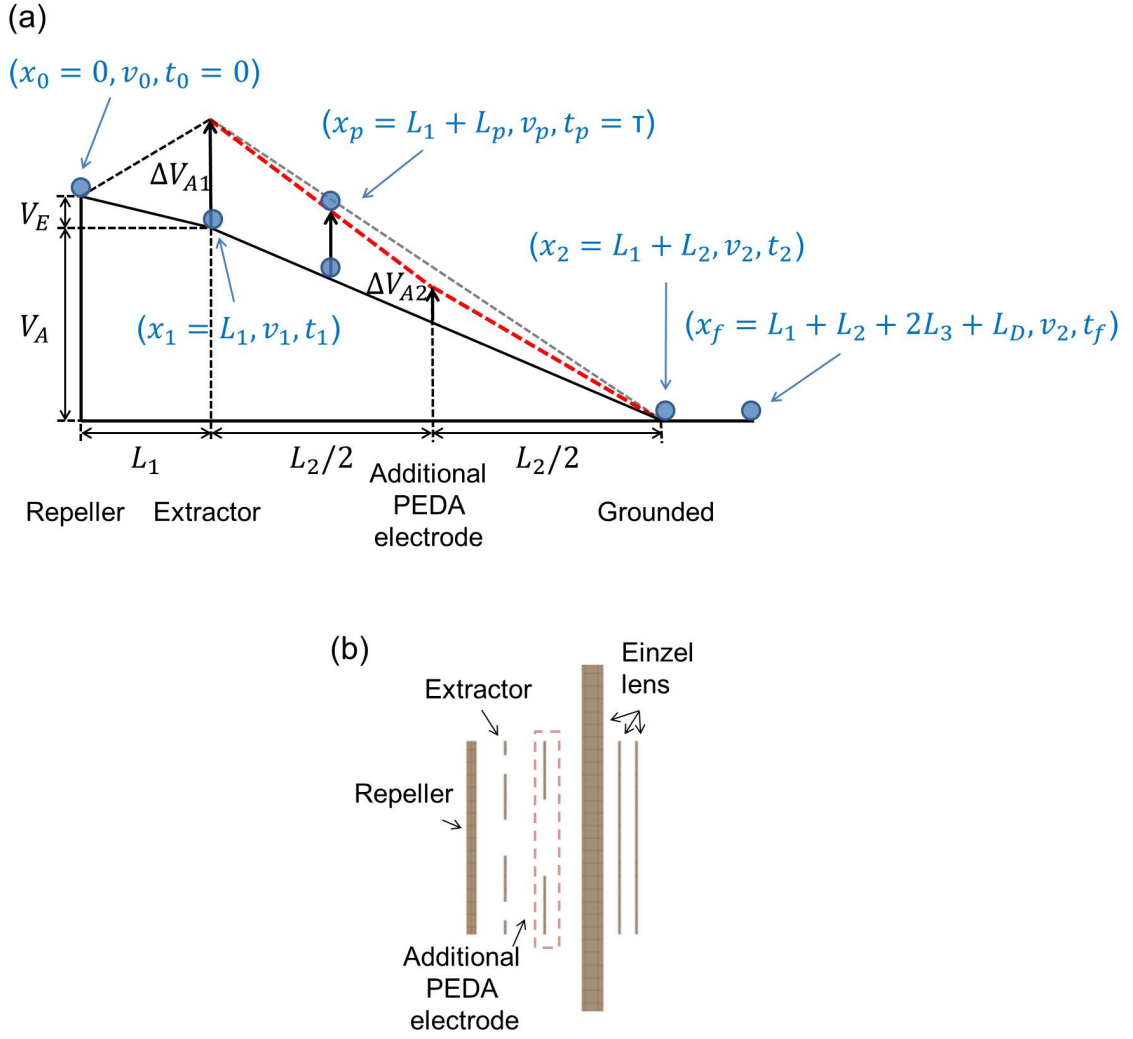


Figure 4.5: (a) Schematic of the double PEDAs method. The solid line is the initial potential surface, the red thick-dashed line the pulsed-up potential surface, and the thin-dashed line the pulsed-up potential surface in the original PEDAs method, (b) schematic of the ion optics for double-field PEDAs.

reaches the extractor electrode at $x = L_1$,

$$v_1 = \sqrt{\frac{2}{m} \left(\frac{mv_0^2}{2} + qV_E \right)}. \quad (4.2)$$

The flight time over this region is described by t_1 ,

$$t_1 = \frac{mv_0 L_1}{qV_E} \left(-1 + \sqrt{1 + \frac{2qV_E}{mv_0^2}} \right). \quad (4.3)$$

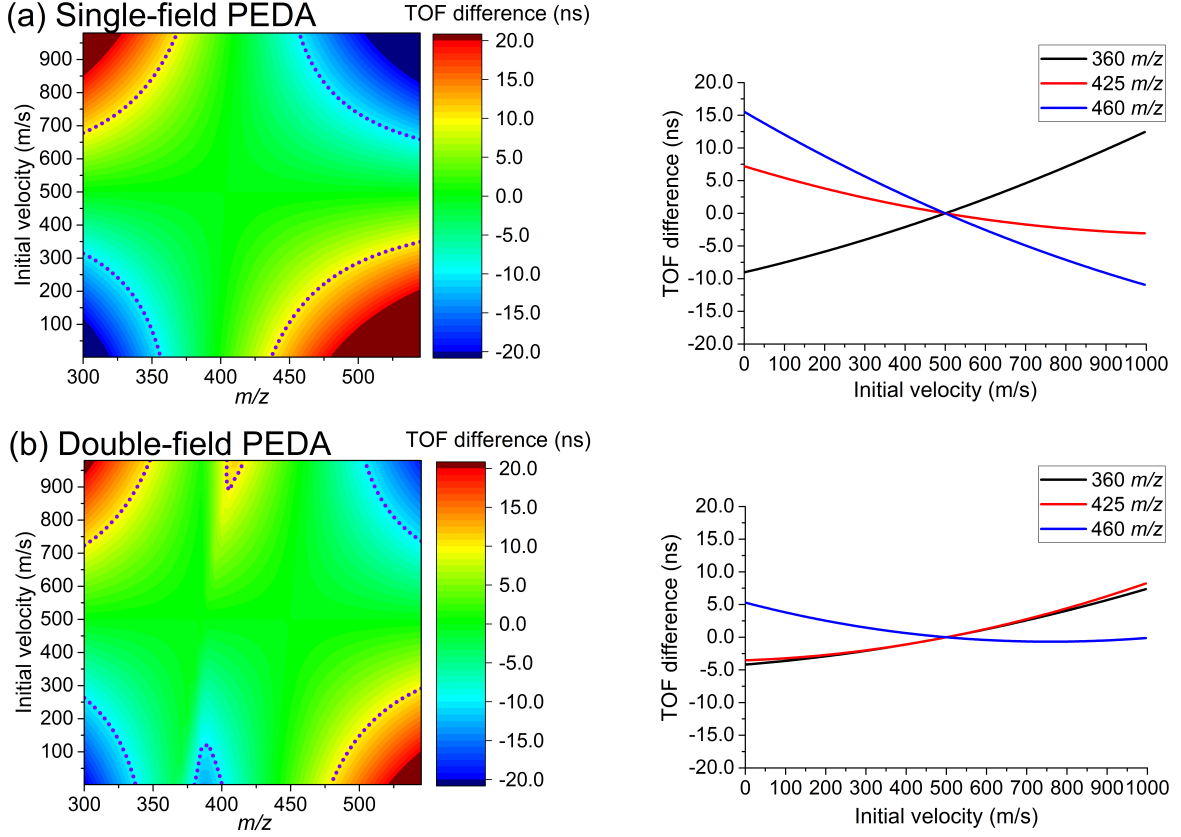


Figure 4.6: Calculated time variance $t_d = t_f(v_0, m/z) - t_f(500 \text{ m/s}, m/z)$ as a function of m/z and v_0 for: (a) original PEDA and (b) double-field PEDA.

The ion then enters an acceleration region between the extractor electrode and einzel lens entrance with potential difference V_A and length L_2 . The additional PEDA electrode is placed at $x = L_1 + L_2/2$ with potential $V_A/2$. At a set time τ , the extractor and PEDA electrodes are raised by ΔV_{A1} and ΔV_{A2} , respectively. When these pulses are applied, the ion is located at $x = L_1 + L_p$ with a velocity v_p . These are given by:

$$L_p = v_1(\tau - t_1) + \frac{qV_A}{2mL_2}(\tau - t_1)^2, \quad (4.4)$$

$$v_p = \sqrt{\frac{2}{m} \left[\frac{1}{2}mv_0^2 + q \left(V_E + V_A \frac{L_p}{L_2} \right) \right]}. \quad (4.5)$$

At τ , ions located in the ‘early’ and ‘late’ acceleration region will accelerate differently. For ions at $L_p \geq L_2/2$, the potential increase resulting from the pulsed electrodes is the

same as in the original PEDA case,

$$\Delta V = 2\Delta V_{A2} \left(1 - \frac{L_p}{L_2}\right). \quad (4.6)$$

The potential change for ions at $L_p < L_2/2$ is similar to the above, but accounts for the difference between ΔV_{A1} and ΔV_{A2} .

$$\Delta V = \Delta V_{A2} + (\Delta V_{A1} - \Delta V_{A2}) \left(1 - \frac{L_p}{L_2/2}\right). \quad (4.7)$$

In both cases, the velocity of the ion as it leaves the acceleration region, v_2 , is given by substituting the appropriate ΔV into the following equation:

$$v_2 = \sqrt{\frac{2}{m} \left[\frac{mv_0^2}{2} + q(V_E + V_A + \Delta V) \right]}. \quad (4.8)$$

The time at which the ion leaves the acceleration region also depends on its position. When $L_p \geq L_2/2$, the ion flight time at the einzel lens entrance, t_2 , is:

$$t_2 = \tau + \frac{mv_p}{qE_2} \left[-1 + \sqrt{1 + \frac{qE_2(L_2 - L_p)}{mv_p^2/2}} \right] \quad (4.9)$$

To calculate t_2 when $L_p < L_2/2$, the ion velocity and time-of-flight at the PEDA electrode, v_m and t_m , must also be determined,

$$t_2 = t_m + \frac{mv_m}{qE_2} \left(-1 + \sqrt{1 + \frac{qE_2 L_2}{mv_m^2}} \right), \quad (4.10)$$

$$v_m = \sqrt{\frac{2}{m} \left[\frac{1}{2}mv_p^2 + qE_1(L_2/2 - L_p) \right]}, \quad (4.11)$$

$$t_m = \tau + \frac{mv_p}{qE_1} \left[-1 + \sqrt{1 + \frac{qE_1(L_2/2 - L_p)}{mv_p^2/2}} \right]. \quad (4.12)$$

In the above, E_1 and E_2 are the electric field strengths of the ‘early’ and ‘late’ acceleration

region, and are defined as $\{2(\Delta V_{A1} - \Delta V_{A2}) + V_A\}/L_2$ and $(2\Delta V_{A2} + V_A)/L_2$, respectively.

At this stage, the ion enters a conventional three-electrode einzel lens with a grounded entrance and exit. Each lens electrode is separated by a length L_3 . The ion leaves the einzel lens at time t_3 with the same velocity as it entered with, and passes through a field-free drift region of length L_D before impacting the detector. Its total time-of-flight, t_f , is therefore:

$$t_3 = t_2 + 2 \frac{v_2 m L_3}{q V_{lens}} \left(1 + \sqrt{\frac{1 - 2q V_{lens}}{m v_2^2}} \right), \quad (4.13)$$

$$t_f = t_3 + \frac{L_D}{v_2}. \quad (4.14)$$

As the electrode voltages ($V_{rep} = V_E + V_A$, $V_{ext} = V_A$, $V_{pulse1} = \Delta V_{A1} + V_A$, $V_{pulse2} = \Delta V_{A2} + V_A/2$, and V_{lens}), the voltage switch time τ , and the instrument lengths (L_1 , L_2 , L_3 , and L_D) are fixed, the time-of-flight of a particular ion reduces to a function of its initial velocity v_0 and its mass to charge ratio m/z . The performance of the double-field PEDAs method can therefore be assessed for a specific m/z by calculating its time-of-flight difference t_d from a mean v_0 .

$$t_d = t_f \left(v_0, \frac{m}{z} \right) - t_f \left(v_i, \frac{m}{z} \right), \quad (4.15)$$

where $v_i = 500$ m/s. In these calculations, the initial ion velocities are assumed to be normally distributed with a FWHM of 500 m/s, according to ref. [94, 108, 109, 111, 112]. The electrode spacings are $L_1 = 1.45$ cm, $L_2 = 3.95$ cm, $L_3 = 0.79$ cm, and $L_D = 91.02$ cm. The single-field PEDAs voltages and voltage switch time are: $V_{rep} = 5000$ V, $V_{ext} = 4600$ V, $V_{lens} = 1728$ V, $V_{pulse1} = 5480$ V, and $\tau = 2.8$ μ s. These remain the same for the double-field method, with the exceptions of $V_{pulse1} = 5510$ V and $V_{pulse2} = 2710$ V.

A high mass or time-of-flight resolution means that ions with the same m/z but different v_0 arrive at the detector plane simultaneously. In other words, a smaller t_d means better mass resolution at that particular m/z . Figures 4.6(a) and (b) illustrate this dependence for single- and double-field PEDAs, respectively. In Figure 4.6(a), the t_d

for ions between 367 and 438 Da is less than 10 ns over the v_0 range ($0 < v_0 < 1000$ m/s). This is slightly longer than the 3.7 ns decay time of the scintillator, and can therefore be thought of as the effective mass range [114]. According to Figure 4.6(b), the optimum double-field PEDAs mass ranges using the 10 ns threshold are 348 – 380 Da and 415 – 480 Da, with excellent resolution over the whole range when a narrower distribution of initial ion velocities is considered. The double-field approach is therefore expected to have a wider overall mass range than the single-field method.

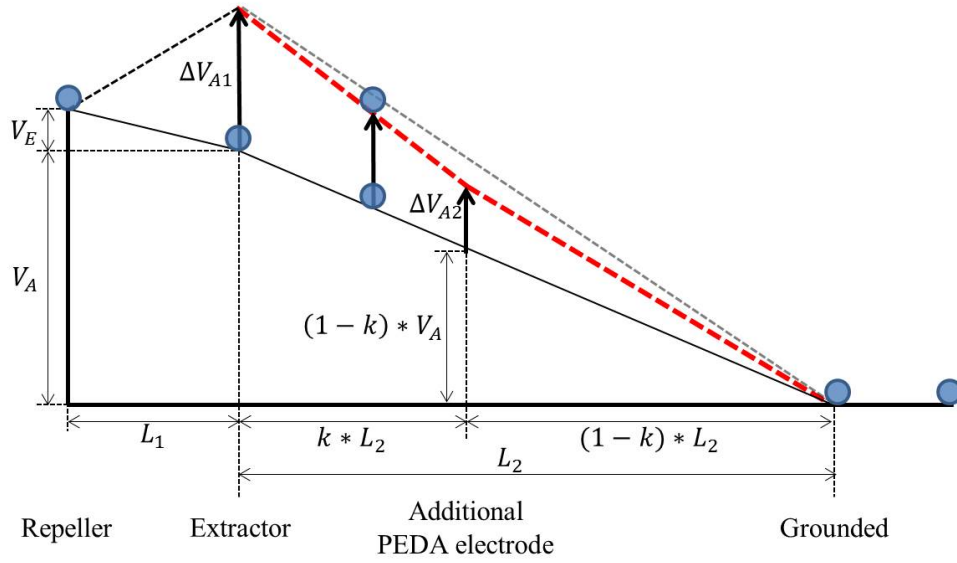


Figure 4.7: Potential surface of double-field PEDAs instrument: $k \times L_2$ ($0 < k < 1$) is the distance between the additional electrode and the extractor.

Varying the position of the additional electrode, or the magnitude of the pulsed voltage applied to it, changes the magnitude of the two pulsed electric fields. As a result, the two optimum mass ranges also shift, either closer or further from each other. However, the overall mass range, where ions are effectively focused by PEDAs, remains roughly the same. As shown in Figure 4.7, $k \times L_2$ ($0 < k < 1$) is the distance between the additional electrode and the extractor. Figure 4.8(a) to (d) illustrate that a smaller k leads to a larger distance between the two optimum mass ranges in double-field PEDAs. However, the mass resolution dip between the two optimum mass ranges also becomes wider and deeper. In addition, if the two optimum mass ranges are far apart, only one of them can

simultaneously possess a relatively good spatial resolution. Figure 4.8(e) shows that the optimum mass ranges of the double-field PEDAs method with different k values can be adjusted to be the same by changing the pulsed voltages ΔV_{A1} and ΔV_{A2} . Figure 4.9 shows that changing the pulsed voltage applied on the electrode can also move the two optimum mass ranges, but the width of the overall mass range basically stays unchanged. In the current setup, the two optimum mass ranges are set close together ($k = 0.5$), so both of them have a good spatial resolution.

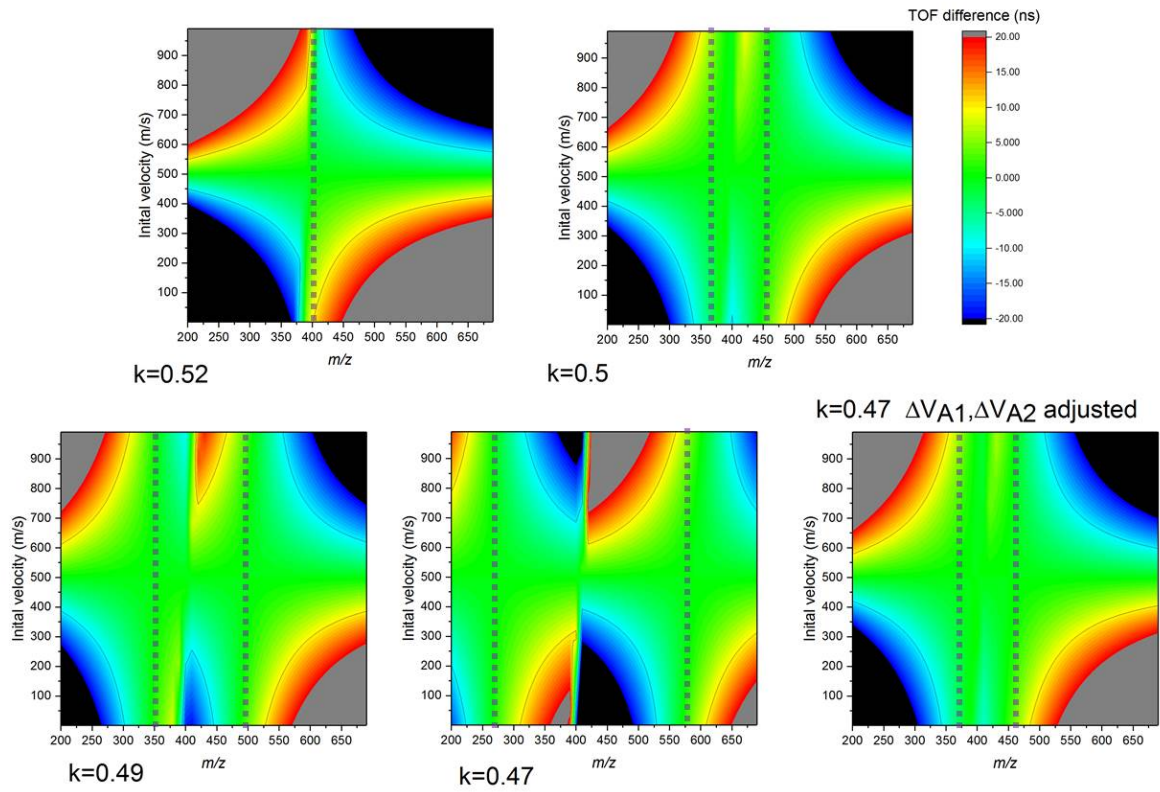


Figure 4.8: Calculated ToF difference function for different k ratios: (a) $k = 0.52$ (b) $k = 0.5$ (c) $k = 0.49$ (d) $k = 0.47$, and (e) $k = 0.47$ (in (e), ΔV_{A1} and ΔV_{A2} are changed from 0.47 to 0.52). The distance between the two optimum mass ranges in double-field PEDAs depends on k . Each dashed-line represents the central m/z of an optimal mass range.

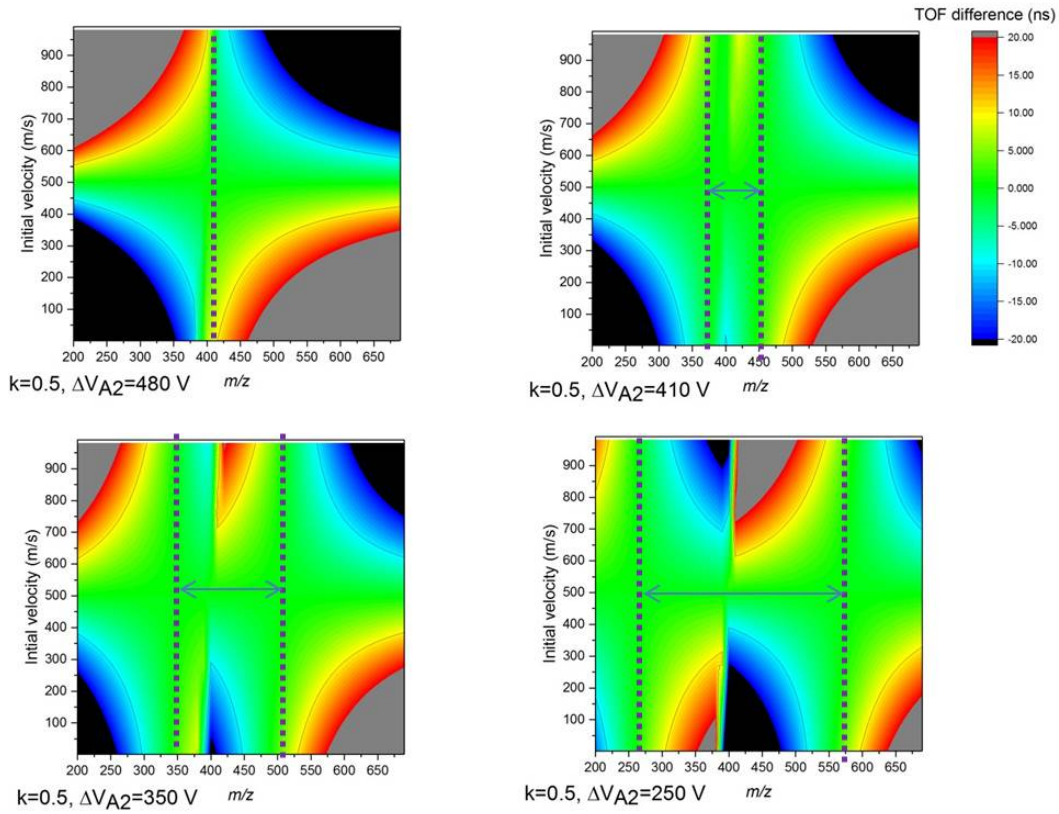
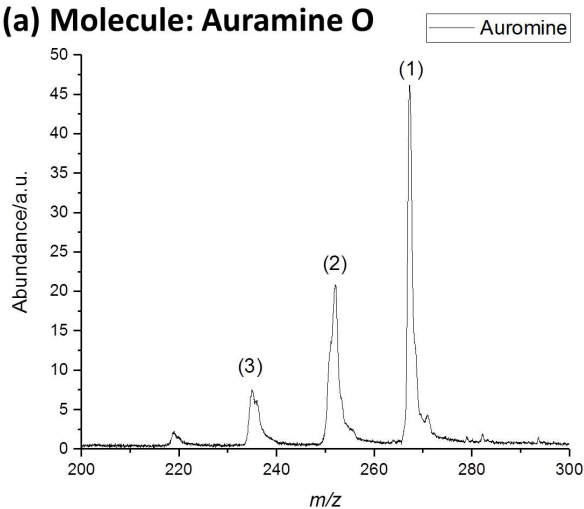


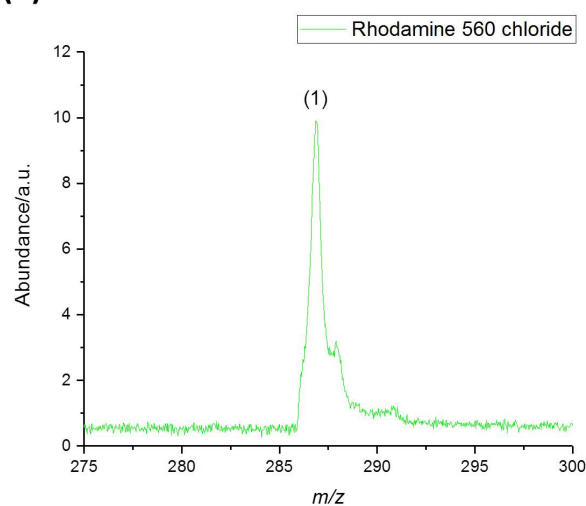
Figure 4.9: Calculated ToF difference functions for different ΔV_{A2} : (a) $\Delta V_{A2} = 480$ V (b) $\Delta V_{A2} = 410$ V (c) $\Delta V_{A2} = 350$ V (d) $\Delta V_{A2} = 250$ V, demonstrating that changing the pulsed voltage applied on the electrode can move the two optimum mass ranges (the two dashed lines represent the central m/z ratios), but the width of the overall mass range stays largely unchanged.

4.2.2 Methods

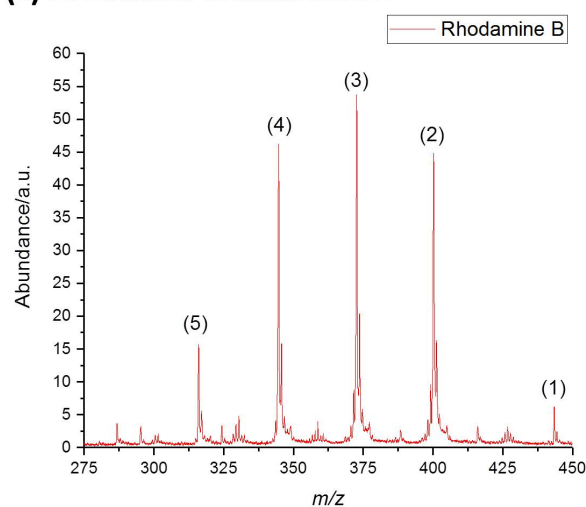
Single- and double-field PEDA were experimentally compared using the instrument described in Chapter 3. Time-of-flight mass spectra and ion images were collected for six commercial laser dyes (Auromine O, Rhodamine B, Rhodamine 560 chloride, Rhodamine 640 perchlorate, BBQ, and Exalite 404) and used to establish the mass and spatial resolutions of each technique over the 200 – 700 Da range. The mass spectra collected for each dye are shown in Figure 4.11. In the light of the studies in ref. [140], the fragment ions presented in the spectra are likely due to the degradation of dye molecules in their solution phase (the methyl, ethyl, or other groups, which are attached as side chains, are respectively replaced by a proton from the solvent as a result of photodegradation). Each

(a) Molecule: Auramine O

Peak	Ion	m/z
(1)	$[M]^+$	267.174
(2)	$[M - CH_2]^+$	253.724
(3)	$[M - (CH_2)_2]^+$	239.708

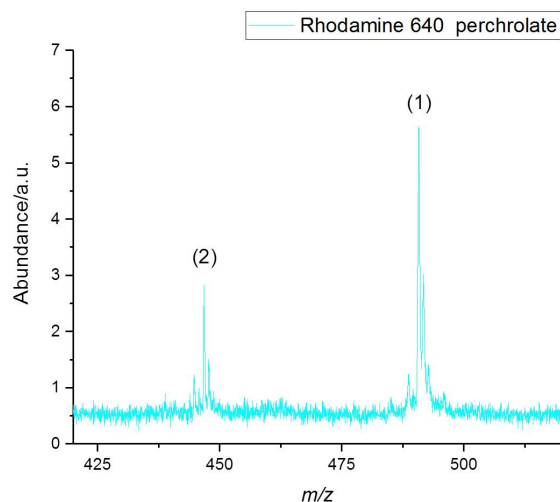
(b) Molecule: Rhodamine 560 chloride

Peak	Ion	m/z
(1)	$[M - CO_2 - Cl]^+$	287.841

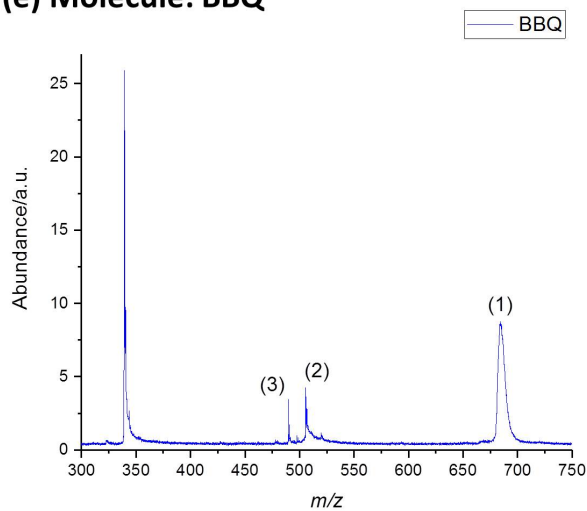
(c) Molecule: Rhodamine B

Peak	Ion	m/z
(1)	$[M - Cl]^+$	443.233
(2)	$[M - CO_2 - Cl]^+$	399.243
(3)	$[M - (CH_2)_2 - CO_2 - Cl]^+$	371.212
(4)	$[M - (CH_2)_4 - CO_2 - Cl]^+$	343.181
(5)	$[M - (CH_2)_6 - CO_2 - Cl]^+$	315.15

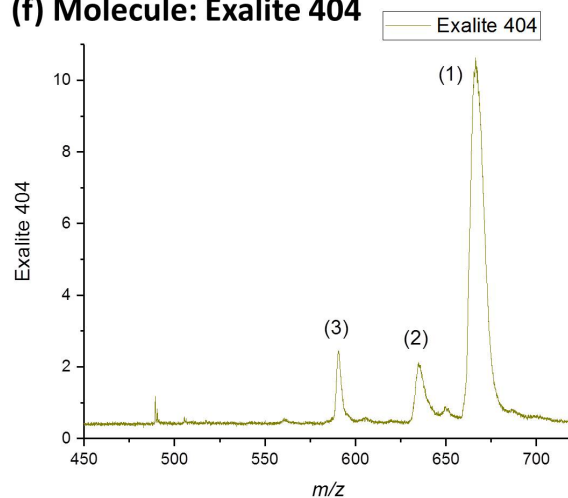
Figure 4.10: Dye sample mass spectra and the corresponding lists of ions assigned to each mass peak: (a) Auramine O, (b) Rhodamine 560 chloride, and (c) Rhodamine B.

(d) Molecule: Rhodamine 640 perchlorate

Peak	Ion	m/z
(1)	$[M - \text{ClO}_4]^+$	491.233
(2)	$[M - \text{CO}_2 - \text{ClO}_4]^+$	447.243

(e) Molecule: BBQ

Peak	Ion	m/z
(1)	$[M]^+$	675.05
(2)	$[M - \text{C}_{12}\text{H}_{24}]^+$	506.862
(3)	$[M - \text{C}_{12}\text{H}_{24}\text{O}]^+$	490.867

(f) Molecule: Exalite 404

Peak	Ion	m/z
(1)	$[M]^+$	658
(2)	$[M - (\text{CH}_2)_2]^+$	629.969
(3)	$[M - (\text{CH}_2)_5]^+$	587.922

Figure 4.11: Dye sample mass spectra and the corresponding lists of ions assigned to each mass peak: (d) Rhodamine 640 perchlorate (e) BBQ chloride (f) Exalite 404.

dye sample was individually electrosprayed through a nickel mesh onto conductive 25×25 mm indium tin oxide surfaces and embedded in the repeller electrode. This produced gridded dye samples with $67 \mu\text{m}$ wire thickness and a $432 \mu\text{m}$ pitch that were used to determine the spatial resolution of the instrument, a process which is illustrated in Figure 4.12.

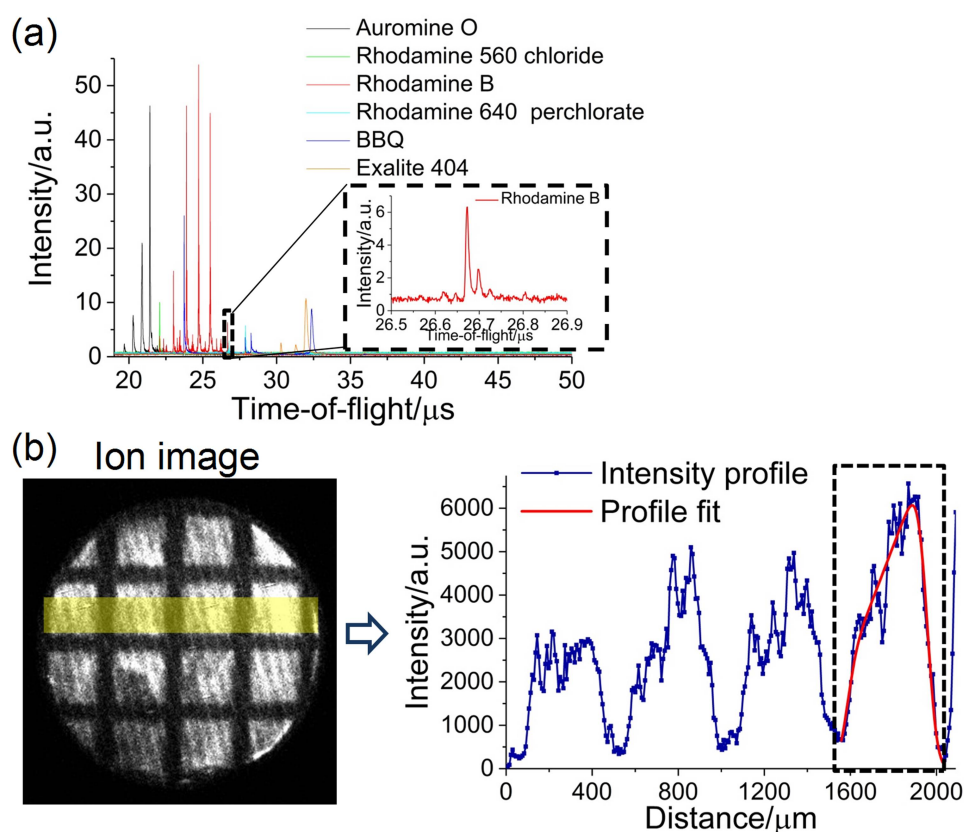


Figure 4.12: Dye samples are used to characterise the: (a) mass and (b) spatial resolution dependence over a mass range from 200 to 700 Da using their ToF spectra and the intensity profiles of the grid patterned ion images, respectively.

Gas-phase ions were laser desorbed from a 10×10 mm surface spot on each dye sample using defocused 355 nm light incident at 20° (1–10 mJ/pulse) from a 10 Hz Nd:YAG laser. Their image plane was then electrostatically transferred onto a two-dimensional detector array using single- or double-field PEDAs optics. The former setup uses the five electrode assembly described previously in Chapter 3. Under single-field PEDAs conditions, the repeller, extractor, and central einzel lens were set to 5000 V, 4732 V, and 1728 V, respectively. At $\tau = 2.85 \mu\text{s}$, the extractor voltage was quickly raised to 5400 V using a

fast Behlke switch (HTS 121). In the double-field PEDA case, the extractor voltage and central einzel lens were changed to 4752 V and 1740 V, respectively, and the additional PEDA lens was set at 2376 V. The extractor and PEDA lens were then raised to 5400 V and 2400 V, respectively, after a delay of 2.85 μs .

The voltages described above were determined in two steps: in the first, all the voltages were set according to the corresponding values predicted by SIMION, which are typically close to, but not exactly, the optimum values. In the second step, the voltages were scanned through a relatively small range around the predicted values and those that delivered the greatest performance (the best mass and spatial resolutions) were used for future experiments. Figure 4.13 illustrates a search for the optimal extractor voltage that best focuses an ion image. In this case SIMION predicted a value of 4646 V, thus the range between 4500 – 4720 V was scanned over. The value that offered the best spatial imaging quality lay between 4660 V and 4680 V. A finer scan with a smaller iteration step would eventually lead to the optimal extractor voltage. Similarly, to achieve the best mass resolution for a certain m/z , one should monitor the width of the corresponding ToF peak on an oscilloscope while adjusting the delay time or magnitude of the voltage pulse of PEDA.

In both cases, ions were detected using the home-built 25 mm detector assembly described previously in Chapter 3, which employed an Exalite 404 scintillator. Experimentally acquired ion images and time-of-flight spectra are presented in the next section, and used to determine the spatial and mass resolution of each PEDA technique. It should be noted that the ion images presented here were processed using the centroiding algorithm to enhance the accuracy of the reported spatial resolution as mentioned in Chapter 3.

4.2.3 Results and discussion

The mass resolution R_m for each recorded dye peak is determined from its full width at half maximum (Δt): $R_m = m/\Delta m = t_f/2\Delta t$. Using this approach, the resolution is independent of the scintillator decay time [95].

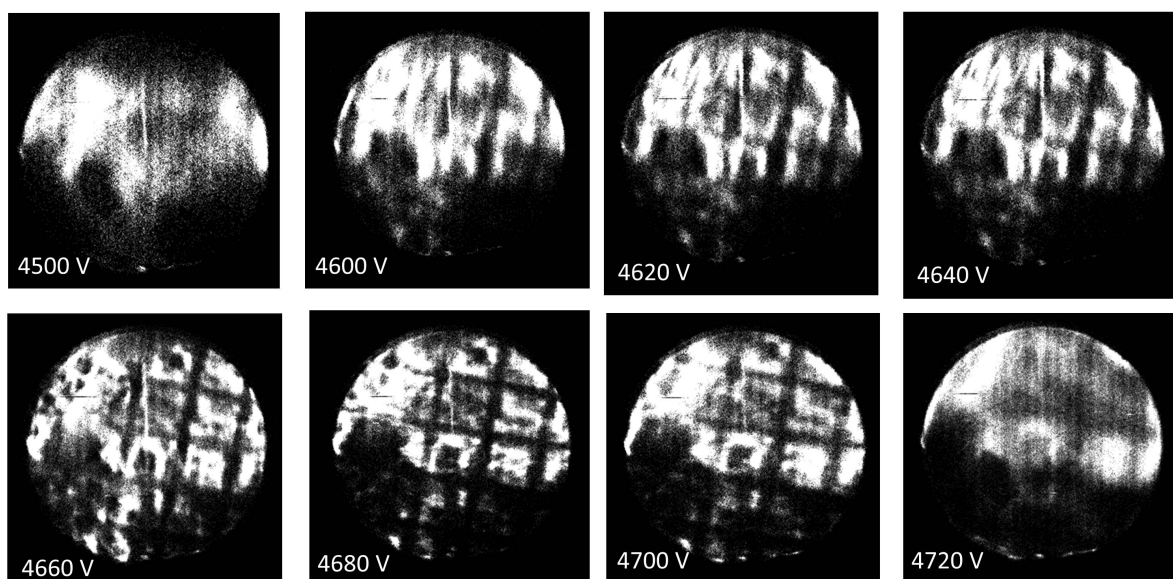


Figure 4.13: The optimum extractor voltage is determined by scanning it from 4500 V to 4720 V while observing the quality of the ion images.

Figures 4.14(a) and (b) illustrate how mass resolution depends on m/z for the single- and double-field PEDAs methods, and compares them to simulations modeled using SIMION 8.1 [141]. The dashed line represents the isotopic limit (i.e. $\Delta m_{50\%} < 1$ Da). In these figures, ion time-of-flights were calibrated with m/z using known peaks in the dye time-of-flight spectra. In each case, the experimental data agrees reasonably well with the SIMION model. The single-field PEDAs optics in Figure 4.14(a) were focused for ions at 410 Da. The effective mass resolution is centred around this value, with heavier and lighter ions having significantly worse resolution. The range of ions with mass resolutions above $1000 m/\Delta m$ is roughly 375 to 440 Da. Figure 4.14(b) illustrates the double-peaked structure indicative of two focused ions. In this example, there are two focused m/z at 360 and 480 Da, and ions between 330 to 465 Da are mass resolved to at least $1000 m/\Delta m$, doubling the range compared to the single-field PEDAs approach.

The key aspect of single-field PEDAs is its ability to preserve image quality while compensating for the initial spread of ion velocities at the sample surface. Therefore, for the enhanced mass range of double-field PEDAs to be advantageous, the technique must similarly maintain spatial resolution. To assess this ability, the spatial resolution

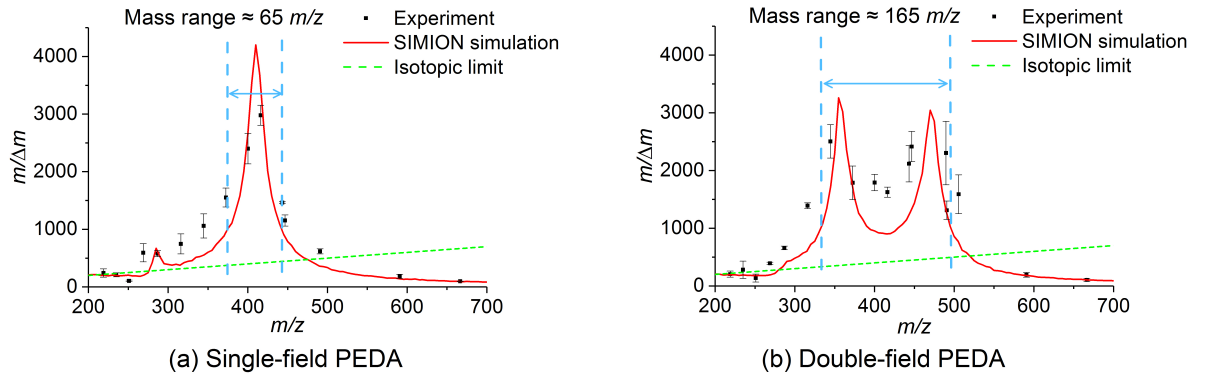


Figure 4.14: The mass dependence of the mass resolution for (a) single-field PEDA and (b) double-field PEDA methods. SIMION simulation results are provided to compare with the experimentally obtained data.

of the ions characterised above were determined from their ion images. Figure 4.12(b) demonstrates a representative example. A gridded dye sample is initially ionised by the defocused laser and acquired as an ion image with the CCD camera. An intensity profile is then integrated across the ion image (see the yellow line in Figure 4.12(b)) to determine the spatial resolution, which is defined as the distance along the profile for which the intensity increases from 20 to 80% of the maximum value (or 30 to 90% of the maximum in instances where the profile baseline is already higher than 20%) [95, 142].

An example of an experimentally obtained intensity profile is shown in Figure 4.15. Contrary to expectations, the intensity profile does not have a flat top. This fluctuation is very likely just a detection artefact, which can also be seen in the raw ion image (Figure 4.12(b)). This artifact probably arises from the image centroiding program employed during data acquisition. In addition, the unevenness is partially due to the 20° incident angle of the laser beam relative to the sample surface, which makes the fluence of the defocussed beam gradually decrease from one side of the sample surface to the other.

Due to the uneven intensity profiles, evaluations of spatial resolution are prone to inaccuracy and inconsistency if the maximum value used to determine the 20 and 80% is chosen arbitrarily. For example, in Figure 4.15 there are at least three candidates for the maximum value on the left shoulder of the intensity profile. The spatial resolutions determined by the distance between 20 to 80% of the maximum value 1, 2, and 3 are 37.1

μm , $64.1 \mu\text{m}$, and $114 \mu\text{m}$ respectively.

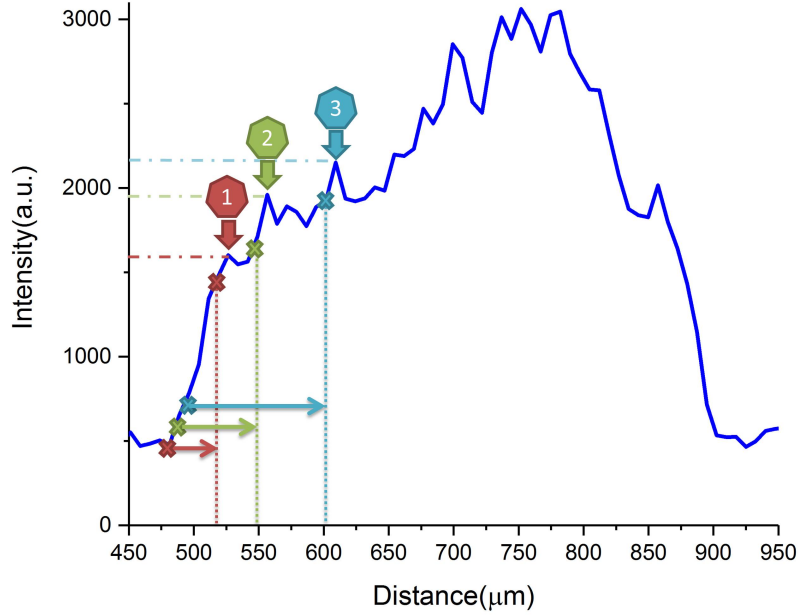


Figure 4.15: The spatial resolution depends on the choice of maximum value. The spatial resolutions determined from the maximum intensity profile values 1, 2, and 3 are $37.1 \mu\text{m}$, $64.1 \mu\text{m}$, and $114 \mu\text{m}$, respectively.

In order to more accurately and consistently characterise the spatial resolution of the instrument using a non-flat intensity profile, the rising and falling edges are fitted with a function where the underlying shape of the grid pattern $g(x)$ is convolved with a Gaussian response function $f(x)$, as shown in Figure 4.16. The distance from 20% to 80% of the maximum intensity of an edge of the fitting function will be used as the spatial resolution. $g(x)$ is a Gaussian distribution that describes the final ion spread on the detector surface when these ions are generated from a point source (x is the displacement from the centre and σ is the standard deviation) :

$$g(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-x^2/2\sigma^2}. \quad (4.16)$$

$f(x)$ is a piecewise linear function. The magnitude of $f(x)$ is proportionate to the amount of ions generated from a point ion source at x :

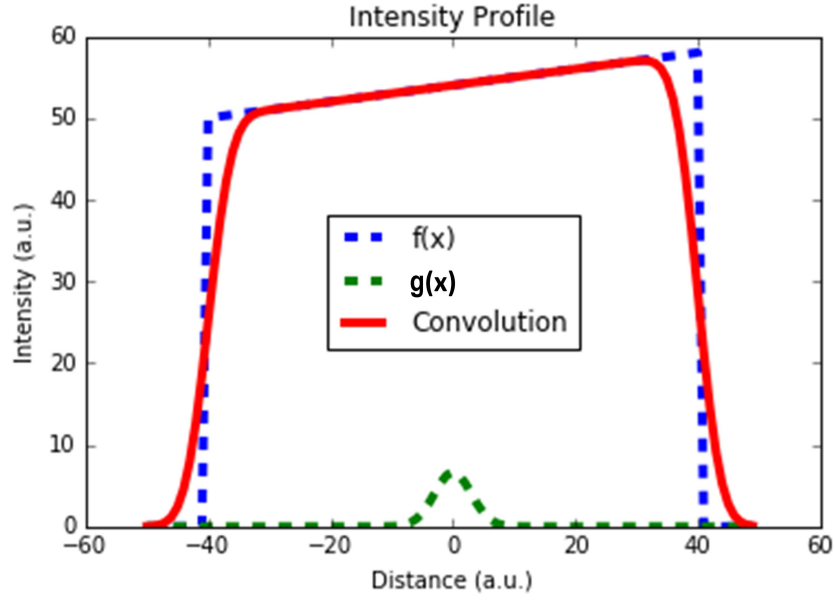


Figure 4.16: The convolution between a normal distribution $g(x)$ and a piecewise linear function $f(x)$ that reflects the underlying shape of the grid pattern.

$$f(x) = \begin{cases} 0 & \text{if } x < a \\ p(x - a) + h & \text{if } a \leq x \leq b \\ 0 & \text{if } x > b, \end{cases}$$

where a and b delineate a region in which sample molecules are deposited homogeneously, the slope p reflects the variation of ion generation due to laser incident angle. The convolution of $g(x)$ and $f(x)$ is therefore

$$y(x) = \int_{-\infty}^{+\infty} f(t)g(x - t)dt = \int_a^b [p(t - a) + h] \left[\frac{1}{\sigma\sqrt{2\pi}} e^{-(x-t)^2/2\sigma^2} \right] dt. \quad (4.17)$$

Parameters to be determined by fitting include a , b , p , h , and σ , but to estimate the spatial resolution what really matters is the value of σ , since the spatial resolution is given by $1.683 \cdot \sigma$. The scaling constant 1.683 was introduced previously in Chapter 2.

In a similar manner to the mass resolution, Figure 4.17 illustrates the spatial resolution dependence on m/z over 200 – 700 Da. The spatial resolution of double-field PEDAs (represented by black dots) is roughly 50 μm over the entire mass range. For comparison,

the mass resolution of the same instrument working under single-field PEDA conditions is also obtained and presented as red triangles, which are very close to the double-field PEDA. The spatial resolution compares favourably with SIMION, which predicts more weakly resolved images outside of the effective mass range, and is comparable to the $20 - 30 \mu\text{m}$ resolutions acquired using single-field PEDA techniques [87, 95]. According to the simulations, the spatial resolution should be below $25 \mu\text{m}$ for ions around $400 m/z$, but both double- and single-field PEDA experimental results show worse resolutions ($\approx 50 \mu\text{m}$). This could be partially due to the fact that the fitting method described above tends to overestimate the resolution, so the experimental data plotted in Figure 4.17 can be seen as the upper limits of the resolution.

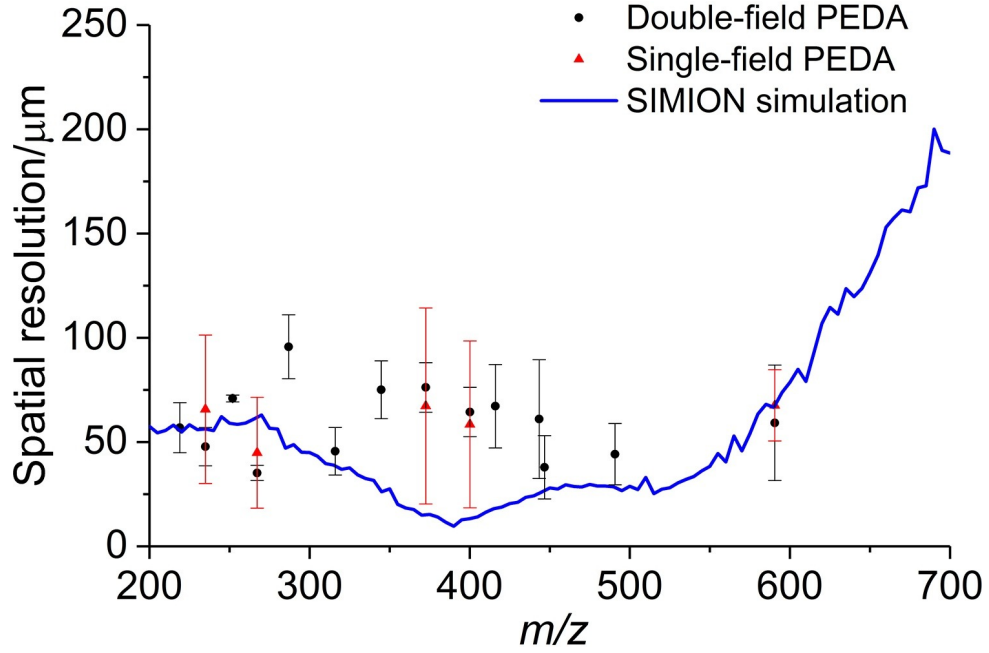


Figure 4.17: Spatial resolutions achieved using double-field (red triangles) and single-field (black dots) PEDA as a function of m/z . The blue trace shows the spatial resolution predicted by a SIMION simulation.

As was shown in Section 4.1, the optimum mass range of single-field PEDA can be tuned using velocity corrected ion extraction [95], where ion optical voltages are simply rescaled to focus ions with different m/z . This effectively moves the mass resolved PEDA window shown in Figure 4.14 to different regions of the time-of-flight spectrum. Figure 4.18 demonstrates that the same method can also be applied to double-field PEDA. In

this example, the double peaks previously observed at 355 and 470 Da were shifted to 470 and 620 Da by multiplying the ion optics voltages by a factor of 1.3. So, the repeller, the extractor, the new PEDA electrode, and the central einzel lens were initially set to 6500 V, 6178 V, 3089 V, and 2262 V, respectively. At $\tau = 2.85$ s, the voltages on the extractor and the new PEDA electrode stepped to 7020 V and 3120 V, respectively. As a result, the mass range of double-field PEDA, where the mass resolution remains above 1000 $m/\Delta m$, is optimised for larger m/z . The capability to adapt the optimal mass range to the m/z of the analytes will be very useful in practical applications where disparate analytes are investigated in different experiments.

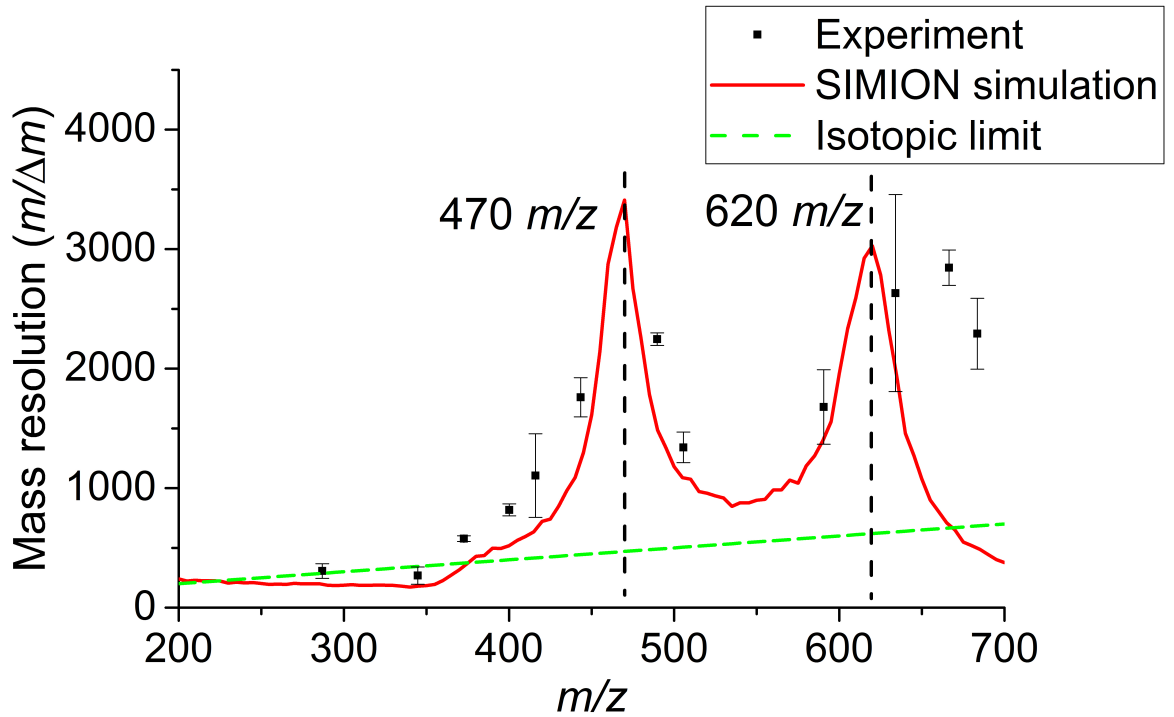


Figure 4.18: The mass resolutions of double-field PEDA plotted against m/z after being optimised for a larger mass range with the velocity corrected ion extraction method.

4.2.4 Conclusions

In PEDA, the voltage on the extractor is pulsed after a predetermined delay time to temporally focus ions of the same m/z , but with different kinetic energies on the detector, to improve the mass resolution. However, only a small fraction of the m/z range can be

perfectly focused by PEDA because the optimal pulsed electric field strength ΔE is highly mass-dependent: an ion of a smaller m/z needs a lower ΔE than that of a larger m/z . Therefore, to extend the effective mass range, a modification of PEDA, called “double-field PEDA”, was proposed, which places an additional pulsed electrode halfway between the extractor and the first grounded plate. If the pulsed voltage on the new electrode is chosen appropriately, two separate ΔE can be created and each is optimised for a different m/z . As a result, the overall mass range where ions have a mass resolution higher than $1000\ m/\Delta m$ is nearly tripled (from $65\ m/z$ to $165\ m/z$) and this is achieved at no cost of imaging quality. Moreover, the focused mass range of double-field PEDA can be tuned by rescaling the ion optical voltages.

4.3 Time-variable PEDA

A logical next step to double-field PEDA is to apply a continuously varying ΔE for a wide range of m/z , so that each m/z is temporally focused at the detector by its corresponding optimum ΔE . Another PEDA variant which incorporates these ideas is studied here both theoretically and experimentally. It provides a reasonably good mass resolution ($> 1,000\ m/\Delta m$) over an even broader mass range than the double-field PEDA, and shares key features in the design and implementation.

4.3.1 Theory

The motion of ions in time-variable PEDA will be modeled in this subsection. Before the delay time τ of the PEDA voltage pulse, ions in time-variable PEDA undergo the same acceleration process as the original single-field PEDA. At τ , the voltage on the extractor in time-variable PEDA $V_A^*(t)$ rises to V_{p0} instantly and then keeps rising exponentially over time until it asymptotically reaches a maximum voltage V_{max} (see Figure 4.19):

$$\begin{cases} V_A & 0 \leq t < \tau \\ V_A^*(t) = V_{p0} + (V_{max} - V_{p0}) (1 - e^{-(t-\tau)/RC}) & \tau \leq t. \end{cases}$$

where R represents resistance and C represents capacitance. In practice, this exponential rise is much easier to implement electronically than other linear or quadratic functions. The product RC is the time constant of an RC circuit, which is used here to generate $V_A^*(t)$. The exponential rise means that ion acceleration $a^*(t)$ becomes time-dependent after τ . This is equivalent to:

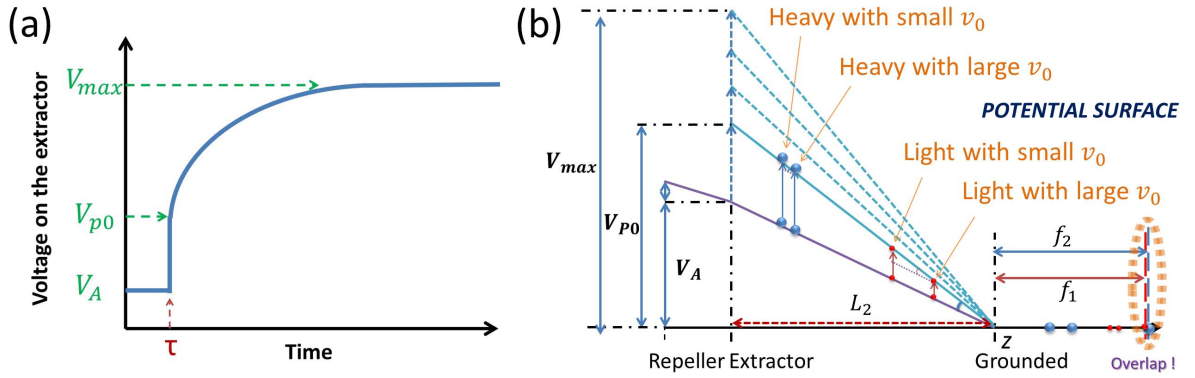


Figure 4.19: Illustration of (a) the exponential voltage rise on the extractor and (2) the potential surface after delay time τ .

$$a^*(t) = \frac{qV_A^*(t)}{mL_2}. \quad (4.18)$$

And their speed $v(t)$ is

$$v(t) = v_p + \int_{\tau}^t a^*(t) dt, \quad (4.19)$$

where v_p is equivalent to Equation (4.5). The remaining flight distance after τ to the end of the acceleration region is therefore:

$$L_2 - L_p = \int_{\tau}^{t_2} v(t) dt, \quad (4.20)$$

where t_2 is the arrival time of the ion at the einzel lens entrance. Substituting Equation (4.18) into (4.19) gives:

$$\begin{aligned}
 v(t) &= v_p + \int_{\tau}^t \frac{qV_A^*(t)}{mL_2} dt \\
 &= v_p + \frac{q}{mL_2} \int_{\tau}^t [V_{p0} + (V_{max} - V_{p0}) (1 - e^{-(t-\tau)/RC})] dt \\
 &= v_p + \frac{q}{mL_2} [V_{max}(t - \tau) - RC(V_{max} - V_{p0}) (1 - e^{-(t-\tau)/RC})].
 \end{aligned} \tag{4.21}$$

By replacing $v(t)$ with Equation (4.21), one can rearrange Equation (4.20) as follows:

$$\begin{aligned}
 L_2 - L_p &= \int_{\tau}^{t_2} \left\{ v_p + \frac{q}{mL_2} [V_{max}(t - \tau) - RC(V_{max} - V_{p0}) (1 - e^{-(t-\tau)/RC})] \right\} dt \\
 &= v_p(t_2 - \tau) + \frac{q}{mL_2} V_{max} \left(\frac{t_2^2}{2} - t_2\tau + \frac{\tau^2}{2} \right) \\
 &\quad - \frac{qRC}{mL_2} (V_{max} - V_{p0}) (t_2 - \tau - RC + RCe^{-(t_2-\tau)/RC}) \\
 &= v_p\Delta t + \frac{q}{2mL_2} V_{max}\Delta t^2 - \frac{qRC}{mL_2} (V_{max} - V_{p0}) (\Delta t - RC + RCe^{-\Delta t/RC}),
 \end{aligned} \tag{4.22}$$

where $\Delta t = t_2 - \tau$ is the flight time of the ion in the acceleration region between the pulse starting time τ and arrival time at the einzel lens entrance t_2 . With the help of python, the value of Δt can be determined by solving Equation (4.22) numerically. This yields the ion arrival time t_2 at the entrance plate of the einzel lens assembly, and hence the speed v_2 :

$$\begin{aligned}
 v_2 &= v(t_2) \\
 &= v_p + \frac{q}{mL_2} [V_{max}(t_2 - \tau) - RC(V_{max} - V_{p0}) (1 - e^{-(t_2-\tau)/RC})].
 \end{aligned} \tag{4.23}$$

From here on, the ion speed and time-of-flight through the einzel lens and drift region can be calculated using the same equations as the single-field PEDAs case (Equations (4.13) and (4.14)). With constant physical parameters and RC lifetime, the total time-

of-flight of the ion t_f is a function of its mass-to-charge ratio (m/z), its initial velocity (v_0), the potentials applied to the ion optics (V_E , V_A , V_{einzel} , V_{p0} , and V_{max}), and the delay time τ . The time-of-flight difference t_d between an ion with an initial velocity v_0 and one with a mean initial velocity can be calculated for a specific m/z . As before, the initial ion velocities are assumed to be normally distributed with a mean of 500 m/s and a FWHM of 500 m/s. The t_d can be determined with Equation (4.15).

In order to evaluate the overall time-of-flight focusing performance of the time-variable PEDDA method, a value S^2 is defined as follows: the square sum of t_d over a range of v_0 ($0 \text{ m/s} < v_0 < 1000 \text{ m/s}$) for a specific m/z ,

$$\sum_{v_0=0}^{1000} t_d^2 \left(v_0, \frac{m}{z}, V_E, V_A, V_{\text{einzel}}, V_{p0}, V_{\text{max}}, \tau \right)$$

is added up for different mass-to-charge ratios ($300 < m/z < 600$):

$$S^2 = \sum_{m/z=300}^{600} \sum_{v_0=0}^{1000} t_d^2 \left(v_0, \frac{m}{z}, V_E, V_A, V_{\text{einzel}}, V_{p0}, V_{\text{max}}, \tau \right). \quad (4.24)$$

It is self-evident that the smaller S^2 is, the better the overall time-of-flight focusing of this mass range will be. S^2 is a function of ion optic voltages and delay time: $S^2(V_E, V_A, V_{\text{einzel}}, V_{p0}, V_{\text{max}}, \tau)$, therefore the goal here is to find an appropriate set of parameters to minimise S^2 .

The Nelder-Mead method [143, 144] (also known as the downhill simplex method) is a widely accepted numerical algorithm used to find the minimum of an objective function in a multidimensional space. It efficiently makes use of simplexes. As defined in ref. [145], a simplex is “an N -dimensional geometrical figure consisting of $N + 1$ vertices and all their interconnecting line segments, polygonal faces, etc.” For example, simplexes are triangles in two dimensional space, and tetrahedrons in three-dimensional space. In each iteration of this method, function values at each vertex are compared, and the vertex with the highest value is rejected and replaced with another vertex. As a result a new

V_{max}/V	V_{p0}/V	$\tau/\mu s$	$S^2/\mu s^2$
7742	5150	2.81	770.6

Table 4.1: V_{max} , V_{p0} , and τ determined by minimising S^2 .

simplex is generated and the search continues. During this process, the function values at the vertices get smaller and smaller until they reach the final minimum. In python, the Nelder-Mead method can be implemented with an optimisation module in the SciPy package (<https://www.scipy.org/>): `scipy.optimize.fmin`.

The combination of V_E , V_A , and V_{einzel} is estimated using the transfer matrix method discussed in Chapter 2 to spatially focus the ion image on the detector. Changing them would bring the image out of focus. So, for the purpose of reducing complexity and computational cost, V_E , V_A , and V_{einzel} are treated as constants ($V_E=400$ V, $V_A=4600$ V, $V_{einzel}=1728$ V) rather than variables when minimising S^2 . Therefore only three variables (V_{p0} , V_{max} , τ) remain to be optimised. The minimisation results are summarised in Table 4.1 and their time-of-flight difference as a function of m/z and v_0 are shown in Figure 4.20(a) and (b). Compared to Figures 4.6, which illustrates the t_d dependences of the single- and double-field PEDAs, time-variable PEDA clearly demonstrates an excellent mass resolution over a wide mass range, since the ToF difference stays less than 10 ns throughout the entire image. A quantitative comparison can be performed by looking at the S^2 values of the optimised time-variable, double-field, and single-field methods, which are $770.6 \mu s^2$, $142420.6 \mu s^2$, and $445546.6 \mu s^2$ respectively.

As mentioned in Chapter 1, the mass resolution of a ToF-MS system can be calculated by the equation $m/\Delta m = t_f/2\Delta t_f$. In this case, t_f is the centre of a ToF peak and Δt_f is defined as its full width at half maximum (FWHM). As before, t_f is estimated as $t_f(v_0 = 500 \text{ m/s})$. The Δt_f of a specific m/z therefore is estimated using the maximum width of the m/z peak. Since the maximum width is larger than the FWHM, the mass resolution can be seen as a lower limit. Figure 4.21 shows the mass resolution dependence on m/z for time-variable PEDA (red line), double-field PEDA (blue dashed line), and single-field PEDA (yellow dash-dotted line), respectively. With the optimised time-variable

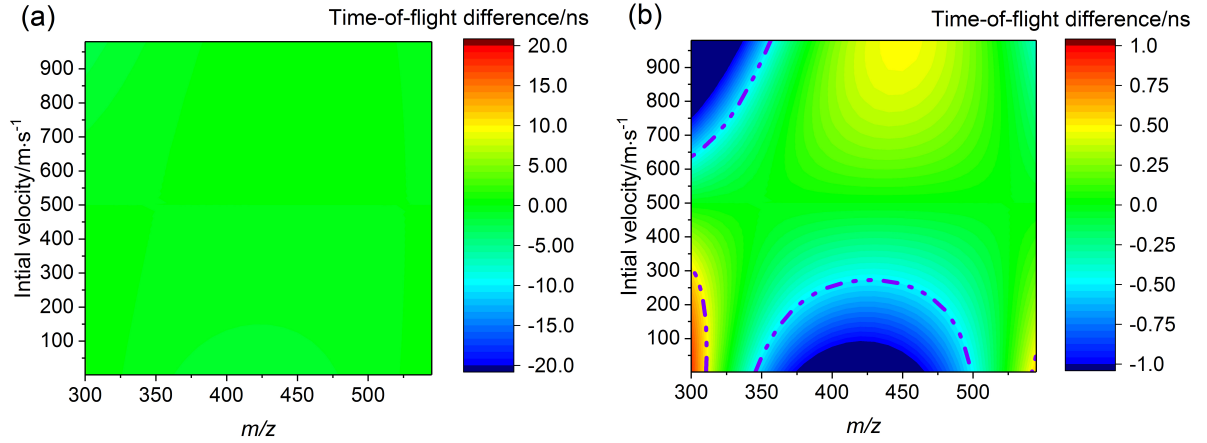


Figure 4.20: Calculated time-of-flight difference $t_d = t_f(v_0, \frac{m}{z}) - t_f(500 \text{ m/s}, \frac{m}{z})$ as a function of m/z and v_0 for time-variable PEDA. (a) Scaling between -20 ns and 20 ns (the same scaling as the single- and double-field PEDA calculation results in Figure 4.6), and (b) scaling between -1 ns and 1 ns.

PEDA parameters, the mass resolution can in principle reach a maximum of more than 18,000 $m/\Delta m$ while staying above 2,000 $m/\Delta m$ over a wide mass range from 310 to 590 m/z . Therefore, the effective mass range of the PEDA MSI instrument is expected to be broadened considerably by this novel PEDA variant.

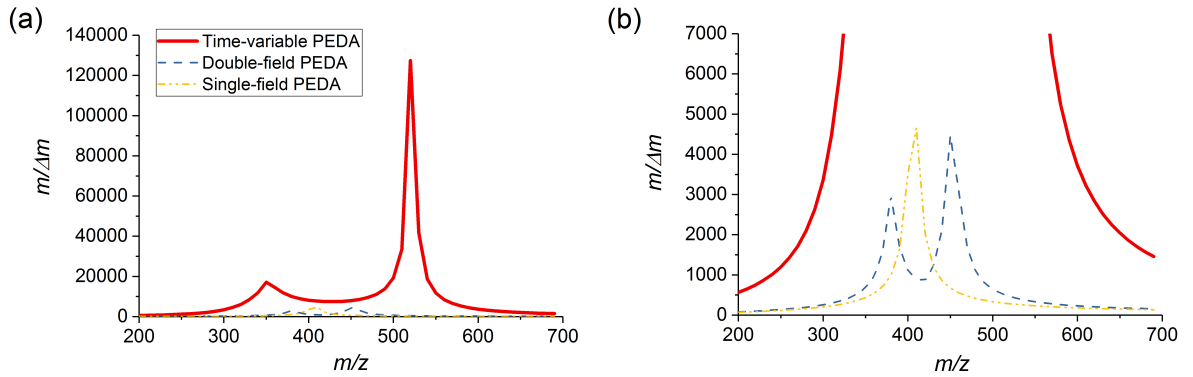


Figure 4.21: Calculated time-of-flight mass resolution dependences on mass-to-charge ratio for time-variable PEDA (red line), double-field PEDA (blue dashed line), and single-field PEDA (yellow dash-dotted line). (a) Full spectrum, (b) same as (a), but with the y-axis rescaled to make the single-field and double-field PEDA curves easier to see.

4.3.2 Experimental results

For practical reasons that will be introduced in Chapter 5, the time-variable PEDAs experiment used the instrument layout as shown in Figure 4.22. As a result, the total flight path was longer than the original layout and the detector was not in line with the original ToF axis. The repeller plate was held at 5 kV, the baseline of the extractor was at 4.561 kV, the step voltage of time-variable PEDAs (V_{p0}) was 4.823 kV, the final voltage of time-variable PEDAs ($V_{\max}$) was 7.7 kV, and the middle electrode of the einzel lens assembly was 1.95 kV. A pair of deflectors (± 157.2 V) was used to direct the ion beam onto the detector assembly. The extractor was pulsed up to V_{p0} after a delay time of $2.6 \mu\text{s}$, which also corresponds with the onset of the exponential rise of the potential on the extractor from V_{p0} to $V_{\max}$ (the delay time τ was set relative to the time zero T_0 of each experiment, which was the moment the laser fires). The laser pulse energy was around 9 mJ/pulse. The angle of the reflectron chamber was about -3° (more details of the reflectron will be introduced in Chapter 5). The mesh used to prepare the array of square patterns had a hole size of $102 \mu\text{m}$ and a wire width of $38.1 \mu\text{m}$.



Figure 4.22: The layout of the MSI instrument for the time-variable PEDAs experiments.

Experimental results achieved under these conditions are shown in Figures 4.23(a) and (b) along with the simulated results. In terms of mass resolving power, an optimal mass resolution higher than $4,000 m/\Delta m$ was obtained, which is slightly greater than the optimal mass resolution obtained with the single- and double-field PEDAs setups. This is probably a result of the longer flight path combined with a better temporal focusing capability of time-variable PEDAs. More importantly, the effective mass range is significantly

broadened compared with the previous methods: a mass resolution above $1,000\ m/\Delta m$ is maintained from 250 to 700 m/z . This is about 7 times broader than the original PEDAs method and 3.3 times broader than the double-field PEDAs case. In Figure 4.23(a), there is some discrepancy between experiments and simulations. The experimentally obtained mass resolutions are higher than the simulated ones for mass ranges less than 300 m/z or around 500 m/z . Similar trends can also be observed in Figure 4.14 where experimental results tend to have better mass resolutions than the simulations. This might imply that the initial conditions of the simulated ions used in SIMION did not match the reality perfectly. The mean ion velocity 500 m/s with a FWHM of 500 m/s and a FWHM of 60° in both the Az and El angle distributions could be too conservative, and simulations confirmed that if the speed spread or the angle spread were set smaller, the simulated mass resolutions would be higher and closer to the experiments.

As seen in Figure 4.23 (b), the spatial resolution stays between 15 and 35 μm throughout the entire mass range. The optimal spatial resolution is slightly better than in the single- and double-field PEDAs. There are two possible explanations for this: (1) the magnification of the new layout was larger than the original one due to a longer flight path; the more an ion image is magnified the less its spatial resolution is limited by instrumental factors such as sensor pixel size; and (2), for the same detector a larger magnification also leads to a smaller field of view, which means less spherical aberration at the periphery of the ion images.

4.3.3 Conclusions

In time-variable PEDAs, the strength of the electric field ΔE becomes time-dependent: the voltage on the extractor is pulsed to a higher voltage over a very short time scale and then continues rising over time. Calculations predicted that such a pulsing scheme could broaden the effective mass range of PEDAs dramatically, and this was corroborated by experimental studies. A mass resolution above $1,000\ m/\Delta m$ was obtained for a mass range seven times wider than the original PEDAs method. Moreover, the spatial resolv-

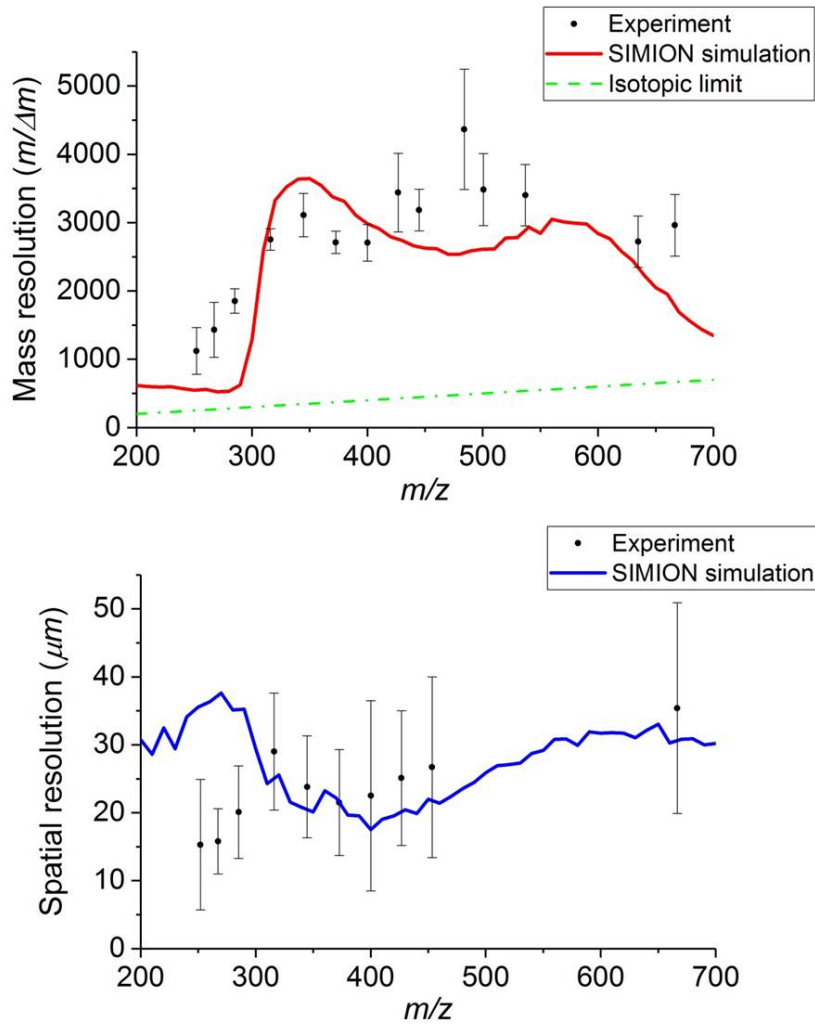


Figure 4.23: Experimental and simulated results for time-variable PEDa: the mass dependence of (a) the mass resolution and (b) the spatial resolution.

ing power of the time-variable PEDa instrument remained comparable with the original design. Time-variable PEDa significantly enhances the applicability of microscope MSI by allowing ions over a wide range of m/z to be simultaneously analysed with high mass resolution.

4.4 Summary

Simultaneously focusing two m/z allows microscope MSI to be performed over a mass range that is roughly double what can be achieved using single-field PEDa. For the home-built mass spectrometer described here, the mass ranges where $m/\Delta m \geq 1000$

were 65 and 165 Da, respectively, for single-field PEDa at a central mass of 410 Da and double-field PEDa at a central mass of 360 and 480 Da. This was accomplished without any comparative loss in the spatial resolution or quality of the associated ion images. The broader mass range of double-field PEDa also extends to velocity corrected ion extraction, which has the benefit of allowing large mass ranges to be sampled and imaged more quickly. Using a dynamic pulsed voltage allows this mass range to be extended further. A mass resolution over $1000\ m/\Delta m$ and a spatial resolution less than $40\ \mu\text{m}$ were measured over 300 to 700 m/z using an exponentially rising voltage pulse. More generally, this chapter points to the fact that the temporal focusing of many ions will likely play a key role in the push to maximise the mass range of microscope MSI while preserving effective mass and spatial resolutions. The performance of different PEDa instruments are summarised in Table 4.2.

PEDA	Optimal mass resolution ($m/\Delta m$)	Mass range where $m/\Delta m \leq 1,000$	Optimal spatial resolution (μm)
Original	$\approx 3,000$	375-440 Da	≈ 50
Double-field	$\approx 2,500$	330-465 Da	≈ 50
Time-variable	$\geq 4,000$	300-700 Da	≈ 20

Table 4.2: Comparison of the original, double-field, and time-variable PEDa methods, as incorporated in the time-of-flight MS described in Section 3.1.

Chapter 5

Reflectron Spatial Ion Imaging

Reflectrons are time-of-flight analysers commonly used in mass spectrometers. They can correct for the initial velocity/energy spread of MALDI-generated ions. B. A. Mamyrin demonstrated the reflectron analysers as a new nonmagnetic high resolution mass spectrometer in 1973 in ref. [146]. A mass resolution of 3,500 $m/\Delta m$ was obtained for ions with a mass-to-charge ratio $m/z \geq 200$, which was about 10 times better than linear ToF analysers at that time [34, 147, 148]. Today, a typical linear ToF-MS has a mass resolution of 5,000 $m/\Delta m$ and its reflectron counterpart has a mass resolution over 20,000 $m/\Delta m$. A reflectron is basically an ion mirror used to reflect ions. It consists of a stack of concentric circular electrodes capped by a gridded electrode and a solid plate electrode. All these electrodes are connected through a chain of equal-value resistors. Ideally, a homogeneous planar electric field is created within the reflectron by the linear voltage gradient on the electrode stack. If the potential on the last electrode is higher than the kinetic energies of the incident ions, the reflecting field can slow down the ions, turn them around, and send them back towards a detector, analogous to rolling a marble up the slope of a hill. As discussed earlier in Chapter 4, ions of the same mass but different kinetic energies/velocities expand in space and therefore arrive at the detector as a distribution of flight times. The reflectron reduces the loss of resolution of ToF mass spectrometry due to the spread of velocities of the ions at the start. It can be shown that motion inside a reflectron can refocus the charged particle packet to a thin plane. After entering the reflectron, ions with higher kinetic energies penetrate more deeply into the reflecting field and therefore possess a slightly longer path length inside the reflectron; if the electrostatic field in the

reflectron (*i.e.* the reflectron voltage) is chosen correctly, high-energy ions will emerge from the reflectron behind the lower-energy ions, but catch up with them right at the detector plane; thus a reflectron can reverse the expansion process of ion packets and improve the resolving power of ToF-MS systems.

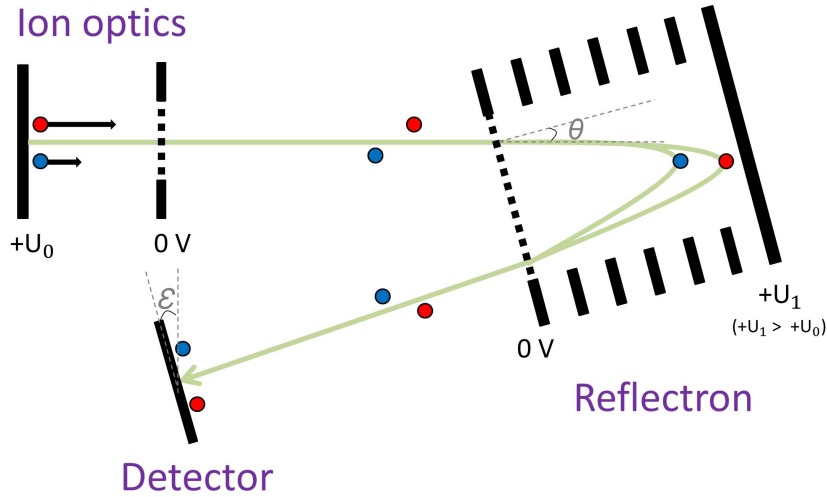


Figure 5.1: The layout of a reflectron ToF-MS system. The red and blue balls represent two ions with the same m/z , but the red ion has a higher initial kinetic energy than the blue one. After they are accelerated by the same electric field in the ion optics, the red ion will travel ahead of the blue one. The potential on the last electrode of the reflectron ($+U_1$) is higher than the acceleration potential ($+U_0$). After entering the reflectron, the red ion penetrates deeper into the reflecting field and therefore possesses a slightly longer path length inside the reflectron. As a result, it will emerge from the reflectron behind the blue ion, but catch up with it right at the detector plane.

Figure 5.1 illustrates the basic layout of a reflectron ToF-MS. The reflectron is placed at the end of the field-free flight tube, opposed to the ion optics assembly and oriented at a small angle θ relative to the initial direction of the ion beam. The detector is adjacent to the ion source and faces the reflectron to collect ions after they are turned around by the electrostatic mirror (the angle of the detector is tilted by ϵ). There is also a coaxial alternative where the reflectron is situated in line with the initial ion beam (the detector is between the ion source and the reflectron in this configuration). In this layout, ions pass through a central iris on the detector to enter the reflecting field. However, this geometry gives rise to considerable sensitivity losses since those reflected ions traveling

close to the centre are discarded (after the ions are reflected, ions at the centre will go through the iris again and miss the detector).

Apart from improving the mass resolution of a ToF-MS system, another important application of reflectrons is to detect the post-source decay (PSD) fragments of metastable ions [149,150]. In a PSD process, the fragmentation of a precursor ion occurs after it leaves the acceleration fields in the ion optics and before it enters the reflectron. So the product ions travel at the same speed as their precursors but have different kinetic energies (a product ion is always lighter than its precursor and thus has a lower kinetic energy). In a linear ToF analyser, any remaining precursor ions, product ions, or neutral fragments will arrive at the detector simultaneously, which means they cannot be discriminated by the analyser. However, in a reflectron ToF analyser, because of their lower kinetic energy, product ions penetrate less deeply and therefore stay in the reflecting field for a shorter period of time than their precursor. As a result, the precursor and its product ions appear as different peaks in a ToF spectrum.

Leskiw *et al.* [151] demonstrated velocity-map ion imaging in a reflectron TOF-MS in 2005. The velocity-resolving capabilities were maintained simultaneously with long flight times and high mass resolutions characteristic of reflectrons. The photodissociation of OCS at 230 nm wavelength was used to characterise the ion imaging performance of their system. If a reflectron is compatible with VMI, it should also work in MSI, as the velocity-focus is merely a point along the ion trajectories before the space-focus.

A commercial fast microprobe MSI instrument, the Rapiflex MALDI Tissue typerTM (Bruker), has a mass resolution $\geq 13,000 m/\Delta m$ for bradykinin 2-9 (m/z 904) [152] when working in reflectron mode. Therefore, to compete with the commercial instrument, a mass resolution around $10,000 m/\Delta m$ is desired for the microscope MSI instrument developed here. This chapter reports the first instrument that incorporates a reflectron into microscope MSI. It begins with a discussion on the principles of reflectrons. The SIMION-assisted design process of the reflectron MSI instrument is then described, and followed by a discussion of the experiments used to characterise the instrument.

5.1 Principle of reflectron ToF-MS

If $t(U)$ is the time-of-flight of an ion as a function of its kinetic energy U , it can be expanded as a Taylor series:

$$t(U) = t(U_0) + \left. \frac{dt}{dU} \right|_{U_0} (U - U_0) + \left. \frac{d^2t}{d^2U} \right|_{U_0} \frac{(U - U_0)^2}{2!} + \cdots + \left. \frac{d^nt}{d^nU} \right|_{U_0} \frac{(U - U_0)^n}{n!} + \cdots, \quad (5.1)$$

where U_0 is the mean kinetic energy of all the ions of the same m/z . To minimise the ToF dispersion due to the kinetic energy distribution, one needs to zero out as many terms in the Taylor series as possible except the first. Since $U \neq U_0$, this means the differentials need to be zero. If only the dt/dU term equals zero, the focusing conditions are first order, if both the dt/dU and d^2t/d^2U terms are zero, the focusing conditions are second order.

Using a reflectron to compensate for the flight time difference caused by the velocity/kinetic energy distribution of ion trajectories was inspired by the simple one dimensional spring system [153]. As shown in Figure 5.2, at t_0 a string at its equilibrium position ($x = 0$) is given a kinetic energy, causing it to move to the right. According to Hooke's law [154], once a string is extended or compressed, it returns to $x = 0$ by a restoring force F . As the displacement from the equilibrium increases, F increases as well. As long as the displacement is not too large, the restoring force F of a spring is proportional to the departure x from equilibrium $x = 0$:

$$F = -kx, \quad (5.2)$$

where the spring constant k is a positive real number that represents stiffness. The minus sign in Equation (5.2) indicates that the direction of the restoring force opposes the displacement from $x = 0$. As the weight moves away from $x = 0$, the kinetic energy is converted into its potential energy P_1 . At $x = x_1$, P_1 can be calculated by the following

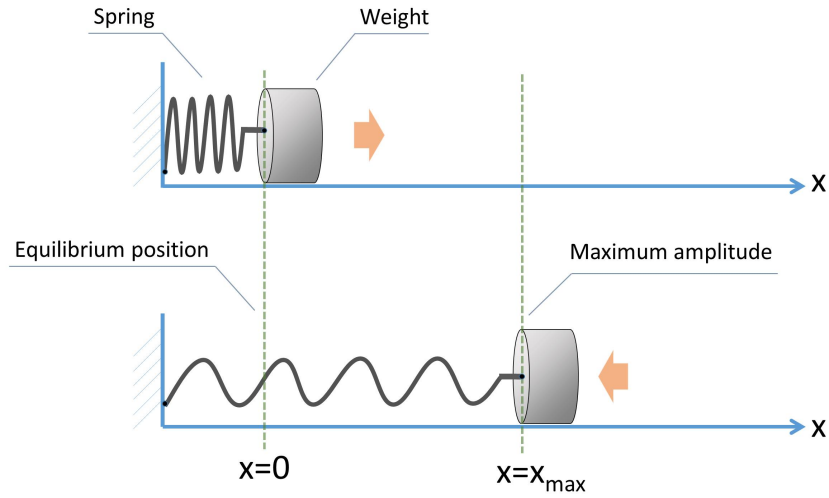


Figure 5.2: A simple one dimensional spring system.

equation:

$$P_1 = \int_0^{x_1} kx dx = \frac{kx_1^2}{2}. \quad (5.3)$$

When the weight reaches its maximum displacement x_{max} , its kinetic energy reduces to zero whilst its potential energy is at the maximum. The spring then turns around and starts to accelerate towards the equilibrium position. The total time required to stop the weight and return it to $x = 0$ is

$$\begin{aligned} t &= 2 \int_0^{x_{max}} \frac{dx}{v(x)} \\ &= 2\sqrt{\frac{m}{k}} \int_0^{x_{max}} \frac{dx}{\sqrt{x_{max}^2 - x^2}} \\ &= \sqrt{\frac{m}{k}} \pi, \end{aligned} \quad (5.4)$$

where $v(x)$ is the speed of the spring at x , and m is the mass of the weight attached to the end of the spring. The first leg of the round trip journey has exactly the same length as the second, therefore a factor of 2 is put in front of the integral to calculate the total time. A remarkable characteristic of the simple harmonic motion of a spring is that its period

(the duration of the round trip) is only dependent on the mass of the hanging weight, but independent on the maximum amplitude of the spring (or the kinetic energy of the weight at equilibrium position $x = 0$). This is exactly what an ideal ToF-MS should achieve; the time-of-flight of an ion should depend on its mass-to-charge ratio but not its kinetic energy. In this way, a thin ion packet created at the sample surface plane will be a thin packet at the detector plane, regardless of its initial energy spread. In order to mimic the harmonic motion of a spring, ions should therefore be retarded and reflected by the following electric field:

$$F = Eq = -kx, \quad (5.5)$$

$$E = -kx/q, \quad (5.6)$$

where q is the charge of the ion and E is the electric field strength that linearly increases along the x -axis. Consequently, the potential surface U becomes parabolic:

$$U = \frac{kx^2}{2q}. \quad (5.7)$$

A quadratic reflectron would be an “ideal” reflectron that provides kinetic energy focusing to infinite order. However, it is not easy to create such a field (or its close approximation) in practice, and the performance reported in previous work [155, 156] cannot justify the efforts. As a result, the harmonic oscillator model has to be modified to accommodate a scenario where charged particles are manipulated with static electric fields. Hence, a uniform reflecting field (potential surface $U = kx$) is used in combination with two field free drift regions, to partially compensate the ToF difference of ions with identical m/z but different kinetic energies.

Figure 5.3 illustrates the motion of a charged particle in a reflectron that is subject to a uniform and planar electric field. The total flight time of the ion from the starting plane to the detector plane is

$$t = t_L + t_R. \quad (5.8)$$

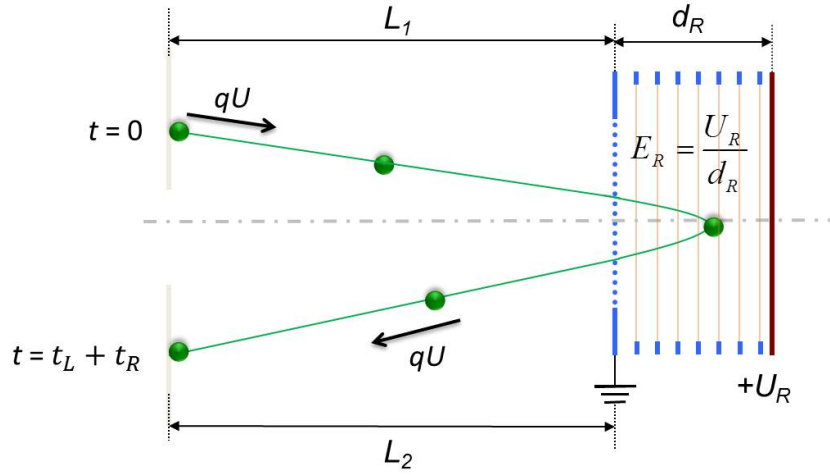


Figure 5.3: Motion of an ion in two segments of field free drift path (L_1 and L_2) and a uniform reflecting field ($E_R = U_R/d_R$).

Here t_L is the total time an ion spends in the field-free drift regions,

$$t_L = \frac{L}{\sqrt{2qU/m}}, \quad (5.9)$$

where $L = L_1 + L_2$ is the sum of the drift length before and after the reflectron, the drift length L can be arbitrarily distributed between the legs L_1 and L_2 ; qU is the total kinetic energy of an ion along the system axis x ; m is the mass of the ion, t_R is the time of flight inside the reflectron. As the ion penetrates into the reflecting field, its velocity along the reflectron axis (as well as its momentum) gradually decline to zero. The ion then reverses direction, accelerates, and leaves the reflecting field with its kinetic energy restored to the same value as when it entered. The time for each leg of this round trip will be $t_R/2$. One can calculate t_R using the relationship between force and momentum:

$$F_R \frac{t_R}{2} = \frac{qU_R}{d_R} \frac{t_R}{2} = m\sqrt{\frac{2qU}{m}}. \quad (5.10)$$

Therefore t_R is:

$$t_R = \frac{2d_R}{U_R} \sqrt{\frac{2mU}{q}}, \quad (5.11)$$

where F_R is the constant electric force present in the reflectron that decelerates and reflects

the ion, d_R is the total length of the reflecting field, and U_R is the potential of the last electrode of the reflectron relative to ground. Equation (5.8) becomes:

$$t = \frac{A}{\sqrt{U}} + B\sqrt{U}, \quad (5.12)$$

where

$$A = \frac{L}{\sqrt{2q/m}}, \quad (5.13)$$

and

$$B = \frac{2d_R}{U_R} \sqrt{\frac{2m}{q}}. \quad (5.14)$$

To minimise the dependence of the total time-of-flight on the kinetic energy, the differential of t with respect to U should be zero when U is close to U_0 :

$$\frac{dt}{dU} = -\frac{A}{2U^{3/2}} + \frac{B}{2U^{1/2}} \Big|_{U_0} = 0, \quad (5.15)$$

Therefore, by substituting Equation (5.13) and (5.14) into Equation (5.15), the field strength inside a reflectron should fulfill the following condition:

$$E_R = \frac{U_R}{d_R} = 4 \frac{U_0}{L}, \quad (5.16)$$

which is equivalent to

$$d = \frac{L}{4}, \quad (5.17)$$

where d is the depth that the ion penetrate into the reflecting field or the minimum length of a reflectron.

Similarly, to achieve second order focusing,

$$\frac{d^2t}{dU^2} = \frac{3A}{4U^{5/2}} - \frac{B}{2U^{3/2}} \Big|_{U_0} = 0, \quad (5.18)$$

which gives:

$$E_R = \frac{U_R}{d_R} = \frac{8U_0}{3L}. \quad (5.19)$$

So, the conditions for first and second order ToF focusing are not simultaneously met. For a single stage reflectron the mass resolution is therefore limited by those ions whose kinetic energies are relatively far from the average. Therefore the total flight time t becomes

$$t = \left[\frac{L}{\sqrt{2U}} + \frac{L\sqrt{U/2}}{U_0} \right] \sqrt{\frac{m}{q}}. \quad (5.20)$$

The total flight time, t , is therefore proportional to $\sqrt{m/q}$, just like a simple linear ToF-MS. Also, comparing Equation (5.9) and (5.20) reveals that $t_L = t_R$ when $U \approx U_0$, which means the time an ion spends in the reflectron is the same as in the field free drift length.

The ToF focusing capability of a reflectron MS instrument can be further enhanced by using a two stage reflectron [146, 157, 158], which enables a first and second order ToF focusing with respect to energy (see Figure 5.4). In a two stage reflectron, one more grid electrode is added in between the entrance grounded electrode and the last plate electrode, creating two successive homogeneous planar electric fields of different strengths. As shown in Figure 5.4, the optimal ToF focusing condition for a two stage reflectron is determined by four parameters d_B/L , d_R/L , U_R/U_0 , and U_B/U_0 , where d_B is the distance between the first and second gridded electrodes, d_R represents the distance between the second gridded and the solid plate electrode, U_B is the potential on the second gridded electrode, and U_R is the potential on the plate electrode.

B. A. Mamyryn reported an experimental configuration of the four parameters [d_B/L ; d_R/L ; U_R/U_0 ; U_B/U_0] in ref. [146]:

$$\begin{aligned} \frac{d_B}{L} &\approx 0.008 \\ \frac{d_R}{L} &\approx 0.06 \\ \frac{U_R}{U_0} &\approx 1.15 \\ \frac{U_B}{U_0} &\approx 0.7. \end{aligned} \quad (5.21)$$

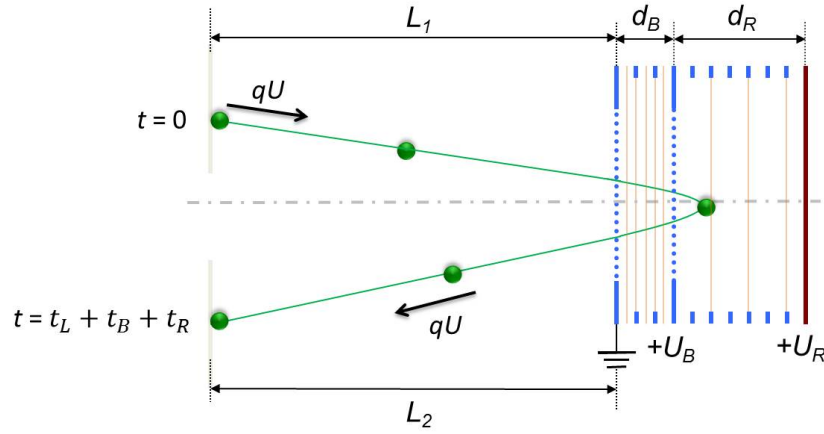


Figure 5.4: Motion of an ion in two segments of field free drift path (L_1 and L_2) and two uniform reflecting fields ($E_B = U_B/d_B$ and $E_R = (U_R - U_B)/d_R$).

Ref. [159] also confirmed that this set of parameters was reasonably close to the optimum physical dimensions and operation voltages determined by the exact analytical solutions to the second-order differential equation describing $t(U)$.

Apart from reducing the ToF broadening to second order, two stage reflectrons are smaller than single stage versions. The first stage of a two stage reflectron is characterised by a strong electric field responsible for the rapid deceleration of ions (the kinetic energy drops by roughly 70%). The electric field in the second stage is much weaker, which determines how much longer high energy ions will stay in the reflectron than low energy ones. As shown in Equation (5.17), the length of a single stage reflectron is at least $0.25L$, while according to Equation (5.21), the length of a two stage reflectron is about $0.068L$, only about $1/4$ of the single stage one. Microscope MSI instruments that incorporate the two-stage and one-stage reflectrons will be investigated by SIMION in the next section.

5.2 Modeling reflectron MSI in SIMION

SIMION [141] was used to model a reflectron for the microscope MSI instrument, with the goal of improving its mass resolving power while preserving the quality of the ion images. The reflectron was added to the five-electrode ion optics system described previously, with PEDAs providing a temporal focus or ‘pseudo-source’ plane for the reflectron. At the tem-

poral focus, ions with the same m/z form a very thin packet. In other words, they arrive at the temporal focus simultaneously regardless of their initial velocities/kinetic energies. After passing the temporal focus, the ion packet expands along the time-of-flight axis due to the kinetic energy spread. As discussed in the last section, the reflectron reverses this expansion and refocuses the ions to a plane after the reflectron that coincides with the detector. Combining delayed extraction (the counterpart of PEDDA in traditional ToF-MS) with a reflectron is routine in modern research-grade TOF-MS instruments [59, 160, 161]. Single-stage and double-stage gridded reflectrons were simulated and analysed before a single-stage gridless reflectron was chosen for the final design. In this section, various aspects of modeling the single-stage and double-stage gridded reflectron MSI systems will be detailed.

5.2.1 Simulating a non-ideal grid

Fine grids are commonly used to approximate homogeneous fields in a reflectron. The only inhomogeneities lie in the vicinity of the mesh wires. As shown in Figure 5.5, if the electric fields on different sides of a grid have different strengths, the stronger field protrudes into the weaker one, giving rise to curved equipotential lines at each opening. Consequently the entire grid will act as an array of diverging lenses, imposing angular deflections on passing charged particles (see Chapter 2 for more details on the lensing effect of an aperture). If U_0 corresponds to the kinetic energy of the ions, s is the spacing between grid wires, and E_1 and E_2 are the electric field strengths on the left and right sides of the grid, the maximum deflecting angle, $\Delta\eta_{\max}$, for paraxial beams can be estimated by the following equation [162]

$$\Delta\eta_{\max} \approx \arctan \left[\frac{s(E_2 - E_1)}{8U_0} \right], \quad (5.22)$$

assuming $s(E_2 - E_1)$ is far less than U_0 and mesh wires are of zero width. As indicated in Equation (5.22), a smaller s helps to mitigate the scattering effect of the grid. This

explains why fine grid meshes are used in reflectrons.

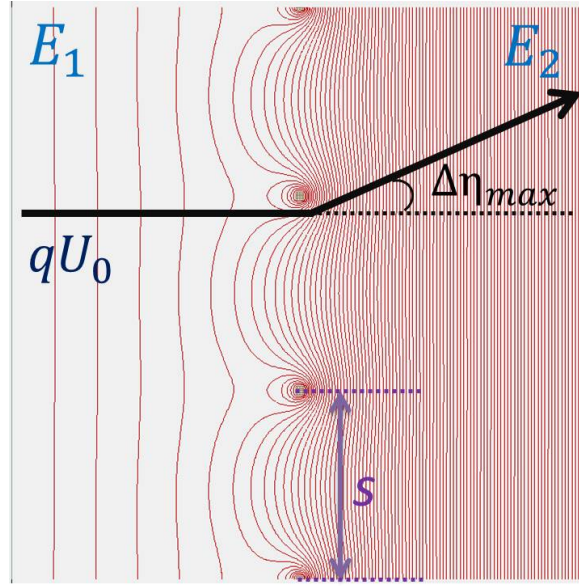


Figure 5.5: Inhomogeneous electric fields around grid openings.

However, using finer meshes to minimise the deflection comes at the price of lower transmission. Typically the transmission of a grid mesh is 90% or less. For a two stage gridded reflectron, where ions pass through a grid four times, one third of the signal will be lost. Therefore one has to balance the drop in sensitivity due to limited transmission and the distortion of ion trajectories due to the inhomogeneous electric field. The density of the most popularly used grid mesh in reflectrons is 70 wires per inch which has a relatively good transmission and a fairly small opening.

The lensing effect of grid openings must also be taken into consideration when incorporating a reflectron into a spatial imaging ToF instrument. Ion trajectories may be distorted in an unfavorable way by these micro-lenses, which could potentially affect both the mass resolving power and spatial imaging performance of the instrument. There exist a few previous studies [163–165] regarding the impact of the grid mesh on the mass resolution of a reflectron ToF-MS. However, little work has been done to investigate the imaging side of its impact since coupling a reflectron with a spatial imaging ToF instrument is very uncommon.

Simulating an ideal grid (100% transmission and no inhomogeneous field at each open-

ing) is straightforward in SIMION. An infinitely thin electrode is defined by setting its thickness as less than one grid-unit. However, simulating a non-ideal grid ($< 100\%$ transmission and an inhomogeneous field at each opening) is not trivial. An intuitive way to do this is to model the entire grid as a large 3D Potential Array (PA), which is only feasible if the size of the grid opening is relatively large. However, as was already discussed, grid meshes used in reflectrons are usually very fine and therefore each opening is very small, which means the array density required to model a non-ideal grid would be impractically high. Moreover, if this same density were applied to the rest of the simulated arrays, ‘impossible’ array sizes would be required. In our case, openings in the 70 lines per inch (lpi) grid are about $360 \times 360 \mu\text{m}$. In order to accurately model such a fine grid in SIMION, at least 200 grid-units are needed to represent a single millimeter in the simulated space (which corresponds to $5 \mu\text{m}$ per grid-unit). A reflectron is at least $10^5 \times 10^5 \times 10^5 \mu\text{m}$ in size, which corresponds to $20,000 \times 20,000 \times 20,000$ grid-units. Each grid point occupies approximately 8 bytes on hard disk or 10 bytes in random-access memory (RAM). So roughly 8×10^{13} bytes or 80 TB is needed in RAM for this non-ideal grid potential array. This is not feasible in practice.

One approach to deal with this challenge is to resort to a so called “particle jump trick”, which allows SIMION to accurately model a very fine non-ideal grid with a reasonable number of grid-units. The particle jump trick was developed by Colby and workers in ref. [166], the work done in this thesis is based on an example project included in the SIMION 8.1 package. In the particle jump trick, a very small volume around a few non-ideal grid openings is modelled accurately with very high array density and a realistic 3D array size, while a very coarse array density is used to represent the rest of the system. There are two parallel boundary planes on both sides of the non-ideal grid and their distance to the grid surface is the same. Electric potentials on these boundary planes are set to match the potentials at the same position as if it were an ideal grid. During a simulated experiment, an ion encounters a grid and jumps from the main volume of the reflectron into the small volume that models the non-ideal grid. Then the ion flies through

the grid wires and gets scattered by the inhomogeneous field. When the ion arrives at the boundary plane on the other side of the non-ideal grid, it jumps back to the main volume with a deflected velocity. Using this strategy, the lensing effect of non-ideal grids can be simulated in SIMION.

The 3D potential array of the small non-ideal grid volume is shown in Figure 5.6 (a) and (b). There are nine grid openings in the potential array with a size of $352 \mu\text{m} \times 352 \mu\text{m}$ and a wire width of $16 \mu\text{m}$. Its array density is sufficiently high to model the

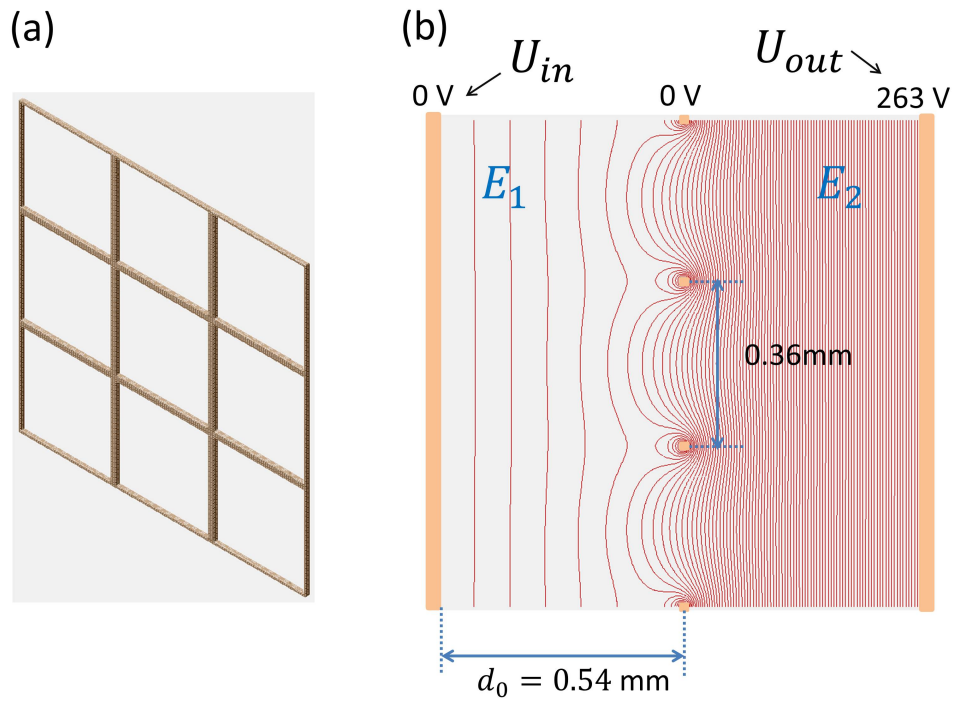


Figure 5.6: (a) 3D non-ideal grid with nine openings; (b) potential array of the non-ideal grid volume.

non-ideal grid effect ($4 \mu\text{m}/\text{grid-unit}$). An entrance boundary plane is on the left side of the grid while an exit plane is on the right. Both boundaries are located $544 \mu\text{m}$ away from the grid surface (the boundary to grid distance is denoted as d_0). The electric field strengths on both sides of the ideal grid are denoted as E_1 and E_2 respectively (as shown in Figure 5.7). The non-ideal grid mesh itself is grounded and the potentials of the two boundary planes can be derived from d_0 , E_1 , and E_2 . The Neumann boundary condition (more details in Chapter 2) is used for the remaining four boundary planes of the small

volume.

The particle jumping trick is implemented *via* the user program interface described in Chapter 2. A single stage reflectron simulation is used here as an example to illustrate how the process works. As shown in Figure 5.7(a), the reflecting field in the main volume is established by an ideal grid and a plate electrode. A small non-ideal grid volume is placed in an empty space far away from the reflecting field and the beam trajectory. An ion initially travels from left to right along the time-of-flight axis and enters the reflectron at position (1). Since an ideal grid is used to separate the reflecting field (E_R) and the field free drift region in the main volume, the ion will go through an abrupt change of electric field strength at the grid plane (from 0 to E_R). SIMION uses this change to detect the grid and trigger the particle jumping process. The ion's flight time and position at (1) are cached as t_1 and x_1, y_1, z_1 before the ion jumps to the area with a dashed border in the figure where the very small non-ideal grid instance is placed. As shown in Figure 5.7(b), which is an enlarged view of the non-ideal grid instance, the ion is randomly positioned over a square area at the centre of the entrance boundary plane, which corresponds to the middle opening of the grid (the dashed area shown in Figure 5.7(b)). The ion's velocity at (2), the position at the entrance plane, remains the same as before jumping, so $v_{2x} = v_{1x}, v_{2y} = v_{1y}, v_{2z} = v_{1z}$.

In terms of the potentials on the entrance and exit boundary planes (U_{in} and U_{out}), they are determined by the electric fields on the entrance (E_1) and exit (E_2) sides of the grid. For the particle jumping process from (1) to (4): $U_{\text{in}} = E_1 \cdot d_0 = 0$ and $U_{\text{out}} = E_2 \cdot d_0 = E_R \cdot d_0$, where E_R is the electric field strength inside the reflectron and d_0 is the distance between the grid mesh and the entrance (or exit) boundary plane (see Figure 5.6).

Then the ion continues to move to the right and pass the non-ideal grid. The deflection angle of the ion depends on two factors. The first is where the ion travels through an opening: the deflection will be small if the ion travels closer to the centre of the opening and be big if it travels closer to the wires. The second is how different the electric fields

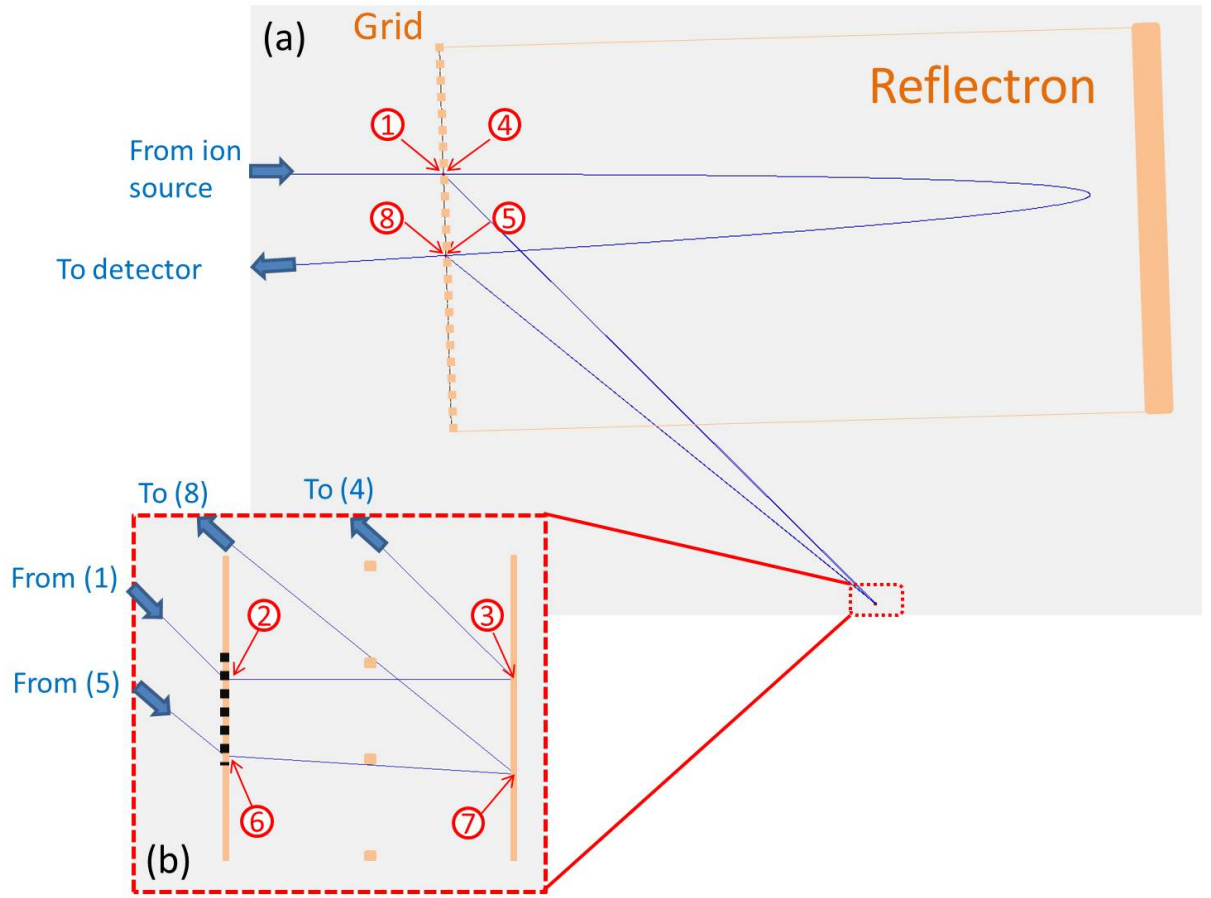


Figure 5.7: Illustration of the particle jump trick: (a) the gridded reflectron in the main simulated space (b) zoomed in view of the non-ideal grid volume (when jumping into the small volume, ions are randomly placed in the dashed area).

on both sides of the grid are: a bigger difference will lead to a larger deflection.

Once the ion arrives at the exit boundary plane of the non-ideal grid instance, it will jump out of the small volume and back to the main volume (from (3) to (4)). The ion's position and flight time (x_4 and t_4) return to the values before jumping in: $x_4 = x_1$, $y_4 = y_1$, $z_4 = z_1$, and $t_4 = t_1$. The ion's velocity at (4) has the following form:

$$v_{x4} = \sqrt{v_{x3}^2 - \frac{2q(U_{\text{in}} - U_{\text{out}})}{m}} \quad (5.23)$$

$$v_{y4} = v_{y3}, v_{z4} = v_{z3}.$$

Ions usually gain or lose extra kinetic energy during the motion within the non-ideal grid instance, so the velocity along the ToF -axis has to be adjusted accordingly after jumping

out.

As the ion penetrates deeper into the reflecting field, it gradually decelerates before it turns around and starts to accelerate towards the exit of the reflectron. When the ion encounters the grid again at (5), the whole particle jumping process will be repeated: the ion jumps into the small non-ideal grid instance, gets deflected by the inhomogeneous field around the opening, and jumps out back to the main reflectron instance. There is one more thing worth noting: the sign of v_x has to be inverted after it jumps from (5) to (6) ($v_{x6} = -v_{x5}$), otherwise the ion will continue to fly to the left and will not go through the non-ideal grid, similarly when the ion jumps out from (7) to (8), the sign needs to be changed again ($v_{x8} = -v_{x7}$) to recover its original velocity along x . Accordingly, in the ion jump process from (5) to (8), $U_{\text{in}} = E_R \cdot d$ and $U_{\text{out}} = 0$.

5.2.2 Two stage reflectron and single stage reflectron

A conventional two-stage reflectron was loaded into a SIMION workbench together with an ion optics chamber and a detector plate. Everything inside the ion optics chamber was identical to the single PEDA simulation in Chapter 4. The two stage reflectron was cylindrically symmetric and consisted of three different electrodes: a round grid electrode at its entrance, a grid that separates the retarding and reflecting fields, and a plate electrode at its end. The radius of the reflectron was 52.5 mm, the length of the retarding region d_b was 7.9 mm, and the length of the reflecting region d_r was 63.2 mm. The reflectron was placed 1075.6 mm away from the last ion optics electrode and tilted by $\theta = 2.35^\circ$. The centre of the detector plate was positioned 378 mm away from the last ion optics electrode and offset by 73 mm from the ion optical axis. To compensate for the flight path difference due to divergent ion trajectories, the detector plane was tilted by $\epsilon = -2.98^\circ$ to maximise the ToF resolution. The repeller and einzel lens were held at 5000 V and 1815 V respectively. The extractor was pulsed from 4690 V to 5660 V after a delay time of 2.8 μs . Under these conditions, PEDA focuses ions ($m/z = 390$) into a thin packet at a distance of 579 mm from the last ion optics electrode, creating the temporal

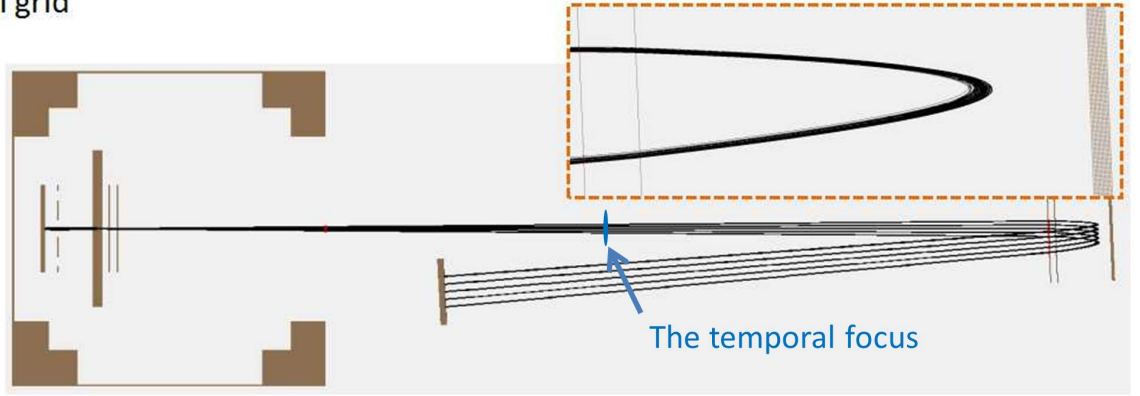
Parameters	Two-stage	One-stage
Repeller (V)	5000	5000
Extractor (V)	4690	4690
PEDA pulse (V)	5660	5660
PEDA delay time (μ s)	2.8	2.8
Einzel lens (V)	1815	1805
Reflectron (V)	3837 (middle grid) 5882 (bottom electrode)	5800 (bottom electrode)
Reflectron anlg (°)	2.35	2.18
Detector anlg (°)	-2.98	-1.8

Table 5.1: The parameters used in simulations of the two-stage and one-stage gridded reflectron MSI.

focus for the reflectron. The entrance grid of the reflectron was grounded, the middle grid was kept at 3837 V, and the bottom electrode was at 5882 V (the parameters used in the simulations are summarised in Table 5.1). A sequence of five point ion sources was simulated with a distance between neighboring sources of 0.4 mm. When ideal grids were used in the simulation (particle jumping turned off), a spatial resolution of $17.5 \mu\text{m}$ and a mass resolution of $20,500 m/\Delta m$ were achieved for the focused mass-to-charge ratio (390 Da). The magnification was 21.9 and the total flight time was $45.5 \mu\text{s}$. Compared to the original PEDA instrument, adding a two stage reflectron with ideal grids improves the mass resolution by nearly seven times, while keeping the imaging quality roughly the same (ion trajectories are shown in Figure 5.8(a)).

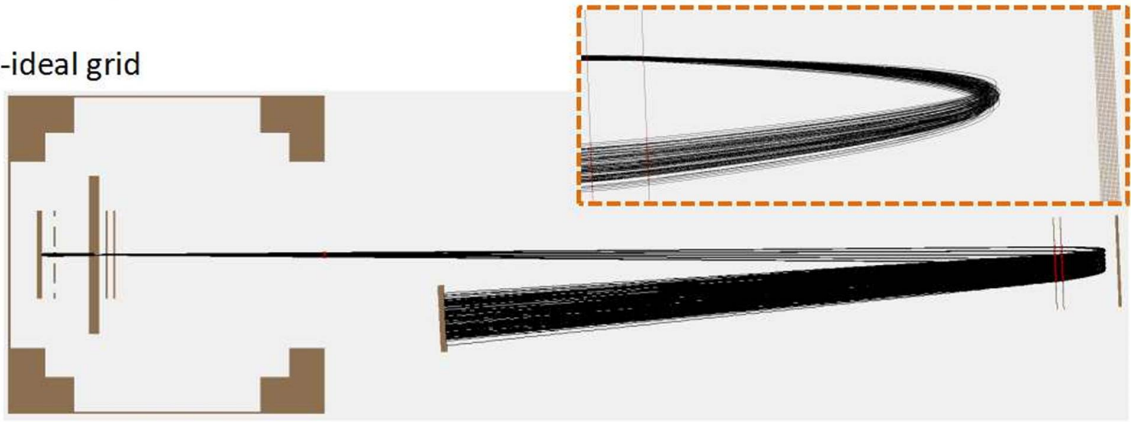
However, once the particle jumping method was activated, as shown in Figure 5.8(b) the lensing effect of each grid opening blurred the ion trajectories significantly. The mass resolution dropped down to $3,460 m/\Delta m$ and the spatial resolution became more than twenty times worse ($490 \mu\text{m}$) than the ideal grid case. Non-ideal grid effects became most pronounced when an ion was slowed down after going past a grid, because the lateral velocity components introduced by the field inhomogeneity became a nonnegligible fraction of the total velocity. As shown in Figure 5.8(b), the ion beams are broadened dramatically when their axial velocity components are close to zero. The perpendicular

Ideal grid



$m/\Delta m = 20,500$, Spatial Resolution = $17.5 \mu\text{m}$

Non-ideal grid



$m/\Delta m = 3460$, Spatial Resolution = $490 \mu\text{m}$

Figure 5.8: Simulated two stage reflectron MSI experiments: (a) with ideal grids and the temporal focus created by PEDAs, (b) with non-ideal grids.

velocity components dominate these trajectories at the rear of the second region of the reflectron. The transmission of ions also declined by 24% with the particle jumping method turned on. The simulation results for two-stage reflectron MSI are summarised in Table 5.2.

The removal of one grid can help to reduce the trajectory distortion. In a single stage reflectron, ions pass through grids twice rather than four times. Moreover, the electric fields on both sides of the grid in a single stage reflectron are more similar than those in a two stage reflectron. In the latter case, the first grid of the reflectron separates the field free drift region (0 V/mm) and the retarding field (485.7 V/mm), and the second grid separates the retarding field and the reflecting field (32.4 V/mm). In the one-stage case,

	Mass resolution ($m/\Delta m$)	Spatial resolution (μm)	Magnification	ToF (μs)
Gridded two-stage reflectron	20,500 (Ideal grid) 3,460 (Non-ideal)	17.5 (Ideal grid) 490 (Non-ideal)	21.9	45.5
Gridded one-stage reflectron	21,780 (Ideal grid) 22,570 (Non-ideal)	22.5 (Ideal grid) 27.9 (Non-ideal)	22.1	61.5

Table 5.2: The optimum specifications of the simulated gridded one-stage and two-stage reflectron MSI systems.

the length of the reflectron was extended to 324 mm and the potential on the bottom electrode was set to 5800 V, which corresponds a field strength of 17.9 V/mm. Therefore the field strength difference of a grid in the one-stage reflectron was about 4% of that in the two-stage case. According to Equation (5.22), a smaller difference between E_1 and E_2 will lead to a smaller deflection (or distortion) of ion trajectories.

The radius of the one-stage reflectron was a bit wider than the two-stage one (89.4 mm). The reflectron was also placed slightly further from the last electrode of the ion optics (1078 mm). The angle θ was set to 1.8° . The repeller and extractor voltages as well as the PEDDA voltage pulse and its delay time were kept the same as in the two-stage case. The middle electrode of the einzel lens assembly was tuned to 1805 V to accommodate a longer flight path. The detector angle ϵ was -2.18° (see Table 5.1 for a summary of simulation parameters). As illustrated in Figure 5.9, in the one-stage reflectron, the effect of non-ideal grids on ion trajectories becomes much smaller: the mass resolution for the ideal grid was $22,570 \pm 920 m/\Delta m$, while for the non-ideal grid case it was $21,780 \pm 700 m/\Delta m$, not a very obvious decline (see Table 5.2). The spatial resolution did decreases from 22.5 μm to 27.9 μm , suggesting that the inhomogeneous field at each grid still has a tiny blurring effect on the ion trajectories. The magnification was about 22.1 and the total flight time was about 61.5 μs for the one stage reflectron instrument.

In theory, single stage reflectrons are able to correct the flight time dispersion due to

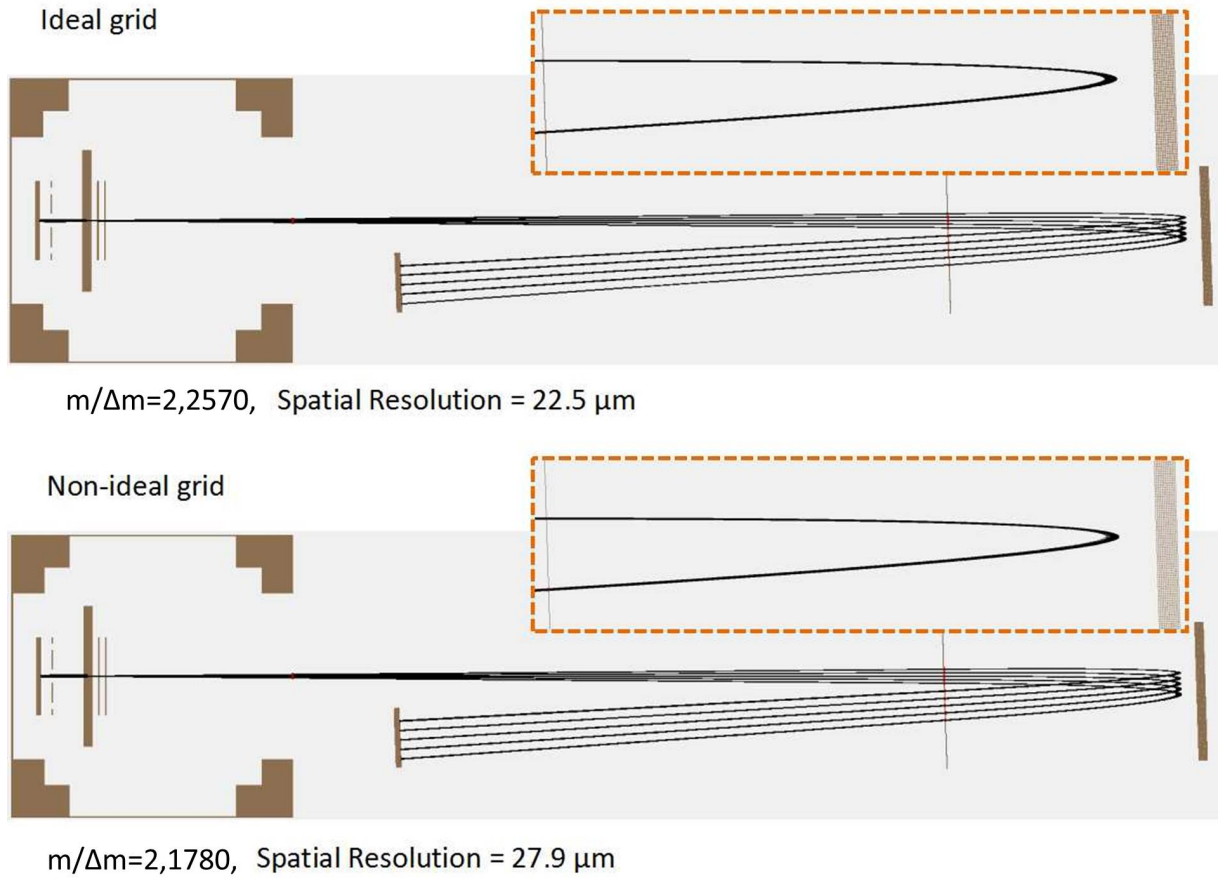


Figure 5.9: Simulated single stage reflectron MSI experiments: (a) with ideal grids, and (b) with non-ideal grids.

kinetic energy spread without the help of PEDDA. For our instrument the kinetic energy spread of ions with the same m/z mainly originates from their initial velocity distribution, which is inherent to the MALDI ionisation process. Contributions from non-flat sample surfaces are negligible. Assume there are two 390 Da ions generated by MALDI, both of them are ejected normal to the sample surface, but ion A has an initial speed of 900 m/s while ion B travels at 100 m/s. The extractor potential is kept static (4.6 kV) meaning PEDDA is inactive. If the repeller is held at 5 kV and the einzel lens at 1.8 kV, then according to SIMION, ion A will be 5.7 mm ahead of ion B at the exit of the ion optics chamber, and their velocities will be $49.745 \text{ mm}/\mu\text{s}$ and $48.736 \text{ mm}/\mu\text{s}$ respectively. Suppose there were a virtual temporal focus that ion A and B would reach simultaneously, then this focus would be at least 31.483 m before the reflectron entrance (which would also be the lower limit of the field free drift length L). According to Equation (5.17), in

order to correct the flight time difference with a single stage reflectron, the reflectron would have to be more than 7.871 m in length. However, with PEDDA a real temporal focus point can be created before the reflectron, allowing the field free drift length L to be significantly reduced (<4 m). This corresponds to a much more affordable and practical reflectron length (<1 m).

As discussed in this section, it appears that with ideal grids the two-stage reflectron analyser does not outperform the single stage one in terms of mass resolution. This is because the ToF of ions in the two-stage reflectron instrument ($t_{\text{two-stage}} \approx 45.5 \mu\text{s}$) is much shorter than in the one-stage reflectron instrument ($t_{\text{one-stage}} \approx 61.5 \mu\text{s}$). As a result, although the Δt of the two-stage instrument ($\Delta t_{\text{two-stage}} \approx 1.0$ ns) is smaller than that of the one-stage instrument ($\Delta t_{\text{one-stage}} \approx 1.4$ ns), the mass resolution of the two-stage instrument is still slightly worse than the one-stage instrument. Simulations confirmed that if the two-stage reflectron is placed further from the ion optics (1,379 mm from the last ion optical electrode) to increase $t_{\text{two-stage}}$ to the same value as $t_{\text{one-stage}}$, the mass resolution will rise to $29,930 m/\Delta m$; about 40% better than the one-stage instrument. Also, the second-order correction of flight time becomes important only when ions have a relatively large kinetic energy spread. However, in this case, a typical kinetic energy spread is less than 1% (as determined by SIMION). Compared with other factors that may affect the mass resolution (such as detector response time), contributions from the second-order term appear to be negligible and therefore adding the extra stage in a reflectron becomes unnecessary.

5.3 Gridless reflectron

Another straightforward way to avoid the effect of real grids is to replace them with open lenses. By introducing multiple ring electrodes with relatively small diameters into the reflectron, the field inside the reflectron becomes mostly uniform (see Figure 5.10(a) and (b)). In the following simulations, the outer and inner diameters of a reflectron ring were

Parameters	Gridless (simulation)
Repeller (V)	5000
Extractor (V)	4712
PEDA pulse (V)	5208
PEDA delay time (μ s)	2.85
Einzel lens (V)	1690
Deflector (V)	± 90
Reflectron (V)	9912 (bottom electrode)
Reflectron anlg (°)	2.18
Detector anlg (°)	-3.37

Table 5.3: The parameters used in simulations of the gridless reflectron MSI.

130 mm and 115 mm, respectively; its thickness was around 1 mm. The distance between the edge of the rings and the inner wall of the chamber that encloses the ring electrode assembly was about 10 mm. The reflectron consists of 65 coaxial rings in total. These rings are parallel and equally spaced with a 10 mm distance between neighboring rings. The total length of the ring assembly was about 640 mm. The first eight rings in the assembly were grounded which served to shield any stray fields outside the reflectron entrance. The potential on the rest of the rings rises following a linear gradient. Curvatures of the field at the mouth of the reflectron did exist, but the potential here was relatively low (< 500 V) and the ions have their highest kinetic energy (> 5 keV) at this point, so the lensing effect was minimal as shown in Figure 5.10(b).

A gridless reflectron MSI system was modeled in SIMION and its layout is shown in Figure 5.11. The detector was moved further away from both the ion source and the ToF axis (912.5 mm from the last electrode of the ion optics and 138 mm off the ToF axis), this was purely for practical reasons: to make sufficient space between the ion optics chamber and the detector chamber for a CCD camera or a PImMS camera. The detector angle ϵ is -3.37° . The reflectron was again at an angle θ with respect to the ToF axis, which was 2.18° in the current setup (the simulation parameters are summarised in Table 5.3). The centre of the first reflectron ring was located 1,514 mm away from the ion optics and displaced 59.2 mm from the ToF axis.

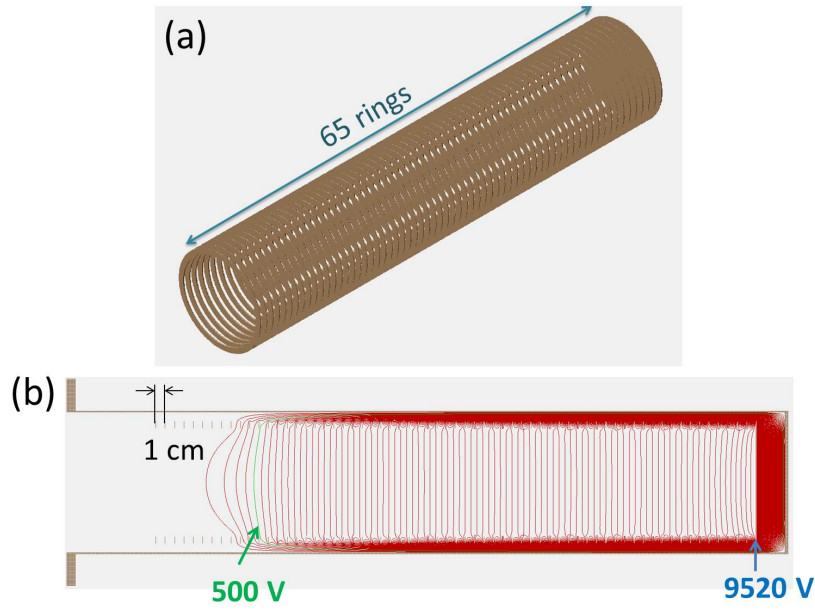


Figure 5.10: Gridless reflectron in SIMION: (a) a stack of ring electrodes (b) equipotential lines inside the reflectron.

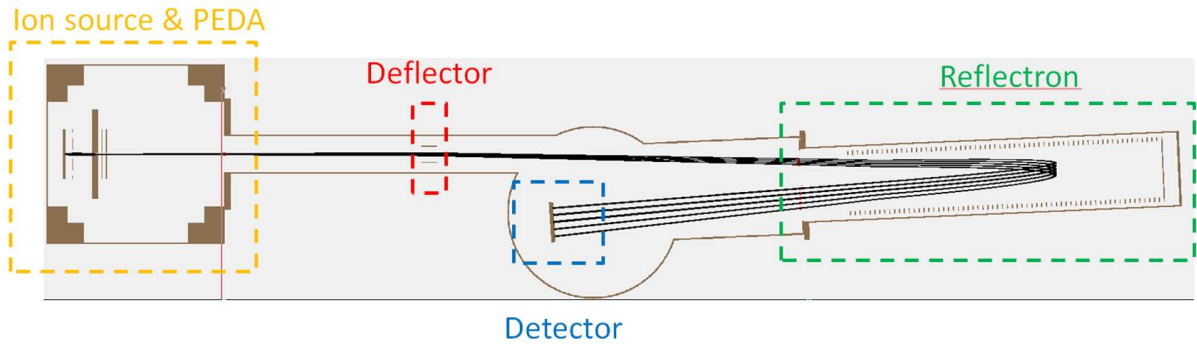


Figure 5.11: The layout of the simulated gridless reflectron MSI instrument

Moreover, two pairs of rectangular deflector electrodes were added into the arm between the ion optic chamber and the detection chamber, which control the incident angle of the ion beam in both y and z directions. The following subsection will discuss how a deflector can correct the trajectory broadening problem inherent to a reflectron MSI instrument.

5.3.1 Correction of ion beam broadening in the reflectron by a deflector

As shown in Figure 5.12, the trajectories of two ions with the same m/z overlap in space before they enter the reflecting field. However, after being reflected by the reflectron, their trajectories start to diverge if the kinetic energies/speeds of these two ions are not identical. Ions of higher energy will stay in the reflectron for a longer time and therefore have a larger S_2 (the distance between the entering and exiting points of the ion trajectory along the entrance grid of the reflectron). The bigger the difference between the higher velocity ion, v_{high} , and the lower velocity ion, v_{low} , is, the larger ΔS_2 and d' will be (ΔS_2 is the difference of S_2 and d' is the corresponding distance between arriving points on the detector). Thus, the width of an ion beam will be broadened by a reflectron and its spatial spread on the detector will be larger, which will inevitably impair the spatial resolution of the instrument.

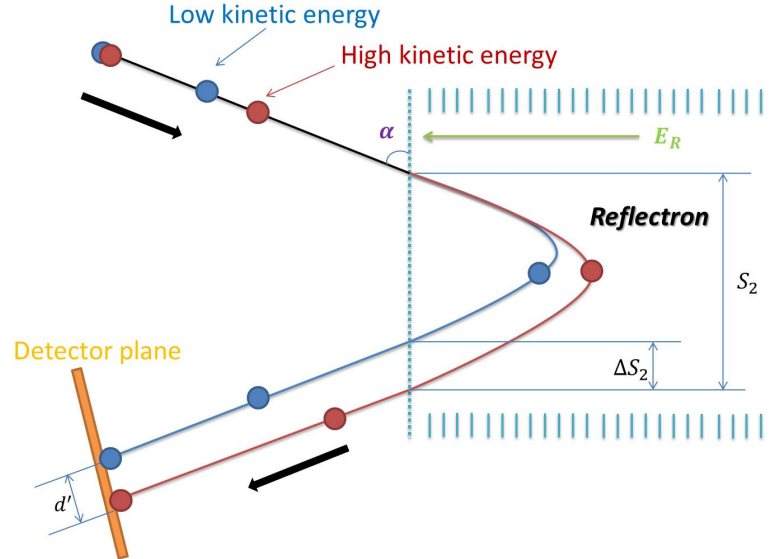


Figure 5.12: Ion beams are broadened as they pass through the reflectron. S_2 is the distance between the entering and exiting points of the ion trajectory along the entrance grid of the reflectron; d' is the distance between the arriving points of ions on the detector due to their different kinetic energies.

One way to correct such broadening is to add a deflector as shown in Figure 5.13(a) and (b). A deflector creates a uniform electric field orthogonal to the incident ion beam.

Therefore, it can transversely deflect those ions that pass through it. Ions of higher speed stay in the deflecting field for a relatively shorter time than those of lower speed, resulting in a smaller deflection angle β . Thus, after a free flight distance L_2' , ions with smaller β (those higher kinetic energies) have a smaller deflection distance S_1 than those with larger β (the difference of S_1 is denoted as ΔS_1).

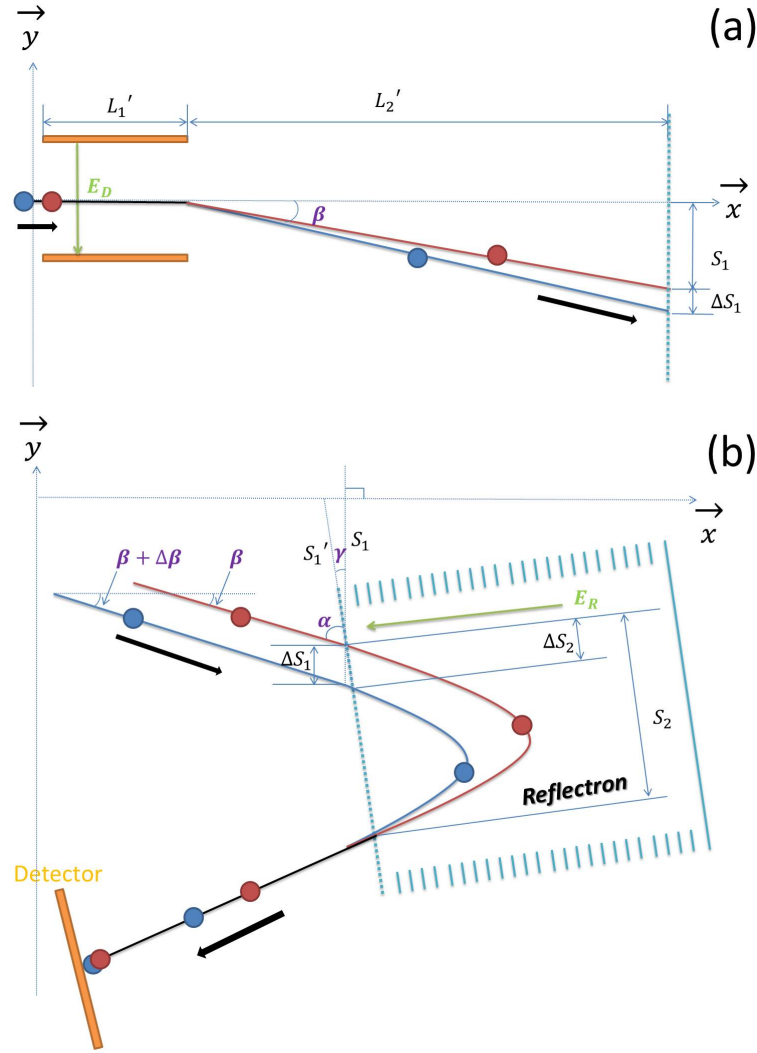


Figure 5.13: (a) A deflector assembly makes lower kinetic energy ions enter the reflectron at a larger distance S_1 along the negative y -axis than higher kinetic energy ions, (b) ΔS_1 compensates for the ion beam broadening d' .

As shown in Figure 5.13(b), if the electric field E_D in the deflector is chosen appropriately, ΔS_2 can be largely compensated by ΔS_1 , which means that ions arriving at the detector plane have no broadening effect ($d' = 0$). The condition to implement this

correction can be derived geometrically, S_1 is:

$$S_1 = L'_2 \cdot \tan \beta = L'_2 \cdot \frac{v_y}{v} = L'_2 \cdot \frac{(E_D \cdot q/m) \cdot (L'_1/v)}{v} = \frac{L'_1 \cdot L'_2 \cdot E_D \cdot (q/m)}{v^2}, \quad (5.24)$$

where v_y is the velocity component along the y -axis imposed by the deflecting field and v is the ion's velocity along the ToF axis after being accelerated by the ion optics (so, v is essentially v_x). S_1 corresponds to a distance along the grid plane S'_1 :

$$S'_1 = \frac{S_1}{\cos \gamma} = \frac{L'_1 \cdot L'_2 \cdot E_D \cdot (q/m)}{\cos \gamma \cdot v^2} \quad (5.25)$$

Before deriving a formula for S_2 , if t_{ref} represents the time that ions spend in the reflecting field and v_0 is the total instantaneous velocity of the ions ($\vec{v}_0 = \vec{v} + \vec{v}_y$) at the entrance of the reflectron, then:

$$t_{ref} = 2 \cdot \frac{v_0 \cdot \sin \alpha}{E_R \cdot (q/m)}, \quad (5.26)$$

so

$$S_2 = v_0 \cdot \cos \alpha \cdot t_{ref} = 2 \cdot \frac{v_0^2 \cdot \sin \alpha \cdot \cos \alpha}{E_R \cdot (q/m)}. \quad (5.27)$$

Therefore, the total distance along the grid plane S_{total} is the sum of S'_1 and S_2 :

$$\begin{aligned} S_{total} &= S'_1 + S_2 = \frac{L'_1 \cdot L'_2 \cdot E_D \cdot (q/m)}{\cos \gamma \cdot v^2} + 2 \cdot \frac{v_0^2 \cdot \sin \alpha \cdot \cos \alpha}{E_R \cdot (q/m)} \\ &= \frac{L'_1 \cdot L'_2 \cdot E_D \cdot (q/m)}{\cos \gamma \cdot v^2} + 2 \cdot \frac{v^2 \cdot \sin \alpha \cdot \cos \alpha}{E_R \cdot (q/m)} + 2 \cdot \frac{v_y^2 \cdot \sin \alpha \cdot \cos \alpha}{E_R \cdot (q/m)} \\ &= \frac{L'_1 \cdot L'_2 \cdot E_D}{2 \cdot U \cdot \cos \gamma} + \frac{4 \cdot \sin \alpha \cdot \cos \alpha \cdot U}{E_R} + \frac{\sin \alpha \cdot \cos \alpha \cdot E_D^2 \cdot L_1'^2}{E_R \cdot U}, \end{aligned} \quad (5.28)$$

where qU corresponds to the average kinetic energy of the ion beam. So the condition for a first order correction of the beam broadening effect is:

$$\frac{dS_{total}}{dU} = 0. \quad (5.29)$$

Based on the geometric relationship shown in Figure 5.13(b), one has the following equation for α , γ , and β (see Figure 5.13 for the respective definitions of α , γ , and β):

$$\alpha + \gamma + \beta = \frac{\pi}{2}. \quad (5.30)$$

Therefore, $\sin \alpha$ equals to $\cos(\beta + \gamma)$ and $\cos \alpha$ equals to $\sin(\beta + \gamma)$. Since the deflection angle β depends on the kinetic energy of the passing ion (qU), $\sin \alpha$ and $\cos \alpha$ in Equation (5.28) cannot be treated as constants. As a result, solving Equation (5.28) analytically is not a trivial job. SIMON was employed instead to search for an optimal E_D by trial and error, given the E_R , L'_1 , L'_2 , γ , and U used in a simulated experiment. In so doing, the spatial resolution was improved from 20.6 μm without deflectors to 17.5 μm with deflectors. It is important to note that the E_D determined by a simulation may not completely match the value found by Equation (5.29). This could be partially due to the various approximations that are employed when deriving a solution to Equation (5.29), and more importantly, for a gridless reflectron, the reflecting field is inhomogeneous at the entrance, while Equation (5.29) assumes a perfectly uniform reflecting field.

The deflectors are also potentially useful if a deflection gate will be needed in the future to filter out ions of no interest. For example, if the reflectron ToF instrument is used to investigate the PSD process, deflectors can be grounded initially to eliminate unwanted ions. When the target ions are passing through the deflectors, appropriate potentials can be applied to direct those target ions into the reflectron. By doing this, one can select a specific precursor ion and analyse only its PSD fragments.

5.3.2 Gridless reflectron simulation results

The optimum specifications of the simulated gridless reflectron MSI instrument are summarised in Table 5.4. The repeller was set to 5000 V, the extractor to 4712 V, the PEDDA pulse voltage to 5208 V, the PEDDA delay time to 2.85 μs , the middle einzel lens to 1690 V, the upper deflector electrode to 90 V, the lower deflector electrode to -90 V, and the reflec-

tron to 9912 V. The mass resolution of the optimal mass peak, $390\ m/z$ in this case, was above $10,000\ m/\Delta m$, which was more than five times higher than the linear PEDDA instrument. Moreover, the spatial resolution was kept under $20\ \mu\text{m}$ by correcting the beam broadening effect of the reflectron with the deflecting field discussed earlier. The magnification was about 80, which is much larger than that obtained with the single stage gridded reflectron described previously (≈ 20). The flight path of ions in the current layout is longer, which naturally results in a larger magnification. The curved potential surfaces around the reflectron mouth may also diverge ion beams, which can be analogous to the diverging effect of a convex mirror. The average ToF of a simulated $390\ m/z$ ion package is about $72.3\ \mu\text{s}$ and its FWHM is about $2.3\ \text{ns}$. As already discussed in Chapter 2, if the arrival time FWHM is comparable with the response time of the detector (which is estimated to be around $5\ \text{ns}$), the response time will limit the mass resolution. If one recalculates the Δt by convoluting the $5\ \text{ns}$ response time with the FWHM of an ion package, the mass resolution drops to $6600\ m/\Delta m$.

	Mass resolution ($m/\Delta m$)	Convolved + 5 ns ($m/\Delta m$)	Spatial resolution (μm)	Magnification	ToF (μs)
Gridless reflectron	13800	6600	17.5	85	72.3

Table 5.4: The optimum specifications of the simulated gridless reflectron MSI system.

The optimal temporal and spatial focusing conditions are unfortunately highly mass dependent, that is to say both the mass and spatial resolutions decline dramatically for ions lighter or heavier than the optimal m/z . This is mainly due to the fact that PEDDA is employed here, which only works properly for ions within a small mass range (more details in Chapter 4). The mass-dependent mass resolution of the simulated instrument is summarised in Figure 5.14, convoluted mass resolution above $3000\ m/\Delta m$ was achievable between 345 and $435\ m/z$ (the error bar was estimated by the standard deviation of resolutions in three repeated simulated experiments). However, by simply adjusting the delay time of the voltage pulse on the extractor in PEDDA, one can tune the optimal mass

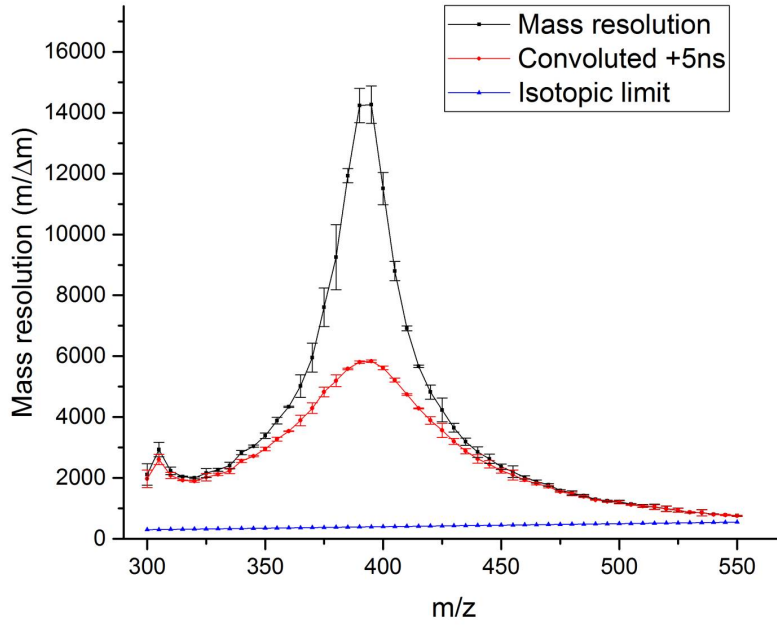


Figure 5.14: Mass-dependent mass resolution of the reflectron MSI instrument. The convoluted curve is for the convolution with the detector response time. The isotopic limit line shows the mass resolving power required to differentiate adjacent isotopes as a function of m/z .

range of the reflectron MSI instrument. As shown in Figure 5.15, by stitching together four different effective mass ranges (delay times of PEDAs are set as 2.8, 3.16, 3.49, and 3.78 μs , which correspond to optimal mass peaks at 390, 500, 610, and 720 m/z respectively), the reflectron MSI can cover a mass range from 300 to 820 m/z , with convoluted mass resolutions between 2000 and 7100 $m/\Delta m$ and spatial resolutions between 10 and 39 μm as shown in Figure 5.16. Of course, the double-field PEDA [167] and time-variable PEDA techniques, both of which deal with the mass dependence problem of PEDA, can also be used with the reflectron. Simulation results shown in Figure 5.17 suggest that the mass resolution can be maintained above 3000 $m/\Delta m$ over a broad mass range (350 to 700 m/z) by applying the time-variable PEDA technique in the gridless reflectron MSI instrument.

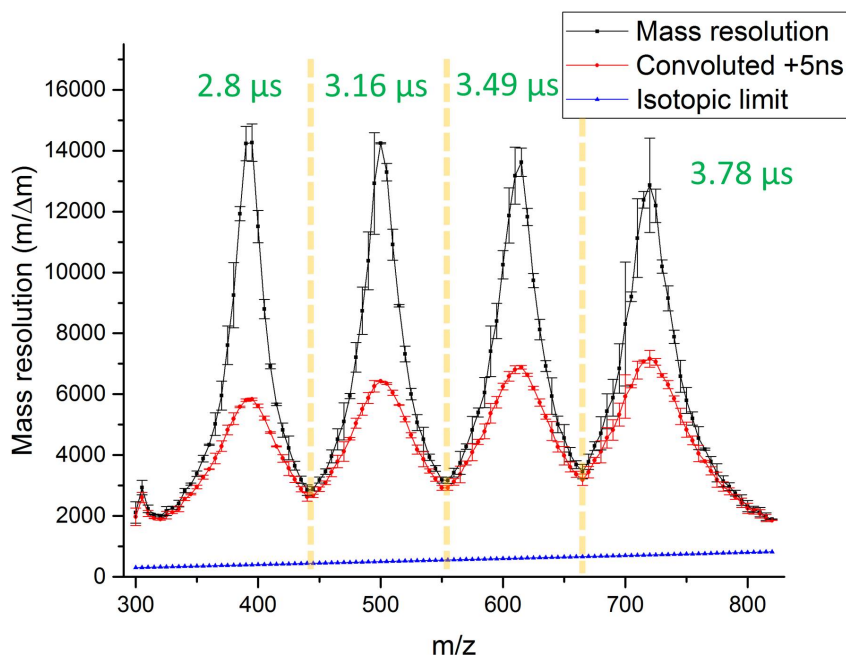


Figure 5.15: Mass-dependent mass resolution of the reflectron MSI instrument after stitching together four different mass ranges (the delay times of PEDDA are 2.80, 3.16, 3.49, and 3.78 μs , which correspond to optimised mass peaks at 390, 500, 610, and 720 m/z respectively).

5.4 Reflectron MSI Experiments

A microscope MSI instrument with a gridless reflectron was built based on the design described in the last section. A logical and systematic approach was employed to troubleshoot such a complex system. This is a critical part of MS instrument development. The troubleshooting process of the reflectron MSI system will be detailed in this section. It consisted of four major stages: (1) identify a malfunction; (2) isolate and pin down the specific part of the instrument that is faulty; (3) establish a theory of probable cause and test it; (4) develop and implement a plan to fix the problem. At the end of this section, preliminary results of the properly running instrument will be presented, which agree reasonably well with the predictions made by SIMION.

Figure 5.18 shows the reflectron and detector chambers of the MSI instrument. There are two things worth noting here. The first is the metal bellows used to connect the

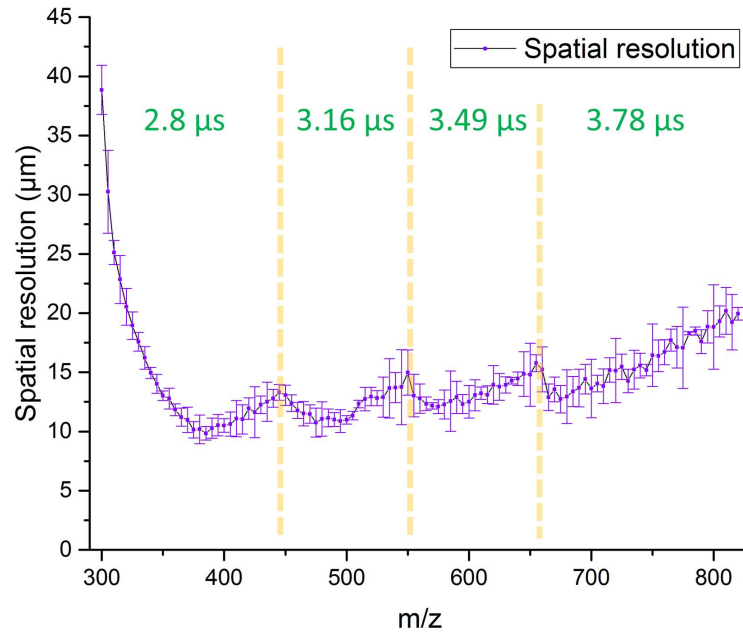


Figure 5.16: Mass-dependent spatial resolution of the reflectron MSI instrument after stitching together four different mass ranges (the delay times of PEDA are 2.80, 3.16, 3.49, and 3.78 μs , which correspond to optimised mass peaks at 390, 500, 610, and 720 m/z respectively).

reflectron to the detector chamber, which enables the mechanical adjustment of the angle θ of the reflectron. This flexibility allows a fine tuning of the instrument geometry and proves useful during the troubleshooting process. The second is that the detector assembly is mounted on a translator, which is used to adjust the angle of the MCP (which is ϵ). Simulations show that the mass resolution can be very sensitive to the detector angle: a 0.5° discrepancy from the optimal angle can lead to a 50% decrease in mass resolution (see Figure 5.19). The manufacturing precision accessible to us was by no means enough to meet this requirement, and in many case the optimal angle predicted by SIMION is usually not 100% correct. Thus, it is necessary to have a mechanical control over this angle so that it can be optimised experimentally.

The system was initially set up according to the optimal voltages and geometries determined by SIMION. The baseline voltage of the extractor was scanned from 4300 V to 4910 V to search for an optimal setting that perfectly focuses the ion images on the

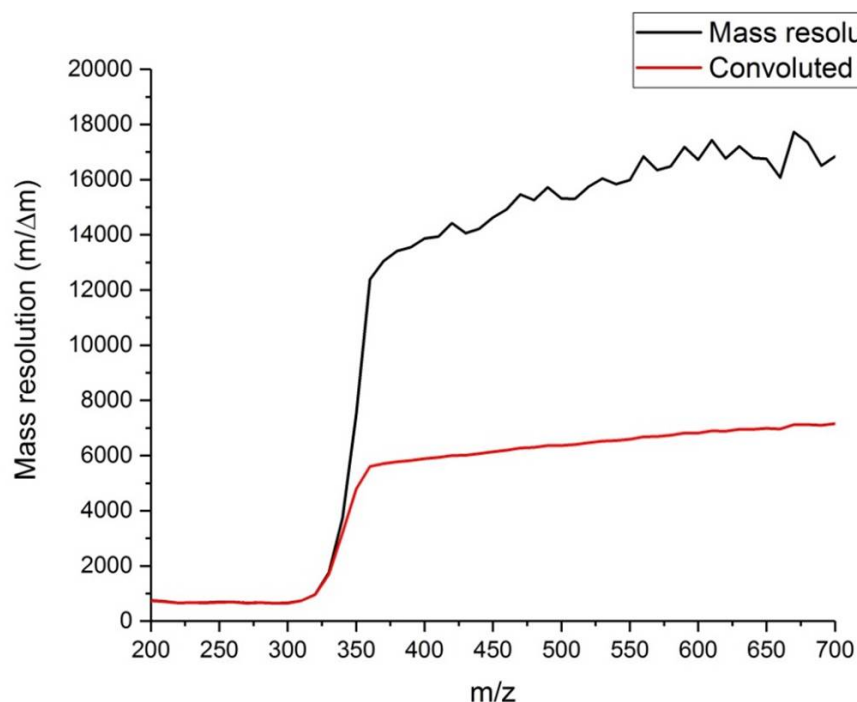


Figure 5.17: Mass-dependent mass resolution of the MSI instrument that couples time-variable PEDAs with the reflectron.

screen. As already introduced in Chapter 3, gridded dye samples were used to characterise the spatial imaging capability of the instrument (dye molecules such as crystal violet and Rhodamine B were sprayed through a mesh stencil over ITO-coated glass slides using our home-built electrosprayer). So, if the instrument works properly, ion images on the detector are expected to match this pattern. However, as shown in Figure 5.20, no regular pattern was observed, irrespective of the extractor voltage employed. Various combinations of voltages were explored on the ion optics, deflectors, and reflectron, but they all lead to nothing, so it was rather evident that something was faulty.

To investigate the potential source of this distortion of ion image, the reflectron MSI system was rearranged. The detector assembly was moved to the end of the reflectron chamber and the last plate electrode of the reflectron was removed. The new layout of the system is displayed in Figure 5.21. The bellows that connected the reflectron chamber to the detector chamber was used to adjust the angle of the reflectron to -5° . By grounding the reflectron rings and keeping the y -axis deflectors at appropriate voltages (± 200 V), this new geometry allowed ion beams to travel along the central axis of the reflectron

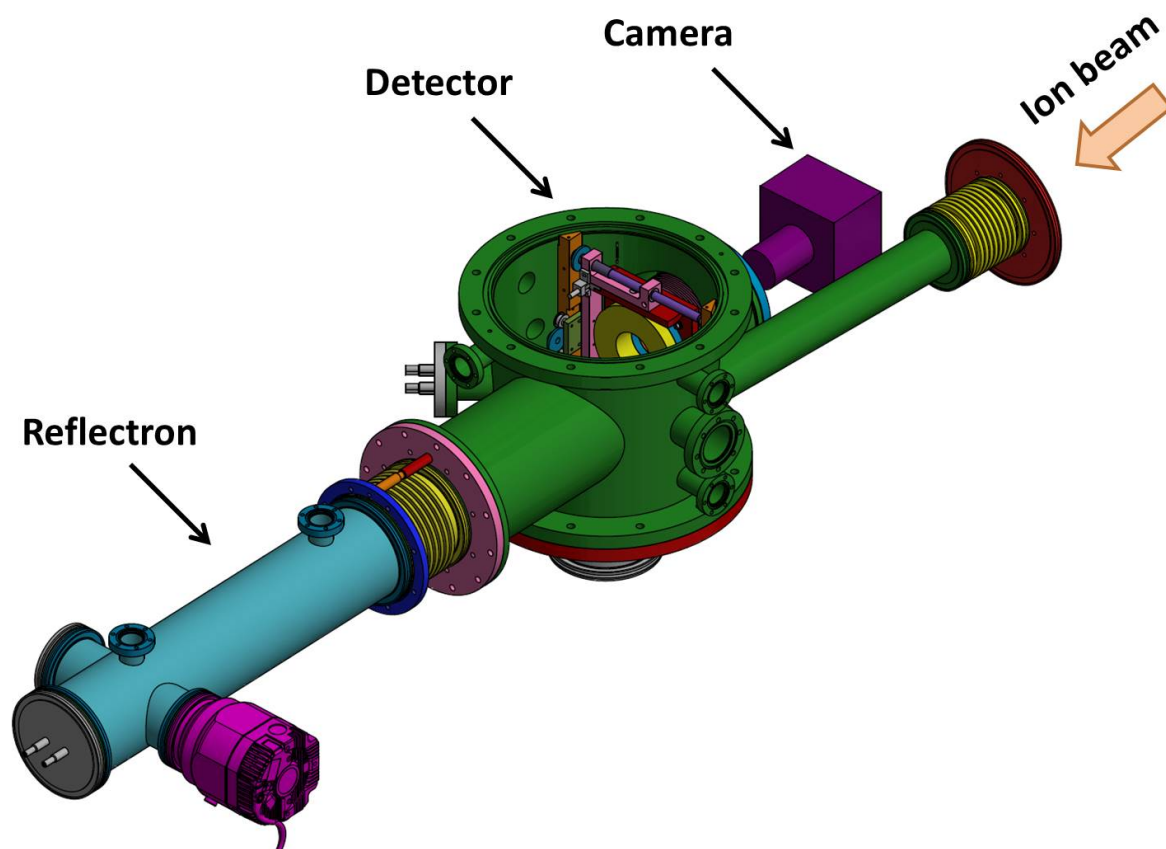


Figure 5.18: The reflectron and the detector chambers of the MSI instrument. The instrument was constructed by the PTCL mechanic workshop, Department of Chemistry, University of Oxford.

rings and eventually arrive at the detector. Although the ion trajectories were bent by the deflectors, this new layout was still an effective linear spatial imaging instrument, like the one used for the PEDDA experiments. If ion images of a clear grid pattern can be obtained with this layout, we can conclude that everything before the reflectron is working properly. Experimental results in the first subplot of Figure 5.22 show that an ion image of a well defined grid pattern can be observed when all the reflectron rings are grounded, which corroborates the assumption that the reflectron was not working correctly.

Since the reflectron was confirmed to be the source of the ion trajectory distortion, the next step was to come up with a theory that may explain such undesired behavior. A possible cause could be stray fields: some external electric fields may penetrate into the reflecting field and badly distort it. As described earlier in this Chapter, most of the

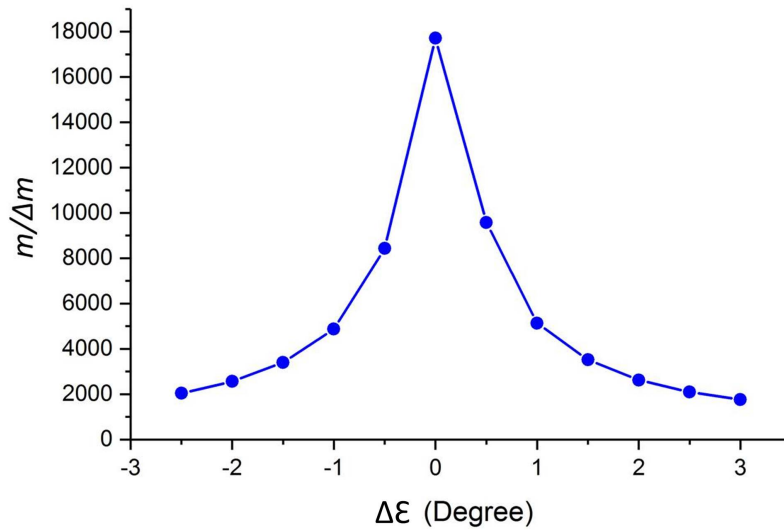


Figure 5.19: Mass resolution as a function of the deviation ($\Delta\epsilon$) from the optimum detector angle.

reflectron rings (from the 9th to the 64th) are connected through a resistor chain. In a normally working reflectron, the first ring of this chain (the 9th ring) is grounded and the last ring is held at a high voltage which is divided evenly across the stack. If the first ring is insulated rather than grounded, the ring stack would be floating and have exactly the same voltage as the last ring.

To test the stray field theory, SIMION was used to model how ion images evolve as the voltage on the ring stack increases. The SIMION results were then checked against the experiments, as presented in Figures 5.22 and 5.23. It should be noted that in the simulations ions are generated from point sources, so a simulated ion image is actually an array of points rather than squares, but this is a reasonable comparison. As the voltage applied on the ring stack rises, the ion images should gradually shrink in size. This is because the ring stack effectively acts as a long einzel lens assembly, which focuses charged particle beams. Apparently, both the experimental and simulated ion images follow this trend, but the simulated square pattern in the simulated images stays well defined and uniform until the ion beam is completely focused down to a single point, while the experimentally obtained images become increasingly distorted as the ring stack

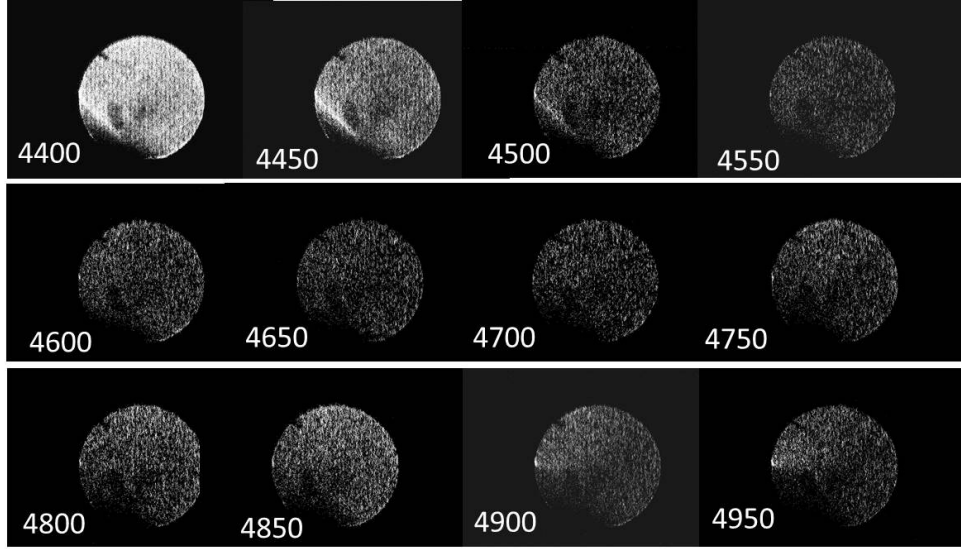


Figure 5.20: Ion images obtained with the reflectron MSI instrument, the extractor voltage was scanned from 4400 to 4950 V.

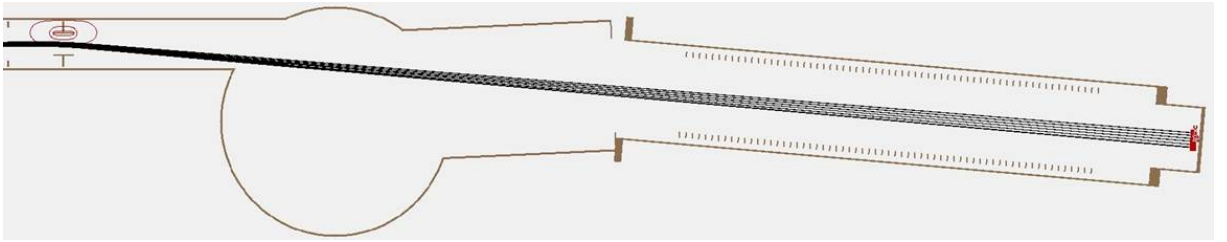


Figure 5.21: The layout of the reflectron MSI instrument in ‘linear’ mode. The detector is moved to the end of the reflectron and ion beams are directed towards the detector using deflectors.

voltage rises. The magnification of the upper part of the experimental ion image also seems bigger than its lower part and the entire image moves downwards rather than shrinks to the centre of the detector like in the simulated image. This discrepancy suggests that the electric field experimentally formed in the reflectron ring stack does not agree with that in SIMION, which is likely due to some unknown external stray field.

5.4.1 Redesign of the reflectron

The most straightforward way to fix the stray field problem is to use “thicker” electrode rings (*i.e.* rings with smaller openings). As shown in Figure 5.24, the ring stack inside the reflectron was rebuilt. Although the outer diameter remains the same as in the original

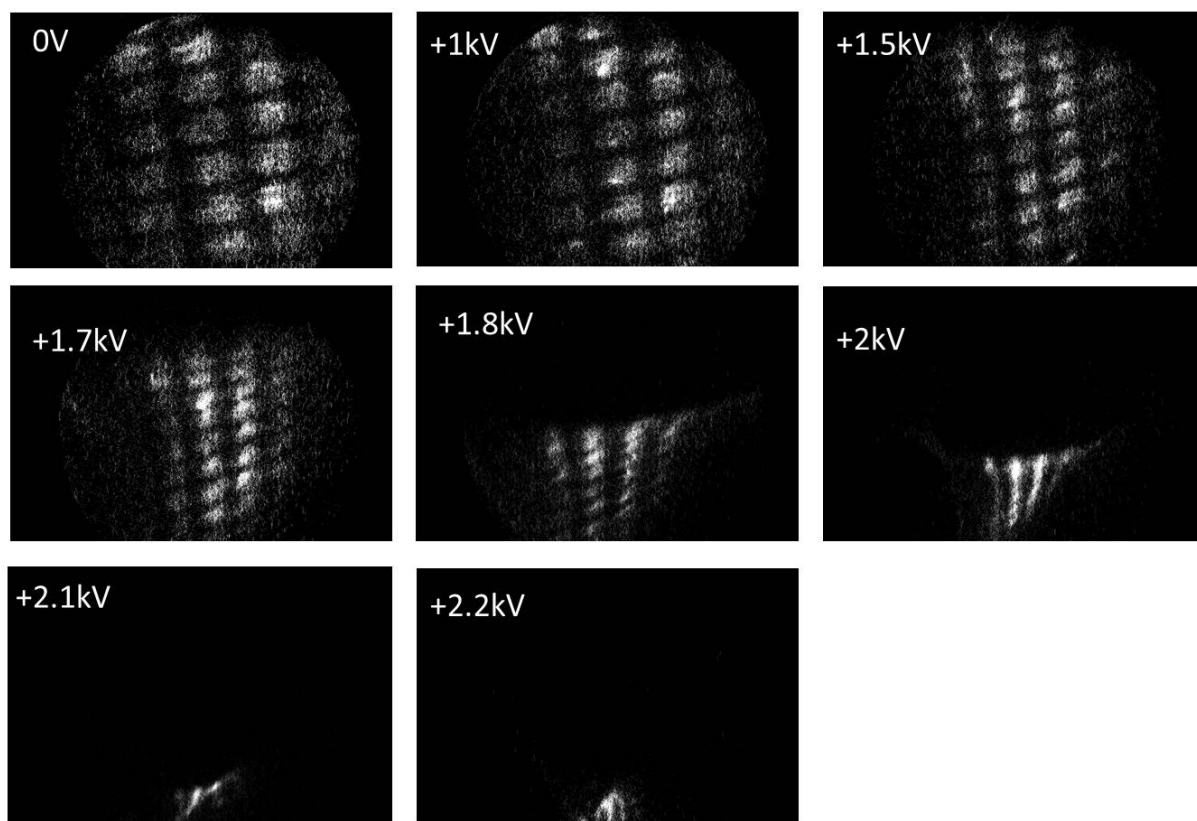


Figure 5.22: Experimental ion images obtained with the reflectron MSI instrument in linear mode. Voltages on the reflectron rings are raised from 0 V to +2.2 kV.

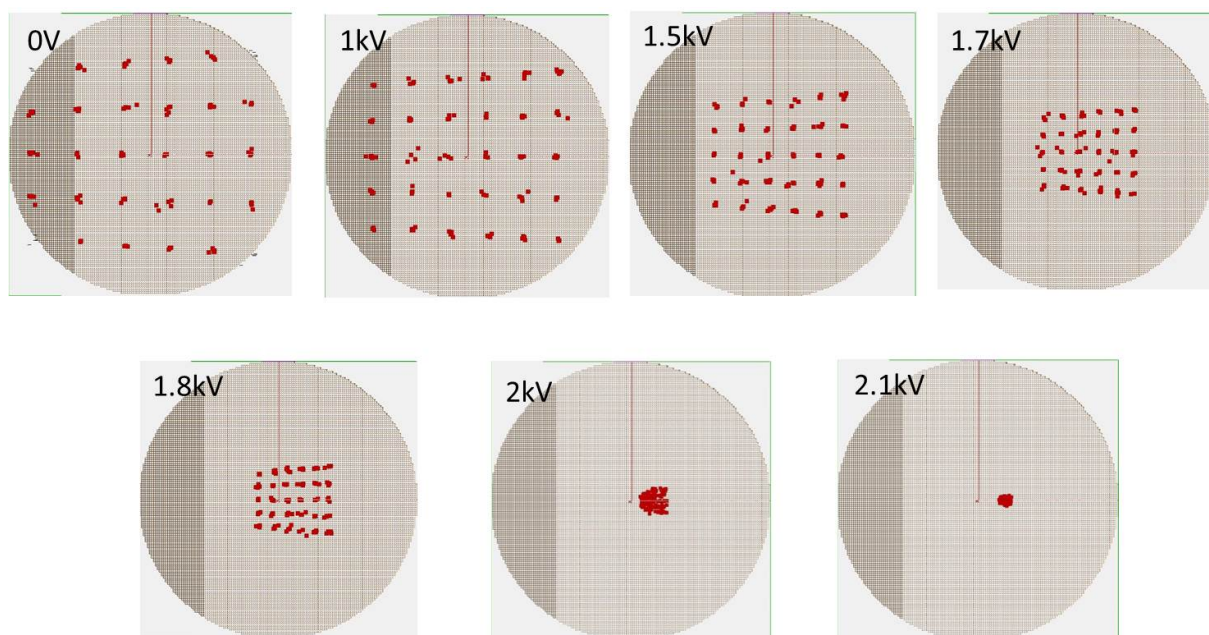


Figure 5.23: Simulated ion images using the reflectron MSI instrument geometry in linear mode. Voltages on the reflectron rings are raised from 0 V to +2.1 kV.

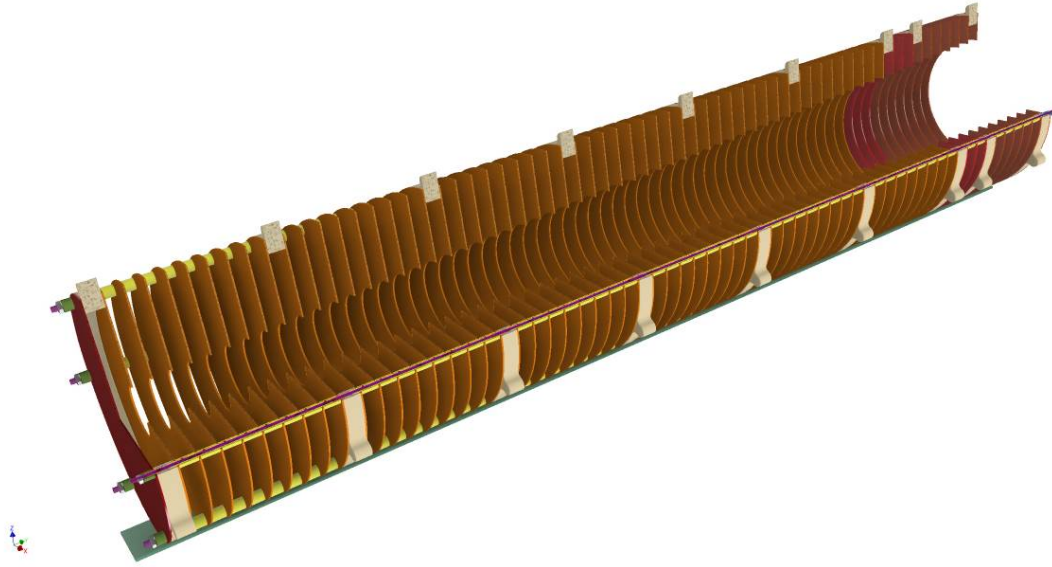


Figure 5.24: Rebuilt reflectron rings: the opening of each ring decreases from 89 to 27 mm. The outer diameter of the rings is 130 mm. The rings were fabricated by A. W. Clarke Engineering Limited (Oxford) through laser cutting.

design, the inner diameter linearly decreases from 89 to 27 mm from the first to last ring. Thicker ring electrodes are in theory better at shielding any external field, so the electric field formed by such an electrode stack should agree better with the simulations. The experiments that tested the stray field theory were repeated with the new rings, as shown in Figure 5.25. Clearly, the experiment results agree much better with the simulations after the ring electrodes have been replaced, the square pattern stays uniform as its size reduces and the final focal point overlaps with the centre of the detector. As the potential of the ring stack continues to increase, the square pattern reappears. This is because ions will start to diverge after passing the focal point as shown in Figure 5.25 above 2 kV. The thicker ring stack therefore solved the stray field problem.

The reflectron was then rearranged back to its original layout (Figure 5.11). The repeller was set to 5003 V and the middle electrode of the einzel lens assembly was set to 1767 V. The single-field PEDDA technique was employed: the voltage on the extractor started at 4673 V and stepped to 5300 V after a $2.85 \mu\text{s}$ delay. The y -direction deflector was set at ± 88.8 V on the upper and lower deflector plate, respectively, and the reflectron angle θ was about 1° (the parameters used in experiment is summarise in Table 5.5). The ex-

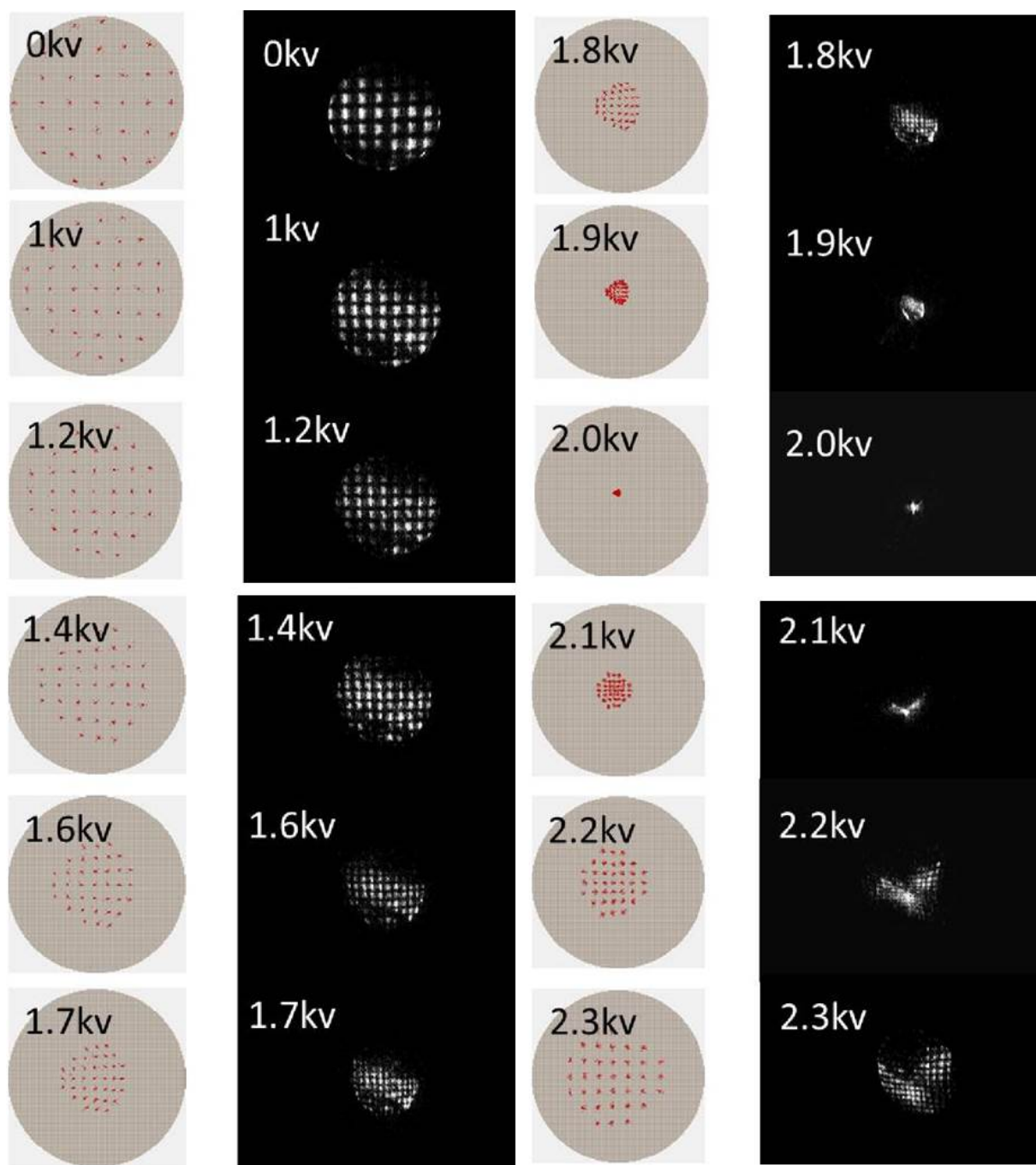


Figure 5.25: Simulated and experimental ion images obtained with the rebuilt reflectron rings. Voltages on the reflectron rings were raised from 0 V to +2.3 kV. Since the reflectron worked effectively as a large einzel lens, the ion image converged into a spot with an increasing reflectron voltage and started to diverge after the focal point was moved in front the detector plane.

Parameters	Gridless (experiment)
Repeller (V)	5003
Extractor (V)	4673
PEDA pulse (V)	5300
PEDA delay time (μs)	2.85
Einzel lens (V)	1767
Deflector (V)	± 88.8
Reflectron (V)	9727 (bottom electrode)
Reflectron angle ($^\circ$)	~ 1
Detector angle ($^\circ$)	~ -3

Table 5.5: The parameters used in experiments of the gridless reflectron MSI.

tractor voltage and the pulsed voltage were determined following the procedures described in Chapter 4: a range of voltages was scanned to find the voltage that gives the best mass and spatial resolutions. Rhodamine B was used to characterise the instrument. It was deposited over an ITO-coated slide as an array of squares with $102\ \mu\text{m}$ width and $38.1\ \mu\text{m}$ gaps. The laser pulse energy was 10 to 15 mJ/pulse, and the CCD camera was gated on the major ToF peaks (various fragments of Rhodamine B). A background-subtracted ion image is shown in Figure 5.26 (a) (the background was obtained by accumulating an equal number of frames without the ionisation laser). A regular pattern with well defined border can be observed with this new ring stack, which confirms that the trajectory distortion problem has been alleviated to a great extent. There is still a small distortion however; the image appears to be an array of rhomboids rather than squares, and the magnification along the horizontal axis (≈ 64.2) is a bit smaller than that along the vertical axis (≈ 85.3), which means the image is either squashed horizontally or stretched vertically. An intensity profile along the yellow band in Figure 5.26 (a) was used to estimate the spatial resolution. By fitting the intensity profile with a convolved function between a Gaussian distribution and the shape of the underlying pattern (more details in Chapter 4), as shown in Figure 5.26 (b) a spatial resolution of $20\ \mu\text{m}$ was obtained, which agrees very well with SIMION. Again, as described previously in Chapter 4, the mass resolving power can be estimated from the ToF spectra acquired with a photomultiplier tube

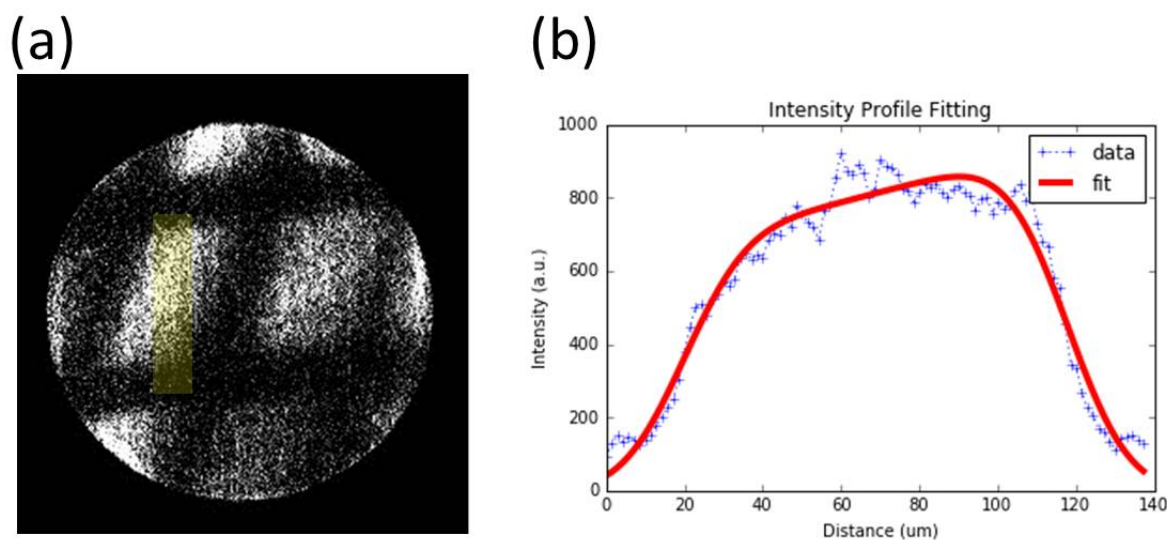


Figure 5.26: (a) Ion images obtained with the rebuilt reflectron rings. (b) The intensity profile was fit with a convolved function incorporating a Gaussian distribution and the shape of the underlying pattern of the image in the yellow band area in (a).

(Figure 5.27). In Figure 5.27 (a), from left to right, the four major fragment peaks correspond to $[M - (\text{CH}_2)_6 - \text{CO}_2 - \text{Cl}]^+$ ($315.15 \text{ } m/z$), $[M - (\text{CH}_2)_4 - \text{CO}_2 - \text{Cl}]^+$ ($343.181 \text{ } m/z$), $[M - (\text{CH}_2)_2 - \text{CO}_2 - \text{Cl}]^+$ ($371.212 \text{ } m/z$), and $[M - \text{CO}_2 - \text{Cl}]^+$ ($443.233 \text{ } m/z$), respectively. The mass resolutions have been calculated for each peak as shown in Figure 5.27 (a). The highest mass resolution obtained so far can reach more than $8,000 \text{ } m/\Delta m$ (around $343 \text{ } m/z$), which is actually higher than that ($6,600 \text{ } m/\Delta m$) predicted by SIMION if a 5 ns detector response time is assumed. This suggests that 5 ns is an overestimation of the detector response time. In conclusion, adding a reflectron to the MSI instrument tripled its mass resolving power while maintaining the spatial imaging capability, which significantly increases the applicability of the instrument especially in the analysis of complex biological samples.

5.5 Discussion

As discussed in Chapter 1, one of the major challenges facing microscope MSI is low mass resolution. The experimental results shown above indicate that incorporating a reflectron

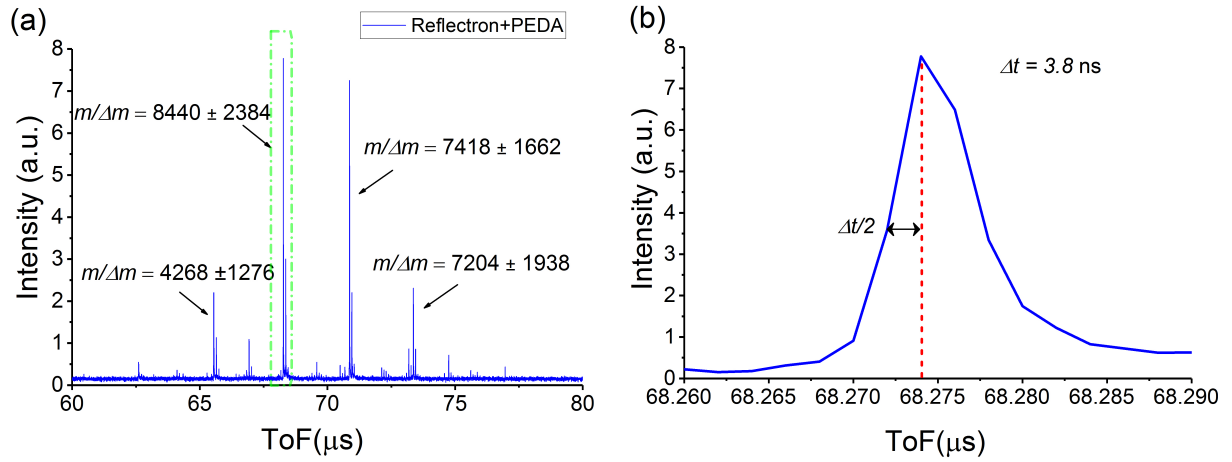


Figure 5.27: (a) A ToF spectrum obtained with the reflectron MSI instrument. The optimum mass resolution is more than 8,000 $m/\Delta m$ around 343 m/z . (b) An enlarged view of the ToF peak in the dashed box in (a). The estimated FWHM (Δt) of the ToF peak is 3.8 ns.

into the PEDDA MSI instrument will meet the goal of improving its mass resolving power without sacrificing its high space resolving power (the mass resolution increases from less than 3,000 $m/\Delta m$ to above 8,000 $m/\Delta m$ and the spatial resolution stays around 20 μm). A high mass resolution will be very useful when dealing with complex biological samples. For example, the $[\text{M}+\text{H}]^+$ ion of methyl-5-acetylsalicylate has a m/z ratio of 195.06519 and the $[\text{M}+\text{H}]^+$ ion of butyl-paraben is 195.10157 Da (M stands for the molecular ions). These two ions appear in the mass spectrum as two adjacent mass peaks which are only 0.036 Da apart. In order to distinguish these two peaks and obtain their respective spatial distributions, the microscope MSI instrument needs a mass resolution above 5,400 $m/\Delta m$. The reflectron MSI instrument is able to perform this task while the PEDDA instrument is not.

Although the preliminary data is very encouraging, there is still room for further improvement. There are two possible sources that cause the ion image distortion in Figure 5.26(a). The first is that since the laser beam illuminates the ITO plate at a small angle, the ion plume generated from the sample surface may be emitted in a direction that lean away from the incident laser beam rather than along the surface normal. This transverse velocity component becomes dominant when the ions are turning around at

the end of the reflectron (where the velocity along the ToF axis is zero). One way to solve this problem is to perpendicularly direct the laser beam onto the ITO plate from behind the sample. The second is the deflector. Simulations show that if the deflector rotates a few degrees around the ToF axis (i.e. the two deflector plates are not vertical), the simulated images will be distorted in a similar way as in Figure 5.26(a). This theory can be easily tested experimentally by adjusting the deflector orientation in the future.

With a 25 mm diameter detector, the field of view of the current instrument is about $390 \times 390 \mu\text{m}^2$. The field of view can be enlarged by using a larger detector, but it is still limited by the opening of the reflectron rings. To have more control over the magnification, an einzel lens assembly, which is able to converge the ion beam passing through it, can be added in front of the reflectron. The first eight rings of the reflectron stack are not connected in the resistor chain, so they are “independent” and could be used as an einzel lens in future experiments.

Compared to the commercial TRIFT-II ToF-SIMS that is commonly used in microscope MSI experiments [64, 77, 91–93] (more details in Chapter 1), the reflectron MSI instrument is much simpler to build and has a comparable mass and space resolving power (the mass and spatial resolutions of the TRIFT II instrument are reported to be $>9,000 m/\Delta m$ [168] and $\approx 4 \mu\text{m}$ [77], respectively.) Moreover, the reflectron instrument reported here can work in the velocity-map imaging mode in a similar way as described in ref. [151]. So, in the cases where ion fragments of similar m/z are created following photon absorption, their respective velocity-resolved images can be obtained without any interference from other species. It is worth noting that the mass resolution of the reflectron instrument reported here is 20 times higher than the one reported in ref. [151].

5.6 Summary

The axial (ToF) velocity spread of ions desorbed from a sample surface sets an intrinsic limit on the resolving power of a linear MALDI ToF-MS. This limit can be overcome by

the use of a reflectron, a relatively long ion mirror. The underlying principle is rather straightforward to understand. An ion of higher velocity/kinetic energy goes into the mirror further and thus spends longer time within the reflectron area. With a proper adjustment of the reflectron voltage, this may compensate for the less time the ion spends in the field free drift. Therefore, the reflectron is a post-source focusing method while techniques such as PEDA and Delayed Extraction are in-source focusing methods, all of which lead to improved resolution.

Ion motion in the homogeneous fields of a gridded reflectron can be handled mathematically in an analytical way. By correctly adjusting the reflecting field strength of a single stage reflectron, the flight time broadening due to kinetic energy distribution of the ions can be eliminated to the first order of the Taylor expansion of $t(U)$. Two-stage reflectrons and parabolic reflectrons have also been developed to achieve higher order focusing.

Reflectrons can be implemented into microscope mode MSI instrument to improve their mass resolution while maintaining a good spatial resolution. However, simulations suggest that the bent electric fields surrounding the micro-openings of the grids in conventional two-stage reflectrons would decrease both mass and spatial resolution dramatically. SIMION also demonstrates that one-stage gridded or gridless reflectrons are more suitable for an MSI system. In addition, by adding a deflector between the ion source and the reflectron, the broadening of the ion beam due to the reflectron can be compensated effectively, leading to an improvement of spatial resolution.

A reflectron MSI was constructed using a gridless design and characterised. The electrostatic field inside the reflectron was initially distorted by an unknown external stray field. As a solution, all reflectron rings were replaced with thicker versions, which effectively shielded any external stray fields. The instrument achieved the predicted mass and spatial resolutions after this replacement.

Preliminary experimental results demonstrated that the reflectron system can provide a mass resolution above $8,000\ m/\Delta m$ with a spatial resolution around $20\ \mu\text{m}$. More

Instrument	Optimal mass resolution ($m/\Delta m$)	Mass range where $m/\Delta m \leq 3,000$	Optimal spatial resolution (μm)
PEDA + Reflectron	$\approx 8,000$	350-435 Da	≈ 20
Time-variable PEDA + Reflectron	$\geq 6,000$	300-700 Da	≈ 20

Table 5.6: Comparison of the PEDA + reflectron and time-variable PEDA + reflectron methods, as incorporated in the time-of-flight MS described in Section 3.1.

experiments are needed to fully characterise the instrument, as will be described in the concluding Chapter. The performance of the reflectron ion microscope is summarised in Table 5.6.

Chapter 6

Analysing Mass Spectra with Machine Learning Algorithms

6.1 Introduction

A fundamental purpose of mass spectrometry is to identify unknown analytes in the presence of other chemical constituents by measuring its mass-to-charge ratio. The performance of a mass spectrometer is therefore gauged by its mass resolution and mass accuracy. As introduced earlier in Chapter 3, mass resolution gauges the capability of a mass spectrometer to discriminate two peaks of slightly different m/z ratios in a mass spectrum. High mass resolution is required to isolate a mass peak of interest from other closely adjacent peaks, otherwise an informative peak could be convoluted with by other neighboring peaks. Adding a reflectron into our microscope mode MSI system proved to be an effective way to improve its mass resolution. Moreover, variants of the Post-Extraction Differential Acceleration (PEDA) method have been introduced that deal with the mass-dependence of mass resolution, considerably expand the m/z range of our system where good mass resolution can be obtained. However, topics regarding mass accuracy have not yet been given equal attention. A comparison between the performance of a mainstream commercial MS system and the microscope mode MSI system reported here is shown in Table 6.1. Unfortunately, commercial non-image time-of-flight mass spectrometer (ToF-MS) instruments significantly outperform the home-built instrument in mass resolution and mass accuracy. There are two reasons why the mass accuracy of our system is not as

good: the first is that poor mass resolution results in poor mass accuracy. This is because with poor mass resolution, neighbouring ToF peaks may be merged together, which makes it impossible to assign a correct m/z value to each individual ToF peak. Figure 6.1 shows a low resolution ($\approx 180 m/\Delta m$) ToF spectrum of the parent ion of crystal violet molecule ($m/z \approx 372$), points 1, 2, and 3 are all candidates when determining the ToF value corresponding to 372 Da, which would certainly lead to a less rigorous calibration equation (a function that converts a ToF spectrum into a mass spectrum) and thus lower mass accuracy. The second reason is that PEDTA methods introduce considerable non-linearity into their calibration functions. Although one may generate a large number of reference ToF peaks with known m/z values and then use a high order polynomial function to fit the underlying calibration function as closely as possible, it is still very unlikely that the mass accuracy can be improved to the 5 ppm level of a commercial instrument.

	MaLinearss resolution ($m/\Delta m$)	Mass accuracy (ppm)
Commercial MS system	$> 100,000$	< 5
Microscope MSI system	$< 10,000$	$> 1,000$

Table 6.1: Comparison of mass resolution and mass accuracy between a commercially available mass spectrometer and our homebuilt mass resolved ion microscopy system. Mass accuracy of the microscope mode MSI system were estimated using the linear PEDTA instrument in ref. [169].)

The consequence of poor mass accuracy is that it would be impossible to identify the chemical formula of an unknown compound purely based on its m/z . As shown in Figure 6.2, to confirm the molecular formula of a small organic molecule (up to 400 Da) by m/z alone, the mass accuracy has to be within 0.5 ppm. Otherwise, the number of possible formulae explodes with increasing uncertainty of actual m/z . Also minor mass changes cannot be detected if the mass accuracy isn't sufficient, *e.g.*, the substitution of one amino acid for another, or a post-translational modification. So with mass accuracy around

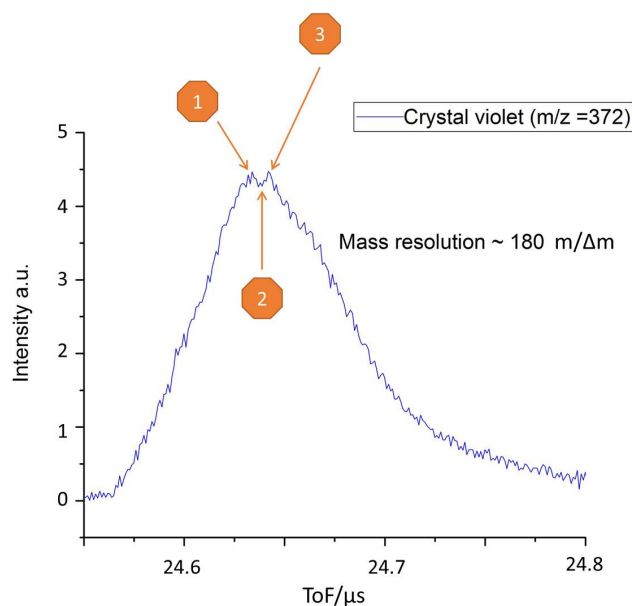


Figure 6.1: A low resolution ToF spectrum of the parent ion of Crystal Violet molecule, 1, 2, and 3 can all be the ToF value that corresponds to the parent ion ($372\ m/z$)

1,000 ppm or 0.1%, our system may seem barely satisfactory at first glance, particularly in the context of analysing complex biological samples. Actually an accuracy of 5 ppm is required for publication in a chemical journal. However, the rest of this chapter will show how machine learning techniques can improve the instrument at practical applications, making it useful even with very poor mass accuracy.

6.2 Background

The concept of machine learning and some common algorithms to implement it will be discussed in this section along with a review of previous works that used machine learning methods to analyse biological mass spectra.

6.2.1 Machine learning

As discussed previously in Chapter 1 time-stamping cameras are able to record the 2D position and occurrence time of an event with nanosecond precision. By coupling the PImMS camera with our microscope MSI instrument, ions generated from a certain point

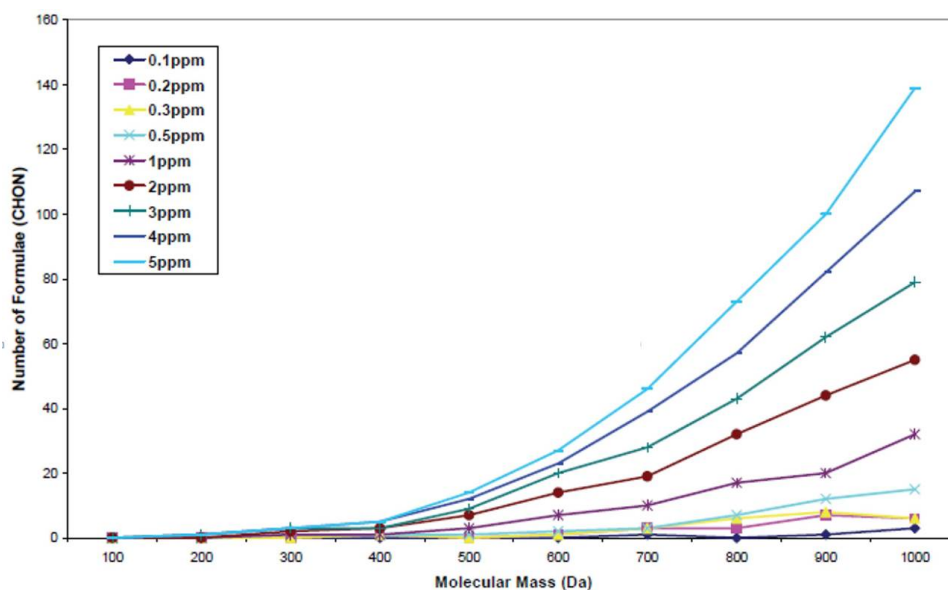


Figure 6.2: The number of possible compound formula as a function of molecular mass for a range of different mass accuracy (0.1 to 5 ppm). For small organic molecules (mass up to 400 Da), a mass accuracy within 0.5 ppm is sufficient to confirm their molecular formulae from mass alone. Adapted from a slide of the Graduate lecture course in Mass Spectrometry provided by Mass Spectrometry Research Facility, University of Oxford.

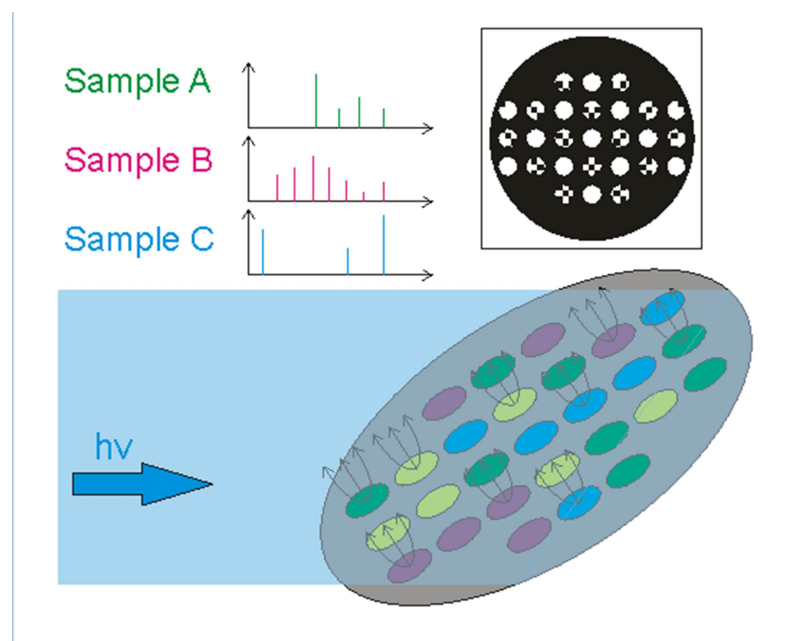


Figure 6.3: Future application: the parallel analysis of bio-array containing multiple biological samples (*e.g.* saliva, blood, urine, and so on). Reprinted from ref. [106].

on the sample plate are projected onto a corresponding point on the camera sensor. Every single pixel or a cluster of neighboring pixels can effectively therefore acts as an independent ToF-MS for a small area of the sample surface. An important future application

of this is the parallel analysis of bio-arrays containing multiple biological samples (*e.g.*, saliva, blood, urine, and so on) as shown in Figure 6.3. For instance, a mass spectrometer may work as a diagnostic tool that detects changes in the chemical composition of blood or other body fluids due to certain disease. The high throughput feature of our system makes it ideal for scenarios where people have to deal with heavy workloads, such as hospitals. If mass spectra are used as a clinical diagnostic solution, it actually becomes a classification problem rather than an identification problem. Classification relies on recognising the patterns or signatures of mass spectra. For example, Figure 6.4 (a) and (b) are the ToF spectra of two types of dye molecules: crystal violet and rhodamine B. Their fragment patterns differ in the number of peaks, their relative intensities, whether there are minor peaks in the middle of two adjacent major peaks, and so on. One can classify the ToF spectra of these two dyes by simply observing the fragment patterns. Therefore in a classification problem, it is not necessary to convert ToF spectra into mass spectra, let alone determine the identity of the fragment ions. However, compared with the mass spectra of relatively pure molecules, those of biological samples are still harder to deal with. The intrinsic complexity, diversity, low reproducibility, and high noise of biological mass spectra all put a question mark over the accuracy and robustness of pattern-based classification methods.

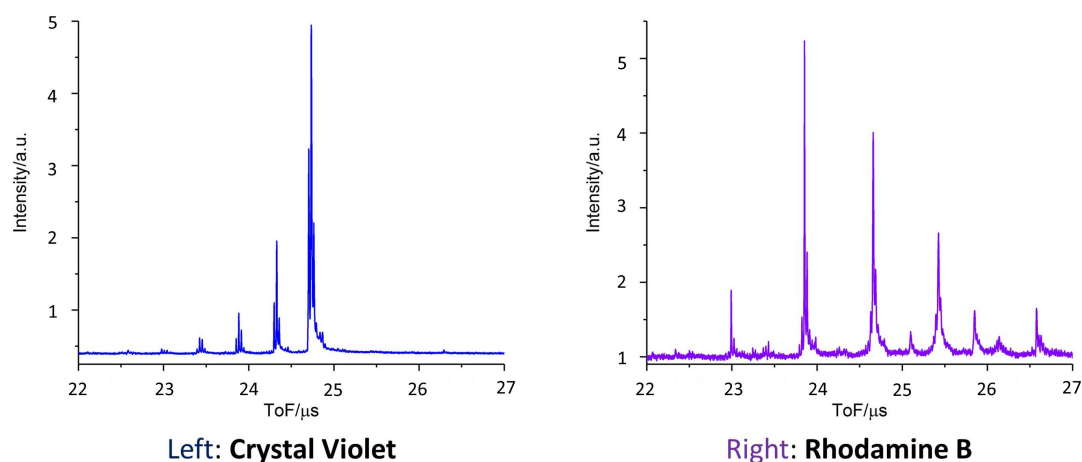


Figure 6.4: The Time-of-Flight spectra of two types of dye molecules: Crystal Violet on the left and Rhodamine B on the right.

Machine Learning algorithms are good at automatically discerning patterns and regularities in complex datasets. In recent years, there have been many amazing technological breakthroughs that encompass all aspects of everyday life, ranging from highly accurate email spam filters to convincing language translation. All of these are powered by rapidly progressing machine learning techniques. In the fields of bio-informatics, pattern analysis of multiple biomarkers has aroused increasing attention as a potential alternative tool for clinical diagnoses, such as early detection of cancer using blood samples [170]. A biomarker is a measurable indicator (mostly biological molecules such as proteins or lipids) that reflects the progress or status of certain diseases. Coupling mass spectrometry with machine learning algorithms offers the potential to perform a comprehensive analysis of complex biological/chemical mixtures rapidly and precisely while extracting valuable information from it. Motivated by such prospects, researchers extensively studied various types of cancer, using MS as a diagnostic tool or as a cancer biomarker discovery platform. Cancers that have already been investigated include ovarian [171–174], prostate [175–177], breast [178, 179], bladder [180, 181], lung [182–185], liver [186], pancreatic [187, 188], renal cell carcinoma [189], colorectal [190], and astroglial tumour [191]. Exceptional sensitivities and specificities have been reported in these studies (both over 80%), which are superior to conventional methods [170]. Although there are concerns questioning the reliability of these results and the feasibility of this method [192, 193], it is still clear that the combination of mass spectrometry and machine learning is one of the most promising new approaches for future clinical diagnostics.

Machine learning is concept proposed at the dawn of the computer age. Arthur Samuel provided a definition to machine learning in 1959: “Machine Learning is the field of study that gives computers the ability to learn without being explicitly programmed.” Tom Mitchell gave a well-known, more formal definition in 1997: “a computer program is said to learn from experience E with respect to some task T and some performance measure P , if its performance on T , as measured by P , improves with experience E .” In the case of diagnosis using mass spectrometry, the task T is to correctly classify whether a mass

spectrum belongs to a sick or healthy patient, the performance measure P is the sensitivity and specificity of the classification, and the experience E is a set of mass spectra. The program can learn the bio-marker pattern of a disease by inspecting more and more mass spectra that have been labeled as positive or negative beforehand with human supervision (so called “supervised learning”). These examples are often called training instances or samples.

Machine learning works in the following way:

Suppose a machine learning model is asked to predict whether a digital image is a cat (as shown in Figure 6.5). The first step is to convert this task into a mathematical problem. In this case, the image comprises an array of pixels and each pixel stores three intensity values for the red, green, and blue channels respectively. As illustrated in Figure 6.5, a color image therefore corresponds to three 2D matrices. In the second step, these three matrices are reshaped and connected to form a single vector ($\mathbf{x}$). Elements in $\mathbf{x}$ are called “features” in machine learning terminology, and their number is equal to three times the number of pixels. This image vector $\mathbf{x}$ is fed into a function f , if the output of $f(\mathbf{x})$ is larger than certain threshold (zero for instance), then the image is classified as a cat; if $f(\mathbf{x})$ is smaller than the threshold, the image is classified as not a cat. Supervised machine learning can be best understood as a process during which a desired function f is constructed that maps an input vector $\mathbf{x}$ to an output variable y .

Figure 6.6 shows a simplified case of the classification problem, which illustrates how the function f is determined by training instances. For easy visualisation the image vectors have only two features (x_1, x_2). All “non-cat” instances are plotted as red crosses and “cat” as blue circles. The instances belonging to the two different categories can be separated by the dashed straight line, which is determined by the distribution of instances in the feature space (in this case a two dimension space). Therefore, the formula of this straight line $f(x_1, x_2)$ is a “decision boundary” that partitions the underlying feature space into two sets, one for each class. The value of the threshold is zero. So if a new image vector is located at the green star in this feature space, its output of $f(x_1, x_2)$ will

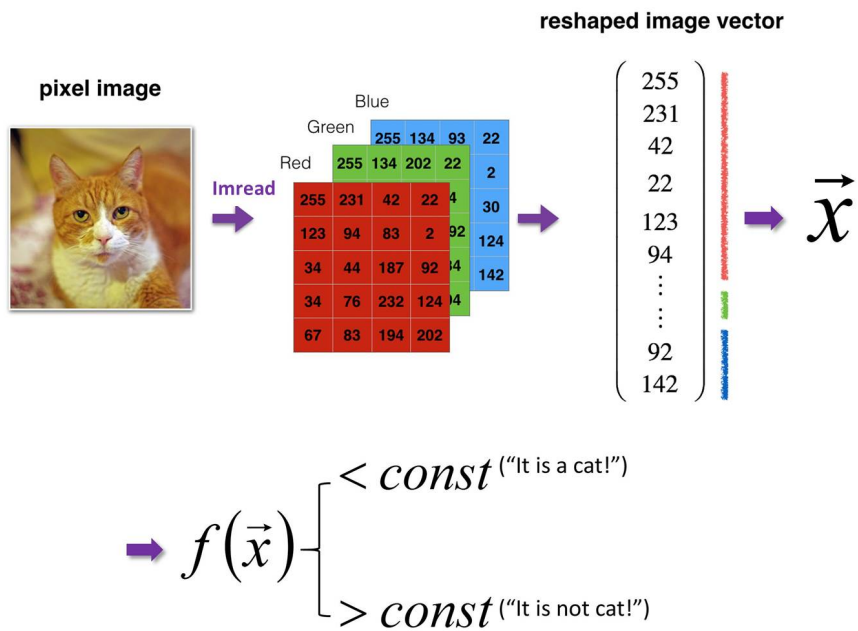


Figure 6.5: Illustration of the machine learning model. Adapted from Coursera.

be more than zero, and it will be classified as a “cat”. Of course, in reality each image may contain thousands of pixels which correspond to 3 times more features (each pixel corresponds to three RGB values), therefore the feature space has many dimensions. In addition, instances belonging to different categories may not be separable by a straight line or other simple decision boundaries, which means the function f could be intimidatingly complex.

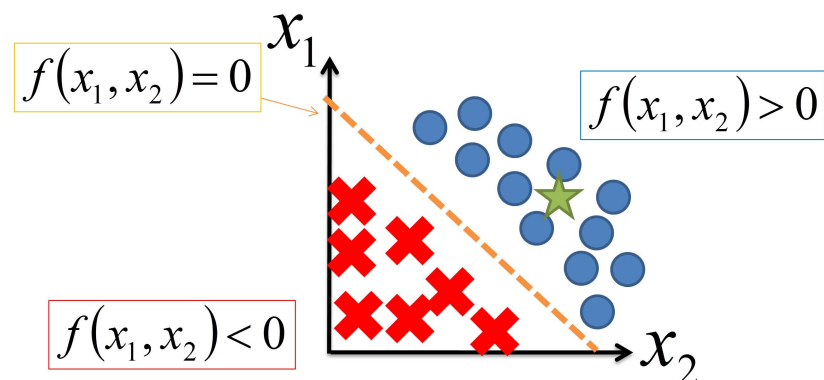


Figure 6.6: A simplified illustration of how to determining model $f(\mathbf{x})$ with training instances.

In summary building a predictive model means finding an appropriate function f . Generally speaking, machine learning algorithms offers us the tools to find such functions.

6.2.2 Application of machine learning algorithms in mass spectra analysis

Soft ionisation methods, such as MALDI or SELDI (Surface-enhanced laser desorption/ionization), are able to generate proteomic profiles of biological samples (tissue cells, blood, saliva, urine, or other body fluids) quickly and with high sensitivity and robustness. Batch analysis of large sets of samples in high-throughput proteomics is therefore possible. Because of this, the use of machine learning techniques to analyse large proteomic mass spectra datasets has started to attract attention in the early 2000s [175, 176, 191, 194]. Based on the belief that proteomic spectra can reflect pathological changes, the goal of such studies was to extract an optimal discriminatory proteomic pattern from known samples, and then to exploit this pattern to predict the status of unknown samples. The potential applications include biomarker discovery and automatic diagnosis.

In 2002, B. L. Adam and coworkers demonstrated for the first time the potential of SELDI mass spectra to be used in the early detection and diagnosis of prostate cancer [175]. SELDI protein profiles of serum from 167 prostate cancer patients, 77 patients with benign prostate hyperplasia, and 82 age-matched unaffected healthy men were used to train and develop a decision tree classification algorithm that used a nine-protein mass pattern that correctly classified 96% of the samples.

In 2003, the performance of common machine learning algorithms was compared with regard to the classification of ovarian cancer based on serum mass spectra by B. Wu and coworkers [195]. The article concluded that random forest (RF) lead to the best classification of MS samples.

Following this, three SELDI mass spectrum datasets of serums were used to identify proteomic patterns that distinguish the serum of ovarian cancer cases from non-cancer controls [196]. In this study the support vector machine (SVM) algorithm was used

to build a classifier and the performance of two feature selection approaches, statistical testing and genetic algorithm-based methods, were compared.

In 2008, G. Ge *et al.* [197] used a pancreatic cancer proteomics dataset to compare the prediction performances of a single decision tree algorithm with six different decision-tree based classifier ensembles (Random forest, Stacked generalisation, Bagging, Adaboost, Logitboost and Multiboost). It was shown that ensemble classifiers generally outperform single decision tree classifier in having lower misclassification rate.

A. Kong *et al.* later presented an innovative algorithm to automatically discover easily interpretable biomarker signatures [198]. This signature discovery algorithm was validated on one new colorectal cancer MALDI-TOF data set, and two widely used ovarian cancer SELDI-TOF data sets.

In 2016, a serum mass profile-based classifier was developed to identify patients with early lung cancer [199]. MALDI-ToF mass spectrometry was used to characterise the ‘low-molecular-weight’ (1,000–14,000 Da) serum fraction of a group of 95 patients with early stage lung cancer and 285 healthy controls. The cases were split into two independent cohorts for signature/pattern discovery and validation, respectively, and analysed separately six months apart by different spectrometrists, but keeping the same protocols and spectrometer settings. Several molecular components of serum (putatively components of endogenous peptidome) with discriminating power were determined in the discovery cohort, which allowed an effective cancer classifier to be built using a weighted multivariable logistic regression algorithm. For the discovery cohort, the classifier has an area under the curve (AUC) of 0.88, a negative predictive value (number of true negatives/number of negative calls) of 100%, and a positive predictive value (number of true positives/number of positive calls) of 48%. However, the classifier significantly underperformed in the validation cohort (AUC 0.73, negative predictive value 88% and positive predictive value 30%).

Recently, metabolite profiles of serum samples were obtained by gas chromatography-mass spectrometry (GC-MS) to study osteosarcoma tumours [200]. Three classification

methods, logistic regression, SVM with SMO solver, and RF, were applied to classify metabolomic data for a healthy control group and for patients with benign tumours or osteosarcoma. All three classifiers were capable of distinguishing between controls and tumour cases, but RF appeared to perform better than the other two in accuracy.

Machine learning-based workflows for the analysis of mass spectrometry datasets are reviewed in ref. [201]. The literature on developing clinical decision support systems for cancer screening based on proteomic patterns of blood mass spectrum are also reviewed in ref. [170] from a machine learning perspective. Ref. [202] provides a brief overview of how machine learning methods have been used to interpret mass spectrometry imaging (MSI) data.

6.2.3 Learning Algorithms

There are several ways to categorise machine learning algorithms, based on different criteria. For example, an algorithm can either be a supervised learning or an unsupervised one depending on whether its training samples are human labeled or not; it can also be characterised as instance-based learning or model-based learning depending on whether it works by simply comparing unseen instances to known ones, or instead recognises underlying patterns in the training instances and generates a predictive model. Most of the algorithms used in this thesis are supervised, model-based learning. Each algorithm is provided with examples of mass spectra as well as their associated class membership (labeled as positive or negative), and the goal is to build a classifier model capable of predicting the class membership of an unseen mass spectra. In the field of biological mass spectra classification, various algorithms have been studied previously, including artificial neural networks (ANN) [184, 186, 191, 203–205], K nearest neighbour (KNN) [174, 184, 204, 206, 207], logistic regression [179, 196, 208–210], decision trees [175, 178, 184, 189, 204, 209, 211, 212], linear or quadratic discriminant analysis (LDA/QDA) [182, 195, 206, 207, 209, 213–215], support vector machines (SVMs) [194, 195, 204, 206], kernel matching pursuit (KMP) [216], logical analysis of data (LAD) [171], stepwise discriminant analysis [217], partial least

square projection [218, 219], naive Bayes [184], rule induction [184], and ensemble algorithms [195, 220–222].

In machine learning, there is also a so called “No Free Lunch” theorem, which states that no one algorithm is best for all cases. This holds particularly true for supervised learning. So in order to build an appropriate model, one needs to use the right training algorithm and therefore it is worth having an in-depth understanding of how the machine learning algorithms actually work, rather than using them blindly. Moreover, one has to tune the so-called hyperparameters of a model to optimise its performance. An algorithm’s hyperparameters are those whose values are set before the learning process begins and will not be improved during learning. Different model training algorithms require different hyperparameters and choosing a good set of hyperparameters for a task also relies on one’s understanding of the algorithms. In addition, a good knowledge of the algorithms may lead to more efficient debugging and error analysis. Therefore, in this section a few representative machine learning algorithms will be briefly introduced.

6.2.3.1 Logistic Regression

For classification problems, the simplest yet most common machine learning algorithm is called “Logistic Regression”. It was developed by statistician David Cox in 1958 [223, 224], and was borrowed by the machine learning community and extensively used to estimate the probability that an instance belongs to a particular class. A Logistic Regression computes the estimated probability in two steps. In the first step, a weighted sum of the input features is computed, plus a constant called bias or intercept term, θ_0 :

$$\begin{aligned} \text{Sum} &= \theta_0 + \theta_1 x_1 + \theta_2 x_2 + \dots + \theta_n x_n \\ &= \boldsymbol{\theta}^T \cdot \mathbf{x}, \end{aligned} \tag{6.1}$$

where n is the number of features (in our case it is the number of Time-of-Flight or m/z bins), x_i is the i^{th} feature value (in our case it corresponds to the abundance of ions in

the i^{th} ToF or m/z bin), θ_j is the j^{th} model parameter (including the bias term θ_0 and the feature weights $\theta_1, \theta_2, \dots, \theta_n$). The second row on the right-hand side of the equal-sign is the vectorised form of the weighted sum, $\boldsymbol{\theta}$ is the model's parameter vector, and $\boldsymbol{\theta}^T$ is the transpose of $\boldsymbol{\theta}$ which converts a column vector into a row vector:

$$\boldsymbol{\theta}^T = \begin{bmatrix} \theta_0 \\ \theta_1 \\ \theta_2 \\ \dots \\ \theta_n \end{bmatrix}^T = \begin{bmatrix} \theta_0, \theta_1, \theta_2, \dots, \theta_n \end{bmatrix}, \quad (6.2)$$

$\mathbf{x}$ is the example/instance's feature vector, containing x_0 to x_n , with x_0 always equal to 1:

$$\mathbf{x} = \begin{bmatrix} x_0 \\ x_1 \\ x_2 \\ \dots \\ x_n \end{bmatrix}. \quad (6.3)$$

In the second step, the logistic function $\sigma(\boldsymbol{\theta}^T \cdot \mathbf{x})$ of the weighted sum is computed

$$\begin{aligned} \hat{p} &= \sigma(\boldsymbol{\theta}^T \cdot \mathbf{x}) \\ &= \frac{1}{1 + e^{-\boldsymbol{\theta}^T \cdot \mathbf{x}}} \end{aligned} \quad (6.4)$$

to determine the probability $\hat{p}$ that $\mathbf{x}$ belongs to the positive class. The logistic function is a sigmoid that always outputs a number between 0 and 1 regardless of the variable passed onto it. As a binary classifier, an instance is predicted as a positive class ("1") simply when its estimated probability is greater than 0.5 or 50%, otherwise it is predicted as negative ("0").

To train the Logistic Regression model, an appropriate parameter vector $\boldsymbol{\theta}$ for the model must be determined. Ideally the model should estimate high probabilities $\hat{p} \approx 1$ for instances labeled as “positive” ($y = 1$) and low probabilities $\hat{p} \approx 0$ for instances labeled as “negative” ($y = 0$). A “cost function” is defined to evaluate how far away a machine learning model’s predictions are from the targets over the entire training dataset:

$$J(\boldsymbol{\theta}) = -\frac{1}{m} \sum_{i=1}^m [y^{(i)} \log(\hat{p}^{(i)}) + (1 - y^{(i)}) \log(1 - \hat{p}^{(i)})]. \quad (6.5)$$

It can be easily verified that this cost function makes sense: for the i^{th} instance, if $y^{(i)} = 1$, then the i^{th} item of Equation (6.5) will be large if the model produces a $\hat{p}^{(i)}$ close to 0 and will approach 0 if $\hat{p}^{(i)}$ is close to 1, and vice versa. So the smaller $J(\boldsymbol{\theta})$ is, the more accurate the model is. For a particular training set, the cost function is a function of the parameter vector $\boldsymbol{\theta}$. The best $\boldsymbol{\theta}$ should lead to the global minimum of the cost function.

Gradient Descent is an iterative optimisation algorithm that is good at finding this $\boldsymbol{\theta}$. It is generic and straightforward to implement: gradient descent approaches a local minimum of a function by taking steps proportional to the negative gradient of the function at the current point (as shown in Figure 6.7). Once the gradient becomes zero, the function reaches its minimum. For the Logistic Regression model described above, the gradient along the j^{th} model parameter θ_j is the partial derivative of the cost function with respect to θ_j :

$$\frac{\partial}{\partial \theta_j} J(\boldsymbol{\theta}) = \frac{1}{m} \sum_{i=1}^m (\sigma(\boldsymbol{\theta}^T \cdot \mathbf{x}^{(i)}) - y^{(i)}) x_j^{(i)}. \quad (6.6)$$

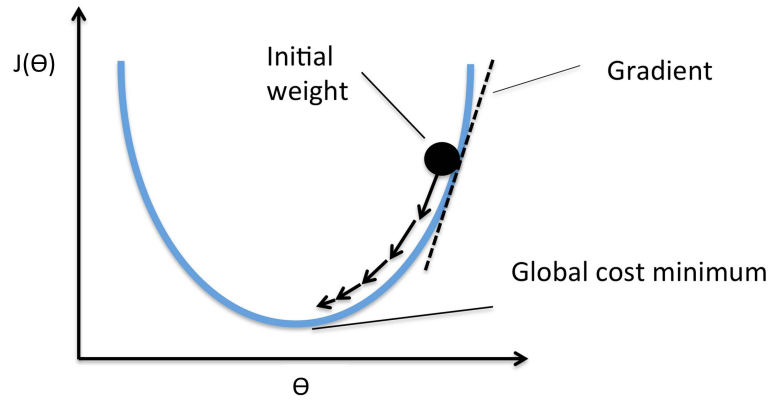


Figure 6.7: Illustration of the gradient descent method, which minimises the cost function by updating parameters in a correct direction during each iteration. Reprinted from Coursera.

Then the entire parameter vector θ is then updated using the following equation:

$$\begin{aligned}
 \theta^{(\text{next step})} &= \theta - \eta \nabla_{\theta} J(\theta) \\
 &= \begin{pmatrix} \theta_0 - \frac{\partial}{\partial \theta_0} J(\theta) \\ \theta_1 - \frac{\partial}{\partial \theta_1} J(\theta) \\ \dots \\ \theta_j - \frac{\partial}{\partial \theta_j} J(\theta) \\ \dots \\ \theta_n - \frac{\partial}{\partial \theta_n} J(\theta), \end{pmatrix} \quad (6.7)
 \end{aligned}$$

where η is called “the learning rate”. It is a hyperparameter of the gradient descent algorithm that controls how much the parameter vector θ changes with each update. This process is repeated until the value of the cost function $J(\theta)$ is zero or close to zero. The Logistic Regression model is therefore improved iteratively. As standard Logistic Regression models like the one described above are typical linear classifiers, they are only useful when working with a dataset where the classes are “linearly separable.” To handle a dataset with more complex underlying pattern (*i.e.* nonlinearly separable), more powerful tools are needed, such as Artificial Neural Networks.

6.2.3.2 Artificial Neural Network

An Artificial Neural Network (ANN) [225] is a biologically inspired machine learning model constituted by a large amount of single nodes or artificial neurons, which are connected with weights to form a network structure. It was first introduced in 1943 [226] and has gone through several cycles of popularity until it recently proved useful by tackling complicated Machine Learning tasks, such as computer vision (*e.g.*, Google Images), Natural Language Processing (*e.g.*, Apples Siri) and Personalised Recommendation (*e.g.*, YouTube). A representative node is shown in Figure 6.8 (a) and a typical ANN that constructed by such artificial neurons is illustrated in Figure 6.8 (b). Nodes are the building blocks of the ANN, and usually convert an input vector into a single output. As shown in Figure 6.8 (a), each element of the input vector plus a bias item is multiplied by its corresponding connection weight and summed. This weighted sum then passes through a so called activation function to generate the output for the neuron. There are three widely used types of activation function: the sigmoid function, the hyperbolic tangent function [227], and the ReLU function [228]. If the sigmoid function is used as the activation function, then the node has the same formula as a Logistic Regression model. Therefore, one can see an ANN as a combination of a large number of simple Logistic Regression models. As shown in Figure 6.8 (b), an ANN consists of three types of layers, an input layer which corresponds to the training instances, one or more hidden layers which are responsible for the pattern recognition tasks, and a final output layer which produces the estimated probability for the positive class. For every node in the hidden layers, the input vector is the output vector of the last layer and its output is fed to the nodes of the next layer. This architecture is called the feedforward neural network, which was the earliest and simplest type of ANN devised. It has been shown that an ANN with just one hidden layer can model even the most complex functions or decision boundaries provided it has enough neurons and the target function is continuous.

Training an ANN means finding an optimal set of connection weights that minimises the cost function. The cost function itself has the same form as the Logistic Regression

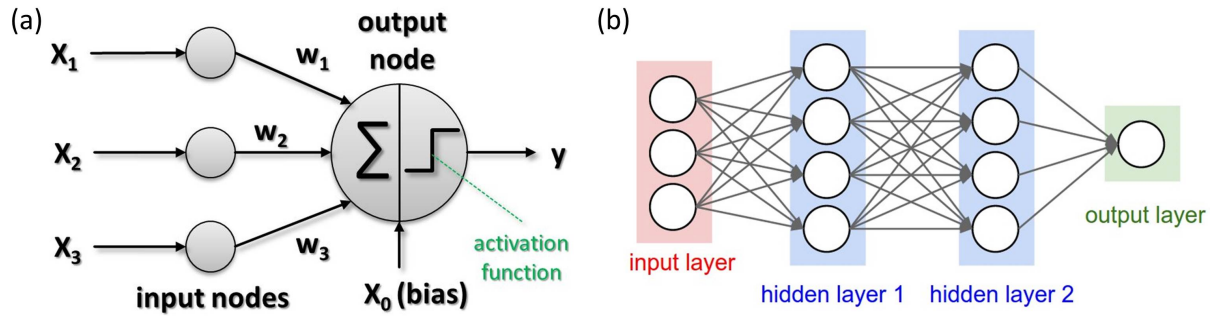


Figure 6.8: (a) Illustration of an artificial neuron or node (b) Architecture of an Artificial Neural Network. Adapted from Wikipedia and Coursera.

model, but the the minimisation process is slightly more complicated since many more parameters are involved. In 1986, D. E. Rumelhart *et al.* proposed the backpropagation training algorithm [229]. In this method the inputs and desired outputs of the problem to be modeled are entered into an ANN. The nodes then process the inputs and run the aforementioned feedforward process (forward pass). The outputs of the network are computed based on the values that reach the last layer. Comparing these outputs with the desired ones, any difference or error is used as a guide to tune all the connection weights, working backwards through the network (reverse pass). Finally the connection weights are updated to reduce the error (Gradient Descent step). This feedforward and back propagation process keeps iterating until the network has learned a reasonably good projection between input vectors and desired output vectors. The weights produced are then determined and the resulting network is ready to be employed for its designed purpose.

6.2.3.3 Decision Tree and Random Forest

Decision tree is one of the simplest supervised learning algorithms, but it is surprisingly powerful. Figure 6.9 describes how a decision tree makes predictions. Suppose one has a mass spectrum and would like to find out its class membership (positive or negative). The first thing to do is to look at the root node (depth 0, at the top), which in this case asks whether the value in the input vector's 1676th feature is smaller than a threshold, 0.507. Here each feature corresponds to a m/z and the input vector's feature values correspond

to the abundance of ions. The nodes “gini” attribute is a metric of its impurity:

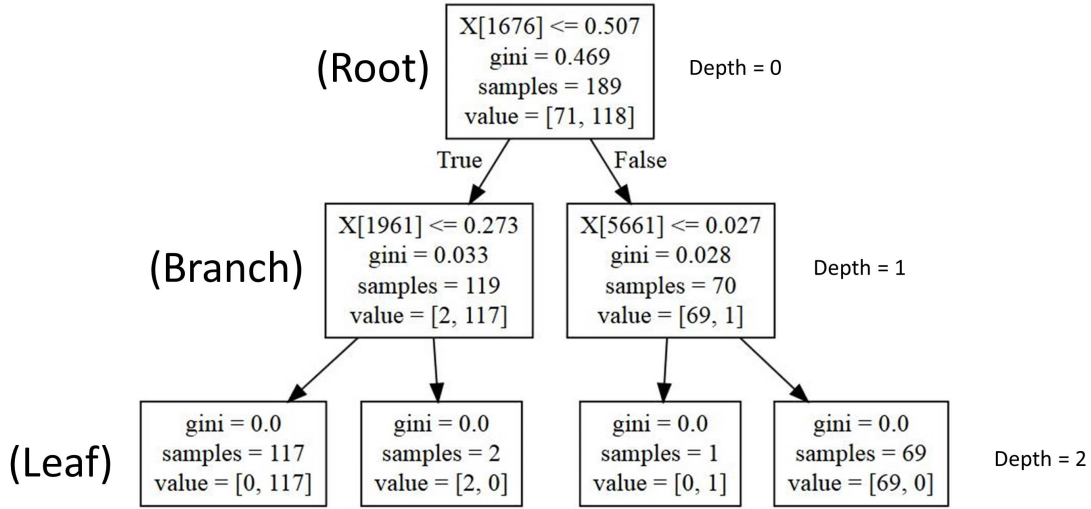


Figure 6.9: The structure of a Decision Tree.

$$G_i = 1 - \sum_{k=1}^n r_{i,k}^2, \quad (6.8)$$

where $r_{i,k}$ is the ratio of class k instances among the training instances in the i^{th} node. So a node is pure ($G_i=0$) if all training instances it applies to belong to the same class. The samples attribute in Figure 6.9 displays the number of instances that have come to this node and the value attribute shows how these instances are distributed between the positive and negative classes (in this case 71 are labeled as positive, 118 as negative). If the value in the 1676th feature is smaller than the threshold, then it moves down to the left branch node of and root (depth 1, left), which checks whether the value in the input vector's 1961th feature is smaller than a threshold 0.273. If this is true, then the instance ends up at the leaf node (a node has no further split) on the left (depth 2) and is eventually classified as negative.

Building a decision tree is simple. At the root node the algorithm first splits the training set in two subsets using the k^{th} feature and a threshold t_k . Then it chooses the pair of k and t_k that generates the purest subsets (weighted by their size). The cost

function $J(k, t_k)$ to minimise for a decision tree model has the following form [230]:

$$J(k, t_k) = \frac{m_{\text{left}}}{m} G_{\text{left}} + \frac{m_{\text{right}}}{m} G_{\text{right}}, \quad (6.9)$$

where G_{left} and G_{right} are the gini values for the left and right subsets, m_{left} and m_{right} are the number of instances in the left and right subsets, m are the total number of instances to be grouped, m_{left}/m and m_{right}/m are just weights. Once the training set is divided into two subsets at the root node, it repeats this process at the children nodes until $G_{\text{left}} = G_{\text{right}} = 0$.

The decision Tree model is easy to interpret. Since it works in such a intuitive way, the importance of each feature can be readily assessed. The model selects the most discriminant features at each stage when growing the tree structure, so important features are likely to appear closer to the root of the tree, while unimportant features will often appear closer to the leaves (or not at all). It is therefore possible to rank features based on their contributions to successful classification by computing their average depths. Generating the feature importance for a predictive model will turn out to be very useful in the mass spectra analysis described later.

If a group of Decision Tree models, for instance 200 independent trees, are bundled together and each tree is trained with a different subset of the entire training set, the aggregated prediction of the group often outperforms the best individual model in terms of generalisation (imagine the models work as a “committee” for decision-making). This method is known as “Random Forest” [231], a type of “ensemble learning”. In a Random Forest, if the subset for each tree is sampled from the original whole training set with replacement (each instance could be sampled for a tree more than once), this sampling approach is called “bagging” (short for bootstrap aggregating). Since Random Forest achieve more accurate and stable results than a single Decision Tree, they were used for the data analysis presented here.

6.2.3.4 Support Vector Machine and K Nearest Neighbors

Other learning algorithms are also used in the following analysis, but for the sake of brevity, they will not be introduced in detail and only a short description of their respective unique features will be presented here.

Support Vector Machine (SVM) is a high performance learning algorithm that has been popular for classification problems since the 1990s [232]. SVM is good at selecting a hyperplane line that splits the positive class instance and negative class instance in the high dimensional feature space with largest margin. A margin characterises the distance between the hyperplane (*i.e.* boundary) and the closest data points. So the decision boundary of an SVM classifier separates the two classes and stays as far away from the closest training instances as possible, which is good for generalisation. Another important advantage of SVM is that a mathematical technique called the “kernel trick” [233, 234] can be applied to the algorithm, making it capable of fitting highly complex non-linear functions without actually incurring much more computational complexity.

Unlike all previous learning models, K Nearest Neighbour is an instance-based learning algorithm. Therefore no training process is required and the classification is based on similarity with instances in the training set. For example, to classify a new instance, one just finds out the K training instances at smallest Euclidean distance from the new one in the feature space and simply assigns it to the class most common among its K nearest neighbours.

6.3 Crystal violet vs. non-crystal violet

A proof of concept experiment was performed where machine learning algorithms were used to classify ToF spectra of pure dye molecules. Essential procedures of an end-to-end machine learning project will be provided including data preprocessing, training, fine-tuning, and evaluating a machine learning model. The classification results will also be discussed.

6.3.1 Methods

As described in the last few chapters, throughout this thesis various dye molecules have been used to characterise the spatial imaging mass spectrometer. As a result, hundreds of ToF spectra of different samples were accumulated. All of these spectra were obtained with the same home-built linear microscope mode MSI system as described in Chapter 3 and Chapter 4. But the experiments were not necessarily performed under a same condition (for instance, different laser power or ion optical voltages were used during experiments). In total, 723 ToF spectra were acquired, 259 of them were generated by pure crystal violet samples, the most commonly used dye molecule, and 464 were a collection of ToF spectra generated by different pure molecules that weren't crystal violet such as other dye molecules (Rhodamine B, Auromine O, *etc.*), polymers (Polyethylene glycol 400 and 550, *etc.*), and matrix materials (2,5-dihydroxy benzoic acid, α -Cyano-4-hydroxycinnamic acid, *etc.*). Thus, 269 instances are labeled as "1" and 464 are labeled as "0".

In a ToF spectrum each feature corresponds to the abundance of ions that arrive at the detector after a certain flight time. All ToF spectra used in this analysis were acquired by an oscilloscope, which recorded the PMT signal within 1 ns precision. Consequently, each ToF spectrum has more than 50,000 features. Given limited computational resources, it is advisable to reduce the dimension of feature vectors as much as possible. The first step of dimension reduction is feature selection: only the ToF spectrum between 15 and 35 μ s was retained, which corresponds to 200 – 700 m/z , a mass range that encompasses all informative peaks. The second step is to bin the ToF spectrum: the time span between 15 and 35 μ s was divided equally into 200 intervals, ion abundances within each interval were then summed and stored into the corresponding elements of a new 200-dimension feature vector.

The next step prepares the data for machine learning. In a common machine learning project, data cleaning usually takes the majority of the time. However, in our case this procedure can be basically skipped, because the ToF spectra data are well-structured

and there is no missing features or non-numerical attributes in the dataset. So the only preprocessing job we need to do is to rescale each spectrum with the so called Min-Max normalisation method, where each feature value in an instance i is transformed by the following equation:

$$x_k^{(i)} = \frac{x_k^{(i)} - \min(\mathbf{x}^{(i)})}{\max(\mathbf{x}^{(i)}) - \min(\mathbf{x}^{(i)})}, \quad (6.10)$$

where $x_k^{(i)}$ is the value stored in the k^{th} ($k \in \{1, 200\}$) feature of the i^{th} instance ($i \in \{1, 723\}$), $\mathbf{x}^{(i)}$ is the feature vector of the i^{th} instance, $\min(\mathbf{x}^{(i)})$ and $\max(\mathbf{x}^{(i)})$ represent the minimum and maximum feature values in $\mathbf{x}^{(i)}$ respectively. So after this normalization all feature values in each instance are between 0 and 1. Since we only want the model to discern the pattern of relative abundance of fragment ions in different ToF spectra, no information was lost during this normalization. Moreover, most machine learning algorithms reportedly work better if the range of feature values are relatively small. In order to filter noise, feature values less than a threshold of 0.01 are replaced with zeroes.

It is worth looking at the whole dataset and getting an overview of the problem before starting the learning process. The processed dataset is plotted as a heatmap in Figure 6.10. On the left is the crystal violet dataset, which is essentially a 200×259 matrix. Each column corresponds to one single instance, each row corresponds to a ToF bin (a feature), and each element stores the relative abundance of ions recorded within that ToF bin. Therefore, the cross section along a column is a ToF spectrum. The color scale bar indicates the value of each matrix element, which changes from blue to yellow with increasing value from 0 to 1. On the right is a 200×464 matrix of the non-crystal violet dataset. Compared with the crystal violet dataset, the non-crystal violet one looks much messier. This is because it is a mixture of ToF spectra of multiple types of molecules while the crystal violet dataset are all generated from one molecule.

Taking a closer look at the instances: instance 40 is what a typical crystal violet ToF spectrum looks like; there are a succession of nearly equally spaced peaks which result from the subsequent loss of methyl groups. Instance 14 was also generated by a pure

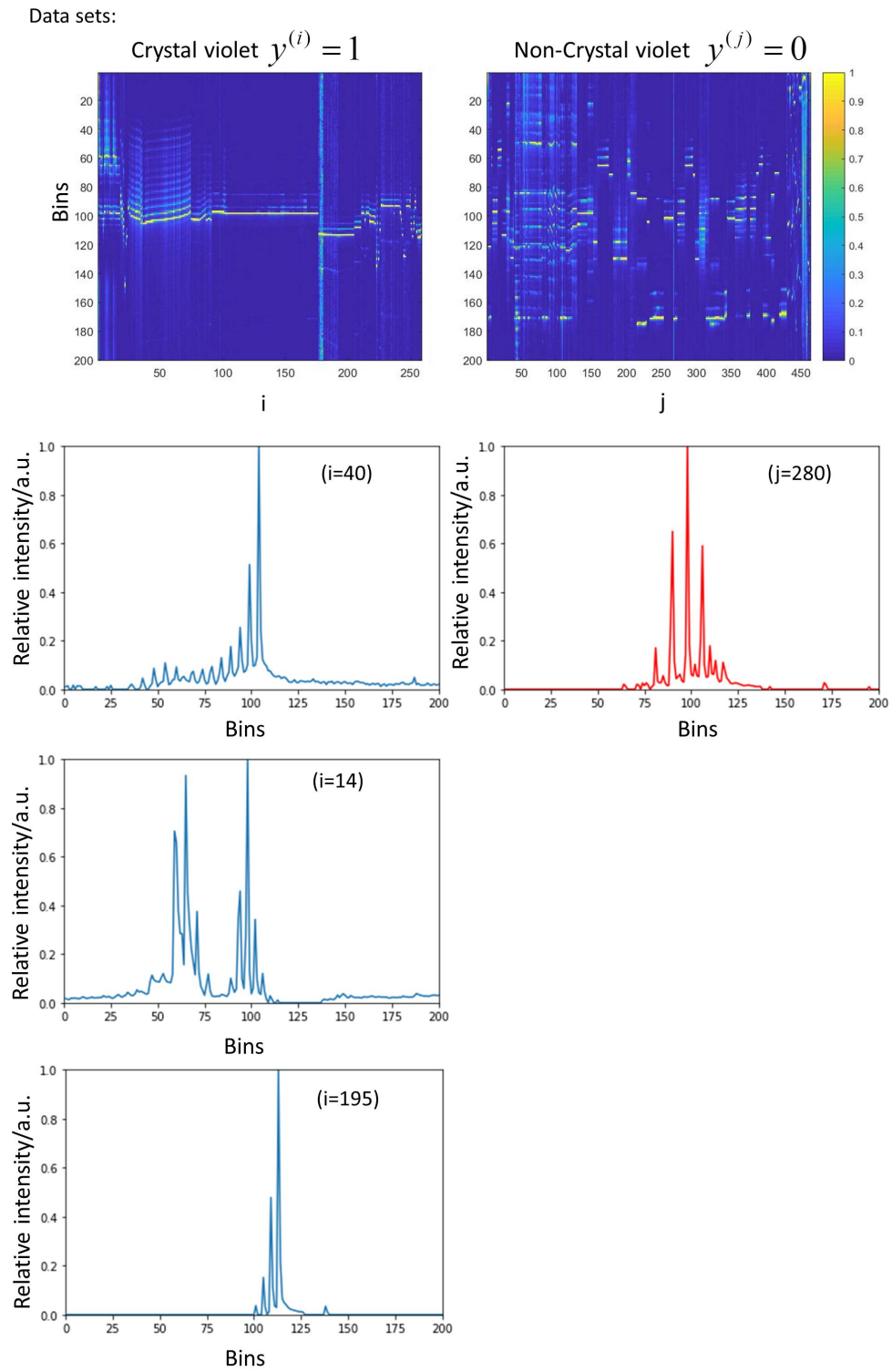


Figure 6.10: The mass spectrum dataset of crystal violet and non-crystal violet classes.

crystal violet sample, but because much higher laser power was used to ionise the sample, its intensity profile is clearly different with instance 40, more doubly charged ions were produced which form a double-peak shape along with the singly charged ones. Also, ions tend to lose more methyl groups due to a higher degree of laser-induced fragmentation (*i.e.* the peak of highest relative abundance no longer belongs to parent ions). Instance 195 has a similar intensity profile to instance 40, which suggests they were acquired using a similar laser power, but all the peaks in instance 195 shift towards higher bin numbers (larger flight time) compared with instance 40. The parent ion of instance 40 is located around bin number 105 while the parent ion of instance 195 is around bin number 115. This is simply because the ion extraction/acceleration field used in instance 195 was weaker than that of instance 40 and thus ions in instance 195 had a lower speed. Apart from discrepancies in laser power and acceleration field, some ToF spectra were acquired with the original PEDA method, some with the double-field PEDA method, and others without PEDA, so the ToF resolution also varies among instances. Instance 280 of the non-crystal violet class also has a succession of peaks around bin number 100 just like a crystal violet spectrum. In order to increase the complexity of classification task, spectra were deliberately selected in such a way, with a purpose of confusing the machine learning algorithms as much as possible.

Besides the 200×723 matrix that contains all the ToF spectra instances, the algorithms also need a 1×723 vector where each element stores a class label of the corresponding instance, for crystal violet ToF spectra it is “1” while for non-crystal violet ones it is “0”. As mentioned in the algorithms section, for supervised learning labels are indispensable components that guide the algorithms to optimise classification models.

The last step is to shuffle and partition the dataset into a training set and a test set. As their names imply, models are trained using the training set, and tested using the test set. The rationale behind this split is that if a model is trained and tested on the same data set, the model could just repeat the labels of the samples as it has already seen. This might give perfect accuracy but would fail to provide useful predictions for

unseen samples. This situation is called overfitting. In order to know how well a model will generalise to new cases, one has to test it on instances it has never seen before (in our case new ToF spectra). The error rate on new cases is called the generalisation error (or out-of-sample error). The split ratio between training and test sets is around 75%/25%.

Usually a model can be improved by tweaking hyperparameters, however it is generally not recommended to do so by evaluating the performance of the model on the test set. This is because if the hyperparameters are adjusted until the model performs optimally on the test set, knowledge about the test set may “leak” into the model and evaluation metrics no longer report a reliable generalisation error. One way to solve this problem is to use another segment of the dataset as a validation set: a model is built by fitting the training set, which is then evaluated on the validation set, and after hyperparameters has been optimised based on the validation set, final evaluation will be done on the test set.

A downside of such an approach is that a considerable part of the precious dataset is “wasted” as validation. A better alternative is k -fold cross-validation, where the training set is equally split into k complementary smaller subsets (k -fold). Learning algorithms use $k - 1$ of the folds as training data and the resulting model is evaluated on the remaining 1-fold of the training set. The overall performance of a model is simply the average of the performance metric of all k models trained with k different combinations of subsets. In addition, cross-validation not only provides an estimate of the performance of a model, but also a measure of how precise this estimate is (*i.e.*, its standard deviation). Then hyperparameter optimisation can be done in a so-called grid search approach, which exhaustively search through a manually specified subset of the hyperparameter space of a machine learning algorithm. Eventually, the final model of optimal settings is trained on the full training set before the generalisation error is measured using the test set.

All the machine learning works in this chapter were performed with the Python scikit-learn package which is a free library for the Python programming language (<http://scikitlearn.org/stable/index.html>). Scikit-learn provides user-friendly Application Programming Interface (API) to perform machine learning related tasks such as cleaning

and preprocessing raw data, building and training a classifier, running cross-validations, outputting learning results, and so on.

6.3.2 Results

Table 6.2 summarises the cross validation results of five classifiers built with different machine learning algorithms (Artificial Neural Network, Logistic Regression, Random Forest, Support Vector Machine, and K Nearest Neighbors). All models were trained with

Learning algorithms	Accuracy (%)	F-1 score
Logistic Regression	96.1 ± 2.8	0.946 ± 0.04
ANN	96.9 ± 2.3	0.956 ± 0.033
Random Forest	96.3 ± 2.2	0.945 ± 0.033
SVM	96.5 ± 2.3	0.952 ± 0.031
KNN	97.8 ± 1.8	0.969 ± 0.025

Table 6.2: 10-fold cross-validation results of crystal violet versus non-crystal violet classification using various machine learning algorithms.

the same training set and hyperparameters for each learning algorithm were optimised by running 10-fold cross validation in a grid search. The performance metrics used here are accuracy and F-1 score. Accuracy is simply the ratio of the number of correctly classified samples over the number of samples in a validation subset. The F-1 score will be introduced shortly. As shown in Table 6.2, the classifiers work well, and an accuracy over 95% can be obtained with all learning algorithms.

The next step is to estimate the generalisation errors of the five final models using the test set. A good way to display the test results is to use the so called “confusion matrix”. The confusion matrix for the Logistic Regression classifier is shown in Table 6.3. Looking first at the row whose actual class is “0” (instances that are non-crystal violet), 117 of them are correctly predicted as “0” and 4 of them are wrongly predicted as “1”. Therefore, the number of true negative samples equals to 117 and false negative number

		Predicted Class		
		0	1	total
Actual Class	0	TN 114	FP 1	115
	1	FN 4	TP 62	66
total		118	63	

Table 6.3: Confusion matrix of the Logistic Regression classifier (TN = True Negative, FP = False Positive, FN = False Negative, TP = True Positive) for the crystal violet versus non-crystal violet dataset.

equals 4. Then the next row corresponds to instances that are indeed crystal violet (actual class=“1”), 3 of them wrongly predicted as “0” and 57 of them are correctly predicted as “1” (3 false positive and 57 true positive). In general 176 (true negative + true positive) of 181 testing instances are correctly classified. In machine learning terminology, the ratio (true positive)/(true positive + false positive) is referred to as “precision”(P) and the ratio (true positive)/(true positive + false negative) is referred to as “recall”(R). So the precision of this Logistic Regression classifier is 0.93 and its recall is 0.95. The F-1 score measures the performance of a model, and is defined as

$$F1 = 2 \cdot \frac{P \cdot R}{P + R}. \quad (6.11)$$

The F-1 score is the harmonic average of the precision and recall, and equals one if a model classifies all test instances correctly. The F-1 score of the Logistic Regression classifier is 0.94. Intuitively the F-1 score is not as straightforward as accuracy, but it usually proves more useful than accuracy because it takes into consideration both precision and recall, especially if one has an uneven class distribution. Since all the datasets to be analysed in this chapter contain more negative instances (controls) than positive ones (patients), the F-1 score will be used as the performance gauge.

Another important thing to consider is the trade-off between precision and recall. When a classifier predicts the class of a new instance, it first computes a score (namely the value of $f(\mathbf{x})$ in Section 6.2.1) for the instance based on a decision function. Once that score is greater than a threshold, the instance will be classified as positive class. For a logistic regression model, this score is the signed distance between the decision boundary/hyperplane and an instance. One may improve the precision of a classifier by increasing the threshold at a cost of decreasing its recall, or vice versa. A 10-fold cross-validation was done here to generate a score for each instance in the training set. Both the precision and recall are plotted in Figure 6.11 as a function of the threshold. Usually the threshold is chosen to be 0 to keep a balance between recall and precision (in this case they are both around 0.95). But sometimes it may be preferable to weight precision over recall or the other way around, so one can simply choose an appropriate threshold accordingly. For example, in diagnostic applications, false negatives could be much more unfavourable than false positives, since a large number of false negatives means potential patients are at a risk of being missed for further screening. So in this scenario, the threshold of the model can be adjusted to achieve higher recall.

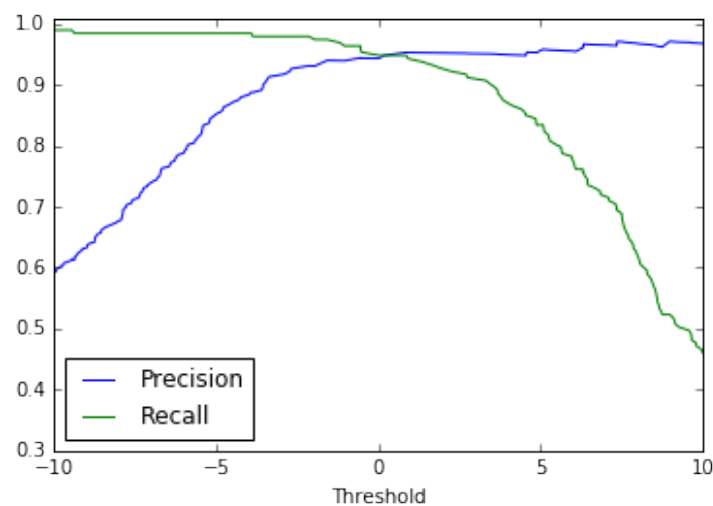


Figure 6.11: The threshold dependence of the precision and recall of the logistic regression classifier for the crystal violet versus non-crystal violet dataset. Thus one may choose a desirable set of precision and recall by changing the value of the threshold.

The receiver operating characteristic (ROC) curve is a commonly used way to compare

classifiers (as shown in Figure 6.12). The ROC curve plots the true positive rate (another

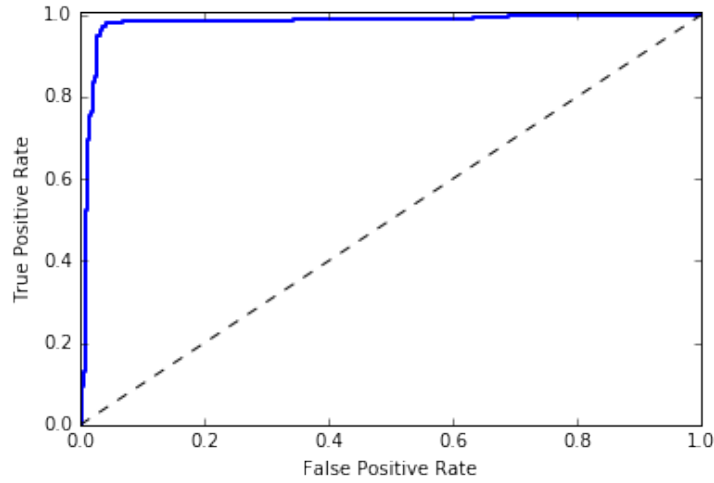


Figure 6.12: The receiver operating characteristic (ROC) curve of the logistic regression classifier for the crystal violet versus non-crystal violet dataset. The area under the curve (AUC) measures the performance of a classifier, which ranges from 0.5 to 1. The closer the AUC is to 1, the more accurate a classifier is. In this case, the AUC equals 0.979.

name for recall) against the false positive rate. Apparently, a better model will lead to a larger the area under the curve (AUC): a perfect classifier has an AUC equals to 1, whereas a purely random classifier has an AUC equal to 0.5. The AUC of our Logistic Regression model is 0.979.

In machine learning, overfitting and underfitting are two common problems that limit the performance of a model [235]. Overfitting occurs if a model is too complex, which is prone to extract patterns of no value, such as noise, outliers, or random fluctuations, from the training data and tries to learn knowledges from them. However, these knowledges do not apply to new data and therefore inevitably increase the model's generalisation error. On the other hand, if a model is too simple, it cannot capture the underlying trend of the data and will have poor a performance on both the training and testing set. A good way to estimate whether a model is overfitting or underfitting the dataset is to plot the “learning curve” as shown in Figure 6.13. The training set is further divided into a smaller training set and a validation set (80:20). By training the model multiple times on different sized subsets of the smaller training set, one can obtain the dependence of the performance gauge on the subset size. For a logistic regression model, the performance

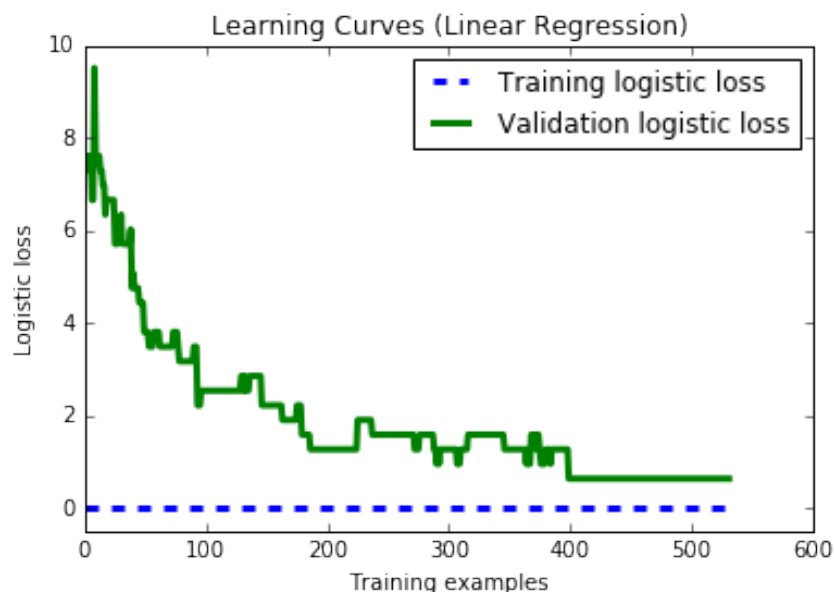


Figure 6.13: The learning curve of the logistic regression classifier: logistic loss as a function of number of training instances. The training logistic loss stays close to zero regardless of the number of training examples, which means the Logistic Regression model is sufficient to perfectly fit the decision boundary. The validation logistic loss decreases as more examples are used to train the predictive model. The gap between the two logistic losses after all training examples are used is a typical symptom of overfitting.

gauge is defined using its cost function (Equation (6.5)). If this is close to zero regardless of the number of training instances, which suggests that the model is complex enough to perfectly fit the training data. However, our model does not perform well on the validation set. When it is trained on very few training instances, the learning algorithm is unable to build a model that generalises properly, which explains why the validation error is so big at the beginning. As more training instances are fed into the model, it learns and thus the validation error gradually declines. But even when the entire training set is used, there is still a gap between the curves. This is a typical symptom of overfitting: the model always works better on the training set than on the validation set. To further eliminate the overfitting problem, one can try to collect more mass spectra, and as Figure 6.13 shows, this may lead to a tiny decrease of the validation error. Other methods such as Early Stopping [235] and Regularisation [236] have also been used to combat the overfitting problem.

The ensemble method is another possible way to deal with overfitting. A group of

classifiers are used as a “committee”, each classifier estimates the class probabilities for a new instance and the class with the highest averaged probability over all the individual classifiers is output as the final prediction. Empirically, such an ensemble model tends to outperform its individual component classifiers. The more diverse the component classifiers are, the better ensemble learning works. Three machine learning algorithms were combined to build the ensemble model, they are Logistic Regression, Random Forests, and K-Nearest Neighbors. Again 10-fold cross-validation was performed to estimate the F-1 score of the ensemble model, a mean of 0.979 and a standard deviation of 0.022 was obtained. This F-1 score is indeed higher than every single classifiers listed in Table 6.2. The ensemble model produces the confusion matrix in Table 6.4, which corresponds to a

		Predicted Class		
		0	1	total
Actual Class	0	TN 115	FP 0	115
	1	FN 3	TP 63	66
total		118	63	.

Table 6.4: Confusion matrix of the ensemble classifier (TN = True Negative, FP = False Positive, FN = False Negative, TP = True Positive) for the crystal violet versus non-crystal violet dataset.

F1-score of 0.98.

6.4 Mass spectra of blood samples

Machine learning algorithms can clearly be used to classify the ToF spectra of dye molecules. However, dye samples are relatively pure and therefore their ToF spectra are very simple. In real world applications, particularly for clinical diagnostics, mass spectra are generated from complex biological samples, which are mixture of proteins,

fats, hydroxylapatite, hydrocarbons, DNA, inorganic ions, radicals, and so on and so forth. For example, in human blood, there are well over 10,000 distinct proteins, and this gives modified products and many other smaller molecules such as peptides and lipids. The concentrations of different chemical components are estimated to span a dynamic range of 10^{12} orders of magnitude [237, 238]. So it is necessary to test the classification capability of machine learning algorithms on real world biological data before further implementing this idea. Therefore, in this section the machine learning method were tested in the classification of the mass spectra of blood samples.

6.4.1 Ovarian cancer and prostate cancer datasets

Two spectra datasets of blood samples were downloaded from the Clinical Proteomics Program Databank from Centre for Cancer Research [239]. These mass spectra were generated by Surface-Enhanced Laser Desorption and Ionisation (SELDI) of serum samples (a liquid derivative of blood) in 2002. SELDI-TOF is a variant of MALDI-TOF in which a modified chip is used to bind only those proteins of interest onto its surface and all other contaminants can be washed out [240]. Dataset 1 included mass spectra from 91 controls and 162 ovarian cancer patients while dataset 2 included mass spectra from 253 controls and 71 prostate cancer patients. Blood is one of the most easily accessible and less intrusive body fluids, which directly communicates with nearly all organs and tissues in the body. Mass spectra based diagnosis is built on the assumption that disease progression can be reflected by the composition change in blood. In the rest of this section machine learning algorithms will be used to build classification models using the ovarian cancer and prostate cancer datasets.

The mass spectrometer (Ciphergen Biosystems) employed to acquire those datasets had a mass resolution of less than $400\ m/\Delta m$ and a mass accuracy of $\approx 0.1\%$ or 1,000 ppm. These are comparable to the home-built MSI system presented here (the mass accuracy of our microscope MSI system is also around 1,000 ppm and its mass resolution is actually better, reaching $10,000\ m/\Delta m$ when working in the reflectron mode). If a

good classifier can be built using the mass spectra obtained with a MS instrument of such mass resolution and mass accuracy, our own instrument will be able reproduce it.

The mass range of each mass spectrum is between 0 to 20,000 m/z , and contains 15,200 m/z values per spectrum, so there are 15,200 features in each input vector. An example of a serum mass spectrum is shown in Figure 6.14, which has significantly more peaks and

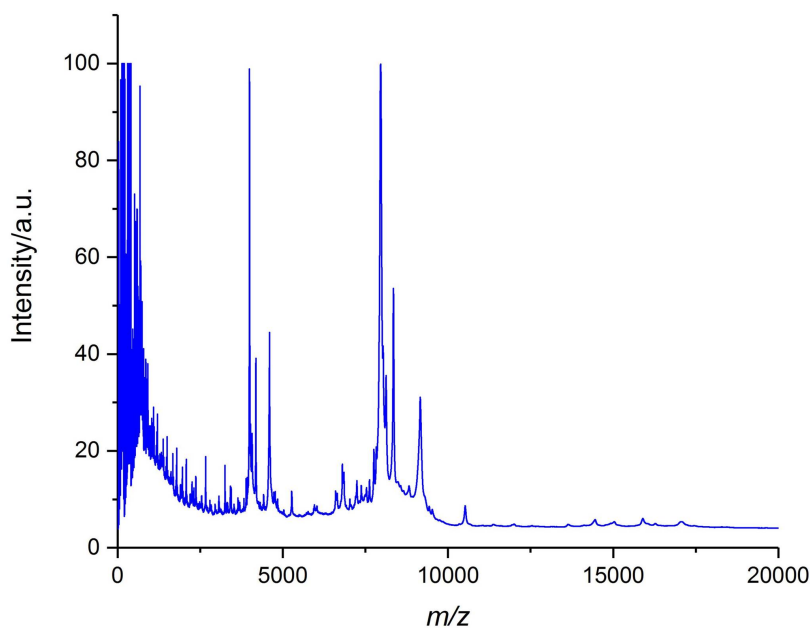


Figure 6.14: SELDI mass spectra of a serum sample. The mass range is between 0 to 20,000 m/z , which contains 15,200 m/z values.

noise compared with those of dye molecules. The process to build a classification model with learning algorithms followed mostly the same procedure as previously described in the crystal violet section. The only difference is that the entire mass range of a mass spectrum was used for training and testing and no binning was done to merge adjacent m/z features, which ensured that no information contained in these mass spectra was discarded.

10-fold cross-validations were again used on the ovarian cancer dataset using the ANN, Logistic Regression, and Random Forest learning algorithms. Their F-1 scores are summarised in Table 6.5 along with their standard deviations. A 100% correct classification

Learning algorithms	F-1 score
Logistic Regression	1.0 ± 0.0
ANN	1.0 ± 0.0
Random Forest	0.992 ± 0.016

Table 6.5: 10-fold cross-validation results of Ovarian cancer versus Control classification using various machine learning algorithms.

was achieved for the Neural Network and Logistic Regression and a 99% F-1 score was achieved using a Random Forest. The Logistic Regression model was further tested on the testing set and its Confusion Matrix is presented in Table 6.6. The Logistic Regression classifier is able to perfectly discriminate a serum mass spectrum based on whether it comes from a ovarian cancer patient or a control.

		Predicted Class		total
		0	1	
Actual Class	0	TN 22	FP 0	22
	1	FN 0	TP 42	42
total		22	42	.

Table 6.6: Confusion matrix of the logistic regression classifier (TN = True Negative, FP = False Positive, FN = False Negative, TP = True Positive) for the ovarian cancer dataset.

Unlike ANN or Logistic Regression, Random Forest can easily evaluate the importance of each feature on a classification task. Features corresponding to the ion abundance of specific m/z ratios are ranked based on their importance in Table 6.7. As discussed earlier, a random forest consists of hundreds of individual decision trees and each node in a decision tree corresponds to a decision rule that splits the dataset in two. The decision

Rank	m/z	Relative importance (a.u.)
1	244.66	0.751784
2	246.12	0.707944
3	244.95	0.697743
4	246.41	0.615104
5	245.24	0.543278
6	246.71	0.540456
7	245.53	0.470134
8	434.69	0.434451
9	245.83	0.420605
10	435.08	0.401695

Table 6.7: Top 10 most important features in a random forest classifier of ovarian cancer dataset. The shaded m/z values cluster around 245.

rule is usually based on a condition arising from a single feature. An optimal condition is chosen by maximizing the decrease of weighted Gini Impurity after each split. Therefore, for a decision tree, it is possible to determine how much impurity can be removed by each feature. For a forest, the impurity reduction credited to each feature is averaged over all its component trees before the features are ranked in compliance with such measure.

For a decision tree model, if a dataset has two (or more) correlated features, all of them will have the same chance of being chosen as a predictor to split the dataset at a node. However, once one of them is used, the importance of other correlated features will be dramatically reduced since effectively the impurity they are able to decrease has already been removed by the first one. Consequently, an unfairly lower importance will be given to the other correlated features. This may provide misleading information when people are trying to interpret the data: one of those correlated features may be seen as a useful predictor while the others in the same group may be overlooked. They may actually be very close in terms of their competence as a predictor. The effect of this phenomenon can be somewhat reduced with a random forest model thanks to its random selection of features when building each node, but in general this inequality is not removed completely. As shown in Table 6.7, among the top ten most important features, eight of

them actually cluster around 245 m/z (shaded in gray). They probably originate from the same molecule or may be different isotopes.

The average abundance and standard deviation of the most important feature 244.66 m/z , are calculated for the positive and negative groups respectively (as shown in Figure 6.15). The difference of the mean abundance between positive and negative instances is statistically significant. It is safe to say ovarian cancer patients have a lower level of 244.66 m/z ion in their serum than healthy women. Does this suggest a bio-marker exists which has a mass-to-charge ratio around 245 m/z ? Cancer is largely caused by genetic abnormalities in protooncogenes and tumour suppressor genes that regulate processes of cellular growth, metabolism, energy production, and lipid metabolism [241]. Lipids are small molecules and 244.66 m/z could be a derivative of one of them. But unfortunately it is impossible to confirm the identity of this molecule because of the poor accuracy ($\approx 1,000$ ppm) of the MS system employed in this experiment. As indicated by Figure 6.2, to correctly identify it, one needs a MS instrument with far higher accuracy (≈ 5 ppm).

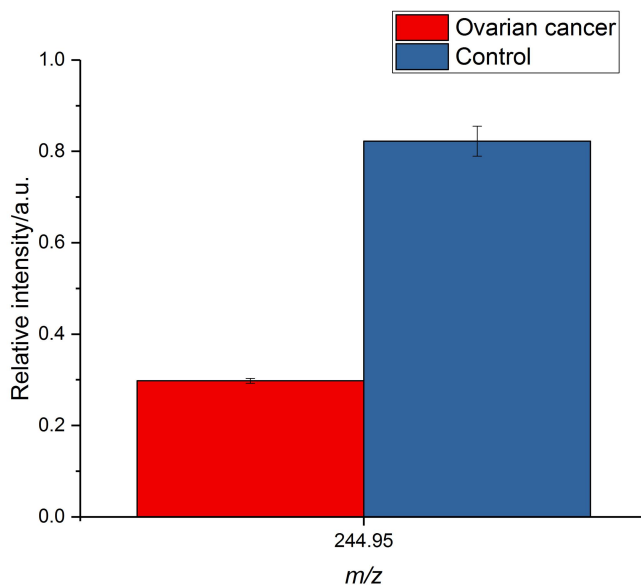


Figure 6.15: Average abundance of ion 244.66 m/z in ovarian cancer and control groups. The control group appears to have a significantly higher abundance of ion 244.66 m/z than the ovarian cancer group, which might indicate that ion 244.66 m/z could be a potential biomarker associated with the ovarian cancer.

Currently, there exist some medical techniques for cancer screening that are effective in reducing the morbidity and mortality [170]: (1) the Pap smear for cervical cancer screening with a specificity of 63% and a sensitivity of 73% (2) mammography for breast cancer detection with a recall of 90% and a precision of 15 – 34%, and (3) fecal occult blood testing for colorectal cancer screening with a fairly high specificity of 96 – 98% and a sensitivity of 40%. Thus, compared with current techniques, the mass spectrometry-based approach may substantially improve the accuracy of cancer screening.

A Logistic Regression model was also optimised to build a classifier that differentiate prostate cancer positive mass spectra from negative ones. Its ten-fold cross-validated F-1 score is 0.87 ± 0.14 , which is not as good as the ovarian cancer classifier. Two steps of feature selection were done to improve its performance. In the first step, features were removed if their abundance variance among training set was lower than a threshold of 0.001. Those discarded features were considered as uninformative noise that might lead to unnecessary overfitting. After doing this, 6982 of 15153 features remained and the ten-fold cross-validated F-1 score was improved to 0.90 ± 0.10 . In the second step, a recursive feature elimination method (RFE) was used to further reduce the number of m/z ratios that would be considered by our classifier. In an RFE process, the classifier is first trained on the initial set of features and the importance of each feature is evaluated. Then, features with the lowest importance are eliminated. This elimination process is recursively repeated on the pruned feature set and the feature set gets smaller and smaller until a target number of features is finally reached. In our case, the 100 most important features were selected to redo the ten fold cross-validation. An F-1 score of 0.94 ± 0.06 was achieved, which confirmed that feature selection was an advisable step when building a mass spectra classifier. Classifiers were also built with other learning algorithms using the same set of selected features, in which ANN and SVM showed the best performance (F-1 score = 0.96 ± 0.05). The ensemble model didn't outperform the SVM or ANN models in this case. The Confusion Matrix of the SVM model is shown in Table 6.8. The recall and precision of positive instances are both 0.82.

Although machine learning models work very well on the ovarian cancer dataset, their performance on the prostate cancer dataset is slightly worse. This is probably due to the difference in quality between the two datasets. For the ovarian dataset, samples were prepared by robotic sample handling (washing, incubation, etc.), while for the prostate case, samples were prepared by hand. The automatic sample preparation process might have improved the reproducibility of the ovarian dataset, which is the key to building a robust classifier. Also, different instruments were used to collect the mass spectra: the prostate dataset was generated using a Ciphergen PBS1 SELDI-TOF mass spectrometer, while the ovarian dataset was obtained with an upgraded PBSII SELDI-TOF instrument.

		Predicted Class		
		0	1	total
Actual Class	0	TN 61	FP 3	64
	1	FN 3	TP 14	17
total		64	17	.

Table 6.8: Confusion matrix of the SVM classifier (TN = True Negative, FP = False Positive, FN = False Negative, TP = True Positive) for the prostate cancer dataset.

6.4.2 Chronic fatigue dataset

Machine learning algorithms have also been used to analyse blood plasma profiles of Chronic Fatigue Syndrome (CFS) patients and controls (m/z features= 11823, control samples= 25, and CFS samples= 48). The dataset, which is still unpublished, was kindly provided by Prof James McCullagh, Department of Chemistry, University of Oxford, whose group curated it from metabolic profiling experiments with a LC-MS system. Cross validation F-1 scores with different machine learning algorithms were summarised in Table 6.9. The ensemble algorithm (which combines pretrained SVM, Logistic Regression, Ran-

dom Forest, K-Nearest Neighbors, and Gaussian Naive Bayes models) shows the highest F-1 score (0.93 ± 0.10). The predictive model trained with the ensemble algorithm was then tested on the test dataset; the confusion matrix is given in Table 6.10, where a recall of 90.9% and a precision of 100% have been achieved. With a rather limited number of examples (< 100), machine learning algorithms still generate a very accurate model that is able to differentiate CFS patients' mass spectra from controls'.

Learning algorithms	F-1 score
Random Forest	0.92 ± 0.10
KNN	0.91 ± 0.11
SVM	0.90 ± 0.12
Gaussian Naive Bayes	0.90 ± 0.13
Logistic Regression	0.90 ± 0.12
Ensemble	0.93 ± 0.10

Table 6.9: 10-fold cross-validation results of CFS versus Control classification using various machine learning algorithms.

		Predicted Class		total
		0	1	
Actual Class	0	TN 8	FP 0	8
	1	FN 1	TP 10	11
total		9	10	.

Table 6.10: Confusion matrix of the Ensemble classifier (TN = True Negative, FP = False Positive, FN = False Negative, TP = True Positive) for the CFS dataset.

The importance of each feature was also extracted from the Random Forest model (see Table 6.11). In Figure 6.16 (a), all instances are plotted in a 2D coordinate system

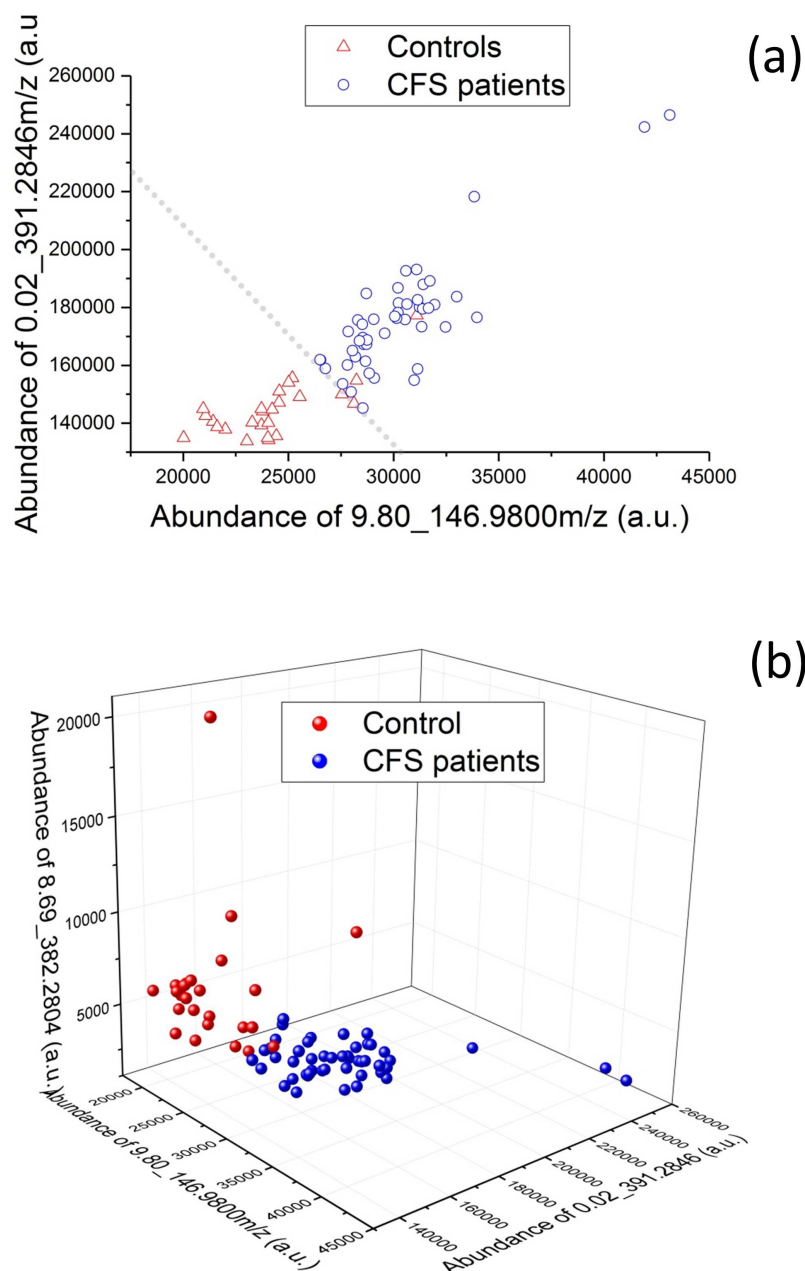


Figure 6.16: Scattering plot of instances in (a) a 2D feature space (coordinates are 391.2846 m/z and 146.9800 m/z), and (b) a 3D feature space (coordinates are 391.2846 m/z , 146.9800 m/z , and 382.2804 m/z).

with the abundance of the top two important features (391.2846 and 146.9800 m/z) as axes (the numbers prior to the m/z values correspond to the retention times of the liquid chromatography). Clearly, the CFS patients can be separated from the controls by a straight line boundary, which may suggest that CFS patients tend to have more 391.2846 m/z and 146.9800 m/z compounds in their blood plasma profile than the controls. The

examples have also been plotted in a 3D coordinate system in Figure 6.16 (b), the extra axis (abundance of 382.2804 m/z) is actually the most important m/z in Table 6.11 which does not highly correlate with the top two features. Moreover, a predictive model can still be built with only the three aforementioned m/z features, its accuracy is 0.90. Table 6.11 lists the ten most important m/z features along with their relative importance. Note that

Rank	Compound Name	Relative importance (a.u.)
1	0.02_391.2846 m/z	0.083021
2	9.80_146.9800 m/z	0.073777
3	9.24_383.1839 m/z	0.061696
4	8.92_207.0951 m/z	0.059671
5	9.96_356.9097 m/z	0.054867
6	10.00_146.9799 m/z	0.053726
7	8.69_382.2804 m/z	0.045251
8	10.00_164.9207 m/z	0.038971
9	9.33_214.2528 m/z	0.035071
10	9.94_88.922 m/z	0.028524

Table 6.11: The ten most important features obtained from CFS dataset using the Random Forest classifier.

actually most of these m/z features are highly correlated (as shown by the correlation coefficient matrix in Table 6.12). This might suggest that these “important” ions originate from the same precursor or were produced by the same mechanism. The cause of CFS is still unknown and there is no cure for it. Since this precursor or mechanism has the power to differentiate CFS patients and healthy people, it may provide deeper insights into the disease.

6.5 Summary

Mass spectrometers are capable of analysing the chemical components of body fluids or organisms, however intrinsic downsides of biological mass spectra such as complexity, diversity, low reproducibility, and high noise are factors that limit our ability to mine

	1	2	3	4	5	6	7	8	9
1	1	0.89871	0.90614	0.87184	0.89579	0.92113	-0.4495	0.90046	0.87125
2	0.89871	1	0.88014	0.84371	0.87909	0.88245	-0.44918	0.89232	0.8189
3	0.90614	0.88014	1	0.81496	0.84768	0.89034	-0.50042	0.8793	0.79673
4	0.87184	0.84371	0.81496	1	0.814	0.77095	-0.40408	0.77524	0.86464
5	0.89579	0.87909	0.84768	0.814	1	0.87352	-0.44401	0.91143	0.82327
6	0.92113	0.88245	0.89034	0.77095	0.87352	1	-0.48046	0.95242	0.80628
7	-0.4495	-0.44918	-0.50042	-0.40408	-0.44401	-0.48046	1	-0.43044	-0.40567
8	0.90046	0.89232	0.8793	0.77524	0.91143	0.95242	-0.43044	1	0.79242
9	0.87125	0.8189	0.79673	0.86464	0.82327	0.80628	-0.40567	0.79242	1
10	0.89224	0.93083	0.85708	0.87819	0.86724	0.87557	-0.41313	0.89746	0.85295

Table 6.12: Correlation coefficient matrix of the most important features.

high value information from them. Moreover, identifying an unknown compound would become impossible if mass spectrometers with low mass resolution or poor mass accuracy are employed in studies. To address these issues, common machine learning algorithms such as Neural Networks, Random Forests, Logistic regression and so on, were proposed here as a possible approach to analyse mass spectra. As a proof of concept "experiment", classifiers able to discriminate mass spectra belonging to different dye molecules were built with a multitude of learning algorithms. Both high precision and recall were successfully obtained, which suggested that machine learning algorithms hold the promise of discerning rather complicated patterns buried in mass spectra dataset through supervised learning. To explore the efficacy of machine learning algorithms in analysing complex biological mass spectra, three pairs of datasets (ovarian cancer, prostate cancer, chronic fatigue and their respective controls) were used to train classifiers capable of differing patients' blood sample from controls. The results demonstrated the great potential of combining mass spectrometry with machine learning algorithms for clinical diagnoses. In addition, possible bio-markers were discovered, which might assist further studies on biological or pathological mechanisms.

Chapter 7

Conclusions

Mass spectrometry imaging (MSI) is able to provide a chemical component analysis of a sample surface along with the spatial distribution of each component. Traditionally, MSI uses an ionising beam to probe each spot on the sample surface, these are then analysed by a conventional mass spectrometer before being reconstructed into an image of each mass peak. Alternatively, MSI instruments working in microscope mode spatially-map ions produced simultaneously over the entire sample surface by projecting their positional information onto a two-dimensional detector using an appropriate ion optical design. By decoupling the acquisition speed from the sizes of the ionisation beam and sample surface, in principle the analysis time of microscope MSI instruments can be significantly quicker than the microprobe MSI instruments. However, to map the spatial distribution of a specific analyte on the surface, the detector is conventionally gated on the corresponding mass peak in the ToF spectrum, which limits the imaging capability of the microscope MSI instrument to one single mass in each analysis. Therefore, if there is a wide mass range of interest, the detector gate has to be scanned across all the mass peaks in that range, which reduces the speed advantage of microscope MSI.

The recent developments of fast, position sensitive detectors such as PImMS reinvigorate the idea of microscope MSI. There are four 12-bit memories in an individual pixel of the PImMS sensor and each of the memories is capable of registering a time stamp with 12.5 ns precision. As a result, the single mass peak limitation has been overcome and imaging of multiple masses can be achieved in a single acquisition [87].

However, there are still three major challenges facing microscope MSI before its power

can be fully exploited for a wide range of applications: low mass resolution, narrow effective mass range, and poor mass accuracy. This thesis proposes effective solutions to each of the challenges and thus significantly improves the performance and applicability of microscope MSI (as shown in Figure 7.1).

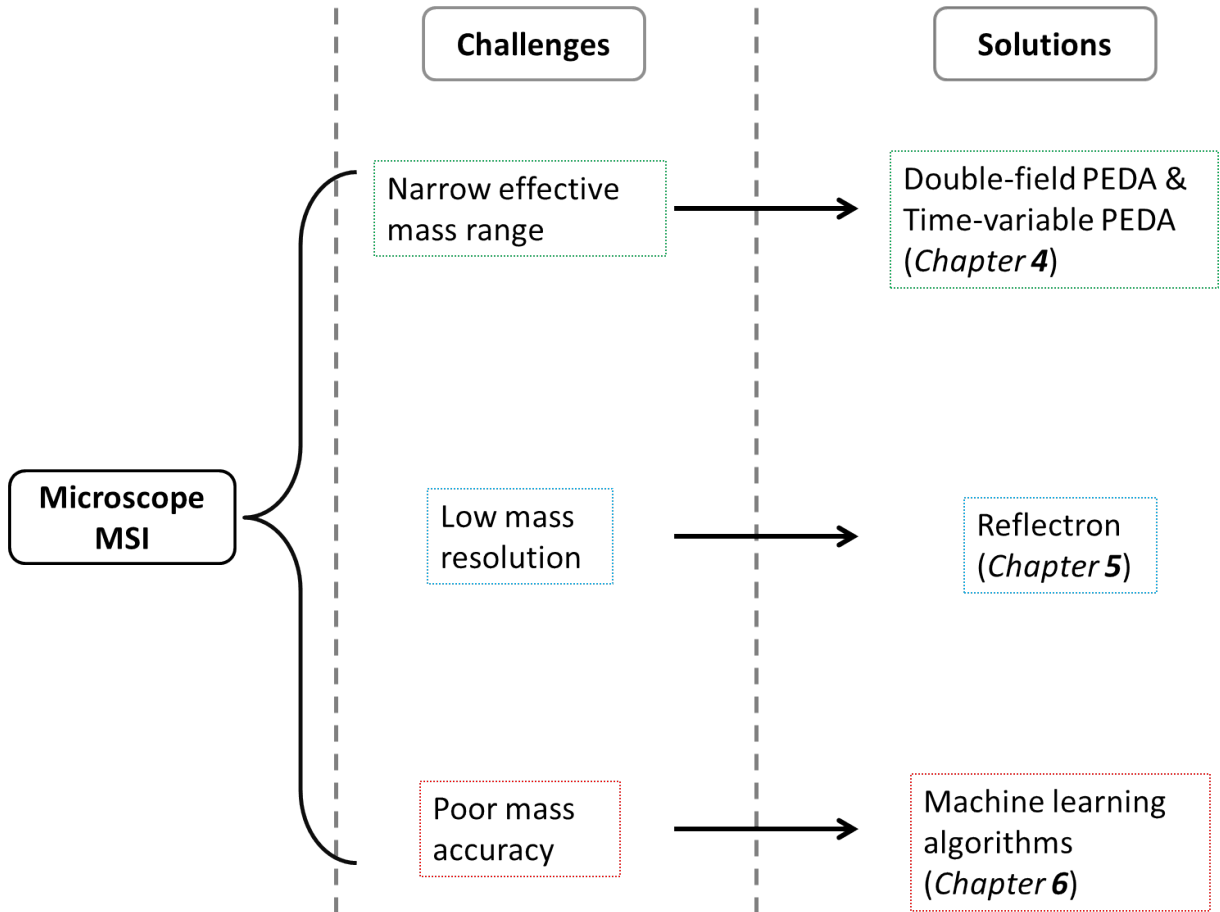


Figure 7.1: The solutions to the three challenges in microscope MSI (1) extending the effective mass range of PEDA by double-field and time-variable PEDA, (2) improving the mass resolving power by adding a reflectron, and (3) avoiding the limitation of poor mass accuracy by analysing the ToF spectra with machine learning algorithms.

Pulsed-Extraction Differential Acceleration (PEDA) improves the mass resolving power of microscope MSI by focusing the ions with the same m/z but different initial kinetic energies at the detector. However, PEDA is mass-dependent and only works well for the mass range around an optimal m/z ratio. To broaden the effective mass range of PEDA, two variants of the PEDA method were studied in Chapter 4: double-field PEDA and time-variable PEDA. In double-field PEDA, an additional electrode was used to create

two pulsed electric fields that temporally focused ions of two different m/z ratios. As a result, the mass ranges where $m/\Delta m \geq 1000$ were widened from 65 to 165 Da. In time-variable PEDDA, a dynamic pulsed electric field was used to maximize the effective mass range of PEDDA. By simultaneously focusing ions between 300 to 700 m/z using an exponentially rising voltage pulse, time-variable PEDDA provides an effective mass range more than six times wider than the original PEDDA method.

A reflectron MSI instrument was designed and implemented in Chapter 5, which tackles the challenge of mass resolution. Reflectrons are able to reverse the ToF expansion of ion packets with the same m/z but different kinetic energies and therefore improve the mass resolving power by extending the overall ToF without increasing the FWHM of the ToF peak. Simulations show that one-stage gridless reflectrons are more compatible with the spatial imaging goal of the microscope MSI instrument than the gridded reflectrons. Preliminary experimental results show that coupling the gridless reflectron with single-field PEDDA achieves a mass resolution above 8,000 $m/\Delta m$ while keeping a spatial resolution of 20 μm . The gridless reflectron therefore triples the mass resolving power without losing any spatial imaging power. Ongoing experimental studies are further characterising the performance of this novel MSI instrument. For example, the mass and spatial resolutions may be further optimised by a fine-tuning of the ion optical and reflectron voltages. The optimal detector angle also needs to be determined by experiments. The mass-dependence of mass and spatial resolutions also needs to be characterised in a similar way as described in Chapter 4. Moreover, as predicted by SIMION, time-variable PEDDA can be used instead of single-field PEDDA in the reflectron instrument to maintain a high mass resolution over a broad mass range.

The last chapter of this thesis deals with the mass accuracy problem of microscope MSI through a novel data analysis method. With poor mass accuracy, it becomes very difficult to identify unknown chemical compositions in a sample using a mass spectrometer. However, one of the potential applications of the microscope MSI instrument is to use it as a high-throughput diagnostic tool, thus analysis becomes a classification problem

(for example, separating healthy people from patients with a disease). Machine learning algorithms, such as Neural Networks, Random Forests, and Logistic regression, can be employed to build models that recognise the peak pattern of biological mass spectra and classify them accurately without knowing the actual mass of each peak. In a proof of concept experiment, the ToF spectra of different dyes collected by the microscope MSI instrument were classified with both very high precision and recall. The idea was then tested on biological mass spectra acquired from blood sample and posted on the Internet (the mass accuracy of these datasets were comparable to the microscope MSI instrument). Again, the classifiers built by machine learning algorithms accurately predict whether a mass spectrum belongs to a patient of ovarian cancer (precision and recall $\approx 100\%$) or prostate cancer (precision and recall $> 90\%$). By evaluating the importance of each m/z feature, possible biomarkers were also discovered, which may be useful for studies on underlying biological or pathological mechanisms.

In future work, the PImMS camera, which is capable of multi-mass imaging, will be coupled with the reflectron MSI instrument. Using this powerful system, MALDI ToF spectra of every single component on the sample surface will be recorded along with its position information in one single experiment acquisition. Then, large disease-related MS datasets will be collected with unprecedented speed using this high throughput system by analysing arrays of biological samples in parallel. Based on the collected datasets, diagnostic models will be built using machine learning algorithms by recognising the patterns of the mass spectra. The end result will be the revival of an MSI technique that complements current analytical methods.

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