

Ion Imaging Mass Spectrometry

A thesis submitted for the degree of Doctor of Philosophy

by

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Abstract

This work investigates the applicability of fast detectors to the technique of microscope-mode imaging mass spectrometry. By ionising analyte from a large area of the sample, and projecting the desorbed ions by the use of ion optics through a time-of-flight mass spectrometer onto a two-dimensional detector, time- (and hence mass-) dependent distributions of ions may be imaged. To date, this method of imaging mass spectrometry has been limited by the ability to image only one mass window of interest per experimental cycle, limiting throughput and processing speed. Thus, the alternative microprobe-mode imaging mass spectrometry is currently the dominant method of analysis, with its superior mass resolution.

The application of fast detectors to microscope-mode imaging lifts the restriction of the detection of a single mass window per experimental cycle, potentially decreasing acquisition time by a factor of the number of mass peaks of interest. Additional advantages include the reduction of sample damage by laser ablation, and the potential identification of coincident co-fragments of different masses originating from the same parent molecule.

Theoretical calculations and simulations have been performed confirming the suitability of conventional time-of-flight velocity-mapped ion imaging apparatus for imaging mass spectrometry. Only small modifications to the repeller plate and laser beam path, together with the adjustment of the accelerating potential field, were required to convert the apparatus to a wide (7 mm diameter) field-of-view ion microscope. Factors affecting the mass and spatial resolution were investigated with these theoretical calculations, with theoretical calculations predicting a spatial resolution of about $26\text{ }\mu\text{m}$ and $m/\Delta m$ of 93.

Typical experimental data collected from velocity-mapped ion imaging experiments were collected, and characterised in order to provide specifications for a novel time-stamping detector, the Pixel Imaging Mass Spectrometry detector. From these data, the suitability of thresholding and centroiding on the new detector was determined.

Initial experiments using desorption/ionisation on silicon and conventional charge-coupled device cameras confirmed the correct spatial-mapping of the apparatus. Matrix-assisted laser desorption/ionisation techniques (MALDI) were used in experiments to determine the spatial and mass resolutions attainable with the apparatus. Experimental spatial resolutions of $14.4\text{ }\mu\text{m}$ and $m/\Delta m$ of 60 were found. The better experimental spatial resolution indicates a higher directionality of initial velocities from MALDI desorption than used in the theoretical predictions, while the poorer mass resolution could be attributed to limitations imposed by the use of the phosphor screen.

Proof-of-concept experiments using fast-framing cameras and the new time-stamping detectors confirmed the feasibility of multiple mass acquisition in time-of-flight microscope mode ion imaging. Mass-dependent distributions were acquired of different pigment distributions in each experimental cycle. Finally, spatial-mapped images of coronal mouse brain sections were acquired using both conventional and fast detectors. The apparatus was demonstrated to provide accurate spatial distributions with a wide field-of-view, and multiple mass distributions were acquired with each experimental cycle using the new time-stamping detector.

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Chapter 1

Introduction

Spatially-resolved imaging mass spectrometry (IMS) involves the determination of the distribution of masses over a two-dimensional sample. Although the technique has been used for more than fifty years [1], it is only more recently, thanks to new-found applications to biological samples [2], that there has been a resurgence in interest in the technique. Consequently, there has been much investigation and improvement in various methods of performing spatial imaging mass spectrometry. The technique has become commonplace in fields in which correlation between chemical and spatial information provides insight.

Recent improvements in silicon technologies have allowed for the production of fast detectors, which may prove beneficial to a method of imaging mass spectrometry: microscope-mode IMS. The combination of these two techniques provides a large number of advantages and possibilities, such as high-throughput sample analysis, improved acquisition and analysis speed, and reduction of null data volume.

This work investigates these possibilities, advantages, and implementation of such a combination of techniques. The remainder of the chapter describes the techniques and applications of imaging mass spectrometry, detailing methods and state-of-the-art. The principles of mass spectrometry and its ion production techniques are first described, followed by methods for mass separation and ion detection. Velocity-mapped and spatial-mapped imaging are examined, together with the application of fast detection systems to such imaging. Finally, the outline of thesis is presented

at the end of the chapter.

1.1 Mass Spectrometry

The essence of mass spectrometry is the identification of molecules by their ratio of mass to charge, m/z , and the determination of the subsequent relative intensities of these ions. If the value of the charge, z , can be well controlled or accounted for, this corresponds to determination of the mass of the ion. Depending on the accuracy of the measurement, the elements, isotopes, and composition of a compound can be unambiguously defined, making it an extremely powerful tool in analytical chemistry. Accurate measurement of the masses to sub-Dalton resolution allows identification of compounds with the same total number of protons and neutrons by the slight differences in the total mass of their nuclei.

The general principle of all mass spectrometric techniques is the same. In each experimental cycle, ions are created from the sample to be analysed, the desorption/ionisation step. These ions are passed to one or more mass separation steps in order to separate them out by mass. Finally, the separated ions must be detected and the information on mass and other properties extracted. There are a number of approaches for each of these steps.

1.1.1 Sample Ionisation

In order for the mass/charge ratio of individual molecules to be determined, they must first be separated from each other. Samples of interest not already in the gas phase must thus be vaporised. Additionally, a charge must be imparted to them in order for them to react to the electric or magnetic fields used in the mass separation techniques. The different techniques have different effects on the ions, such as polarity of the charges, degree of fragmentation and clustering. Some ionisation techniques may be more suitable for certain types of sample or compatible with different m/z separation methods. A summary of the most commonly-used ionisation

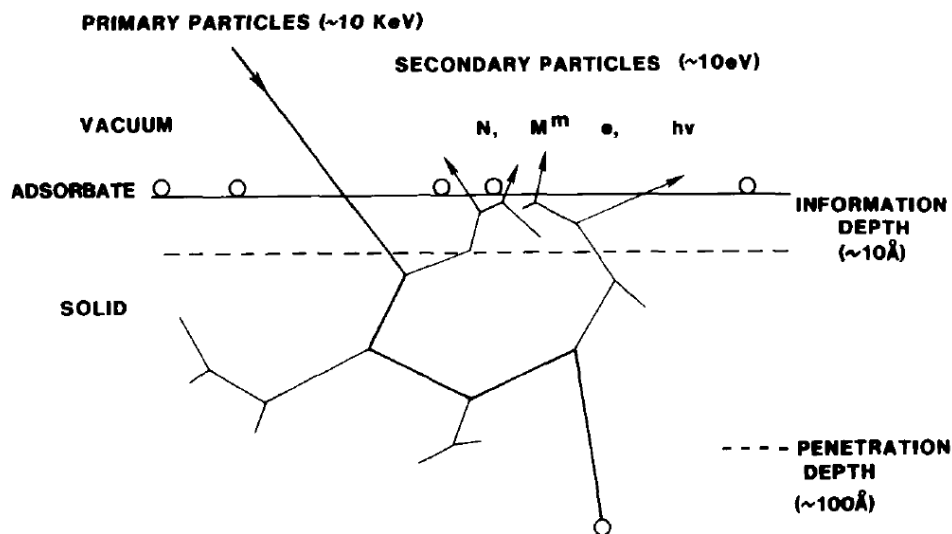


Figure 1.1: Schematic of SIMS process. The impinging primary particle implants itself in the surface, breaking bonds in passing. A variety of particles created near enough to the surface to escape are ejected and may be analysed. Adapted from Ref. [5]

methods is presented here.

Secondary Ion Mass Spectrometry

Secondary ion mass spectrometry (SIMS) is generally used to investigate solids and low-volatility liquids [3]. Ions from these samples are created by sputtering their surface in vacuum with a beam of high-energy (keV) ions. This beam is referred to as the primary beam, and may consist of one of many types of ion, depending on the effect desired. Smaller ions such as Au^+ , Ar^+ , Xe^+ , O^- , O^{2+} , Ga^+ and Cs^+ give a “harder” ionisation leading to more fragmentation of analyte molecules. Clusters such as Au_n^+ , Bi_n^+ , C_{60}^+ and SF_5^+ producing “softer” ionisation with less fragmentation have also been employed [4].

When the ion beam impacts the surface, (Fig. 1.1) the primary ions embed themselves in the sample to a certain “penetration depth”. A collision cascade breaks chemical bonds in the sample, and a variety of secondary particles are formed, including neutrals (N), charged analyte (M^m), electrons (e) and light ($h\nu$) [6]. The limited

depth from which the secondary particles may escape (“information depth”) from the sample ensures the surface specificity of this analytical technique. The softer, less energetic desorption of the cluster primary ions may sometimes be coupled with laser-induced post-ionisation to improve the ion production [7]. The ejected secondary ions in the vacuum may be passed directly to the mass analyser.

This ionisation method causes extensive fragmentation of higher-mass analytes, and the ions generated have a limit of m/z of up to 2000 Da. Hence, SIMS is generally applied to analysis of smaller biological analytes such as phospholipids [8, 9], metabolites [10] or surface material analysis [11, 12]. Additionally, as the ions are created and ejected from the surface in a highly destructive manner, as well as the implanting of the primary ions, the sample is quickly denatured by the process.

(Desorption) Electrospray Ionisation

Electrospray ionisation is a technique particularly suitable to volatile liquids or easily solvated solids. The analyte or analyte/solvent solution is passed out of a charged needle. The solution becomes highly charged from this process, which leads to its dispersion into small droplets and the expansion of the resultant jet by Coulombic repulsion. Subsequent vaporisation of the solvent leaves ionised analytes which may then be passed to the mass analyser. The soft ionisation of this technique makes it particularly suitable to samples of high molecular mass such as proteins [13], which are easily fragmented by harder ionisation techniques.

The related technique of desorption electrospray ionisation (DESI) [14, 15] does not mix the analyte with the solvent in the emitter needle. The solvent itself is ionised, and the sample is sprayed with the resulting ion jet. A nebulising gas is usually added around the emitter to direct the ion jet (Fig. 1.2). An ionised solvent layer is formed on the surface of the sample, and the analyte is solvated in this layer. The resulting charged analytes are then extracted with an electric field and then mass analysed. The main advantage of DESI is that it may be used in ambient conditions, without a need for any sample preparation in the vacuum. However,

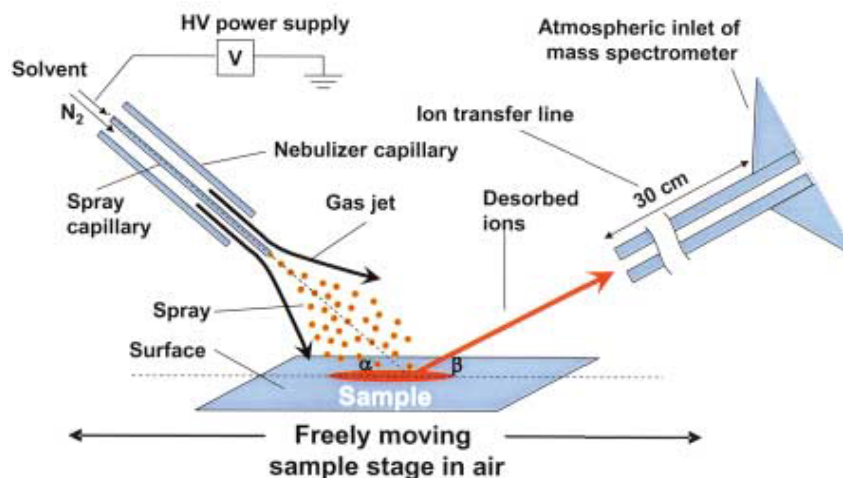


Figure 1.2: Diagram illustrating the principles of desorption electrospray ionisation. A stream of solvent is emitted from a charged needle producing an ionised jet. The charged solvent forms a layer on the sample and solvates a small amount of the sample, passing some charge to the analyte. The charged particles are transferred to the mass analyser. Adapted from Ref. [14]

the ions produced are usually multiply charged, complicating analysis, with larger analytes having even more complex charge distributions. The multiple charges also lead to increased fragmentation of the molecules of interest.

Laser Desorption/Ionisation

Lasers have also been widely used as a method for desorption and ionisation of analytes from a sample. The simplest method of laser ablation (LA) in mass spectrometry involves the total ablation of a sample with a high flux [16]. This results in high fragmentation with low analyte yield, and is generally used for quantitative analysis of elemental analytes. Laser desorption/ionisation (LDI) is differentiated from laser ablation by using a reduced laser flux, producing less fragmented and larger molecular ions at the cost of further reduced signal [17].

The addition of a matrix with a high absorption cross-section at the laser wavelength prevents damage to the analyte, as it absorbs the majority of the power. A carefully-chosen matrix can act to produce ionised analyte on vaporisation. This greatly improves the signal and softness of the ion creation technique, allowing the

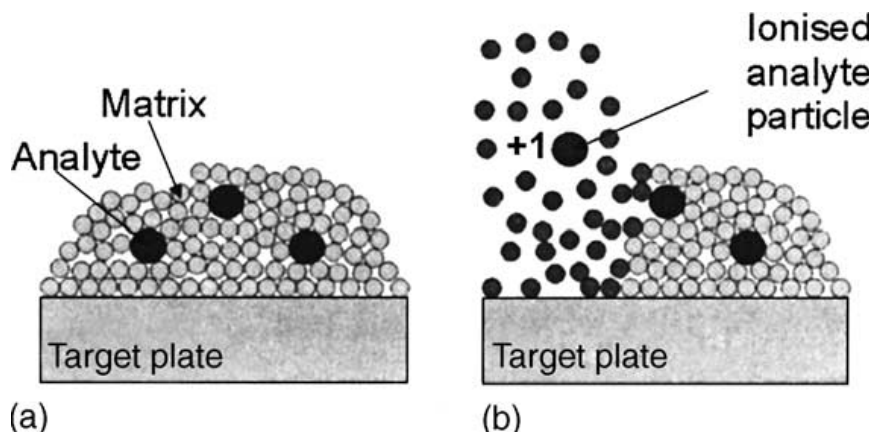


Figure 1.3: A diagram illustrating the principle of MALDI. (a) Analytes are mixed into matrix at a low concentration and crystallised on the surface. Laser light is absorbed primarily by the matrix and some of the sample desorbs from the surface (b). The vaporisation of the sample releases charged analytes. See Sec. 5.1.2 for more details on the MALDI mechanism. Adapted from Ref. [18].

reliable production of singly-charged proteins up to the megadalton mass range. This “matrix-assisted” laser desorption/ionisation (MALDI) is one of the most widely applied techniques for solid-state biological analysis. The mechanisms and applications of MALDI will be discussed in detail in Sec. 5.1.

Another method of reducing the required laser flux to ionise and desorb analytes from a surface have been to make use of surface effects. “Surface-enhanced” laser desorption/ionisation (SELDI) involves the use of specially-prepared surfaces which are highly absorbent at the chosen laser wavelength. A variety of effects, ranging from thermal excitations to surface plasmons facilitate the ionisation and/or desorption. Examples of materials used for SELDI include porous silicon [19], gold nanoparticles [20], and silicon nanowires [21–23]. Applications and mechanisms of surface-enhanced laser desorption/ionisation, for desorption/ionisation on porous silicon (DIOS) in particular, are described in detail in Sec. 4.1.

1.1.2 Mass Separation

Principles of Mass Separation

After the ions have been created, the aim is to separate them from each other by their masses. Many methods and apparatus have been devised, but they all rely on the physical separation of ions based on their mass's characteristic inertia. By applying a force on the ions with an electric or magnetic field, ions with different masses or charges will experience different accelerations according to the Lorentz force law,

$$\mathbf{F} = q(\mathbf{E} + \mathbf{v} \times \mathbf{B}), \quad (1.1)$$

where $\mathbf{F}$ is the resultant force on the ion, q the charge on the ion, $\mathbf{E}$ the strength of the electric field, $\mathbf{v}$ the velocity of the ion, $\mathbf{B}$ the strength of the magnetic field, and $\mathbf{v} \times \mathbf{B}$ the cross product of the two. Combination with Newton's second law gives acceleration, $\mathbf{a}$:

$$\mathbf{a} = \left(\frac{m}{q}\right)^{-1} (\mathbf{E} + \mathbf{v} \times \mathbf{B}). \quad (1.2)$$

It is important to note that without prior knowledge of the charge on the ions, q , the separation will be based not solely on the mass, but instead on the mass to charge ratio, m/q . More commonly, m/z is used, where z is the number of charges on the ion ($z = q/e$; e is the charge of the electron). The advantages of an ionisation method producing mainly singly-charged ions can be seen from this, as analysis would give only mass dependent results.

The types of mass separation methods are too numerous to describe in detail here, but include magnetic and/or electric sectors [24], quadrupole mass filters [25], ion traps [26, 27] and Fourier-transform ion cyclotron resonance (FT-ICR) [28]. These techniques can be combined sequentially, with or without additional fragmentation steps in between the mass separation stages. For example, stereoisomers with the

same m/z ratio may fragment differently, and thus may be differentiated by such MS/MS techniques. Combinations with other analytical techniques are also possible, with the mass analysis of the separate products of separation by methods such as liquid chromatography (LC/MS) common [29–31].

Time-of-Flight Mass Spectrometry

The method of mass analysis used in this work is that of time-of-flight mass spectrometry (TOF-MS). This is the method of choice for ion imaging as it provides a theoretically limitless mass range, while carefully-constructed ion optics may preserve the spatial distribution of the analysed ions.

The ions created in the mass spectrometer are accelerated by an electric field. Different ions reach different final velocities v due to differing m/z ratios. For an accelerating potential difference U , ions gain a kinetic energy $E_k = zeU$. Since $E_k = \frac{1}{2}mv^2$,

$$v = \left(2eU \frac{z}{m}\right)^{\frac{1}{2}}. \quad (1.3)$$

The ions are allowed to fly along a field-free drift region, of a certain known length L . Assuming that the acceleration time is much less than that spent in the flight drift region, this gives a flight time of

$$t = L \left(\frac{1}{2eU} \frac{m}{z} \right)^{\frac{1}{2}} \quad \text{or} \quad \frac{m}{z} = 2eU \left(\frac{t}{L} \right)^2. \quad (1.4)$$

Thus, by measuring the time taken to arrive at the detector after acceleration of the particles, it is possible to determine m/z of the ions. In practice, an oscilloscope or other transient recorder is coupled to the detector to give a direct measurement over the time-of-flight period. A schematic of the process is shown in Fig. 1.4.

In the application to MALDI, the velocity distribution of the ions created by desorption of the sample will have a detrimental effect on the mass resolution. The effect of the spread may be reduced by the increase of the accelerating potentials

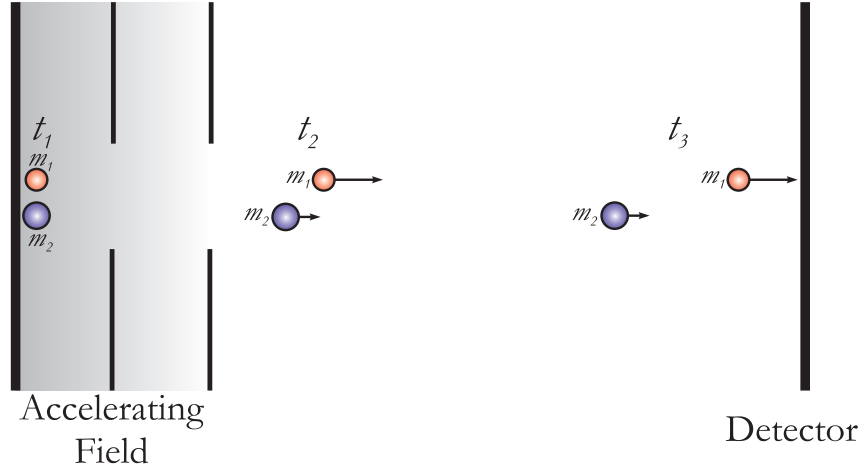


Figure 1.4: A schematic of the TOF-MS process in linear mode. The ions m_1 and m_2 are created at time t_1 within an electric field. m_1 has a lower mass than m_2 . On leaving the accelerating field (t_2), the ions have velocities $v \propto (1/m)^{1/2}$. The ions separate out in space due to different velocities of travel down the flight tube (t_3). The different arrival times at the detector are recorded and used to determine m/z .

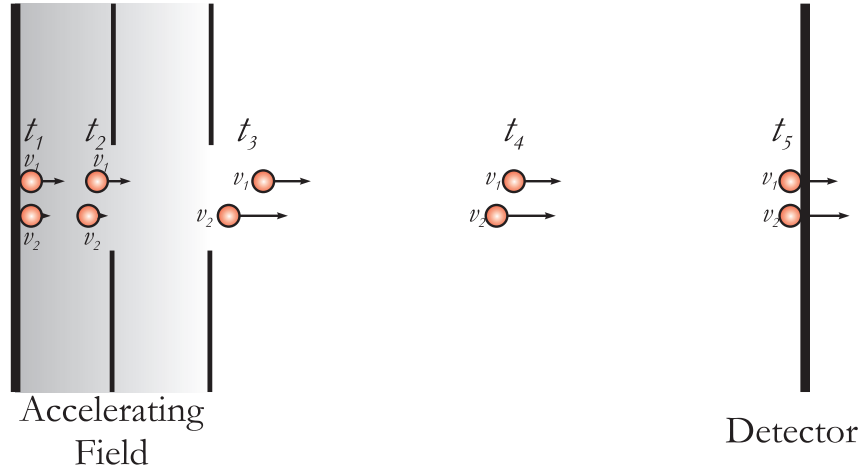


Figure 1.5: Diagram illustrating the principles of delayed extraction. At time t_1 , the nascent ions v_1 and v_2 (with the same mass) have slightly different initial velocities. They are allowed a short amount of time to drift within the interaction region with no field. At time t_2 , the accelerating potentials are turned on and the ions accelerated out of the field. Ions nearer the repeller experience higher potentials and reach higher velocities upon exiting the field (t_3). The ions travel at their given velocities along the drift tube (t_4). The ions will at some point (t_5) reach the time focus plane. The detector would ideally be placed at this point to maximise mass resolution.

(see Sec. 2.1.6). Another technique to compensate for this is the delayed extraction [32] of the ions from the interaction region (Fig. 1.5). In this, the interaction region is kept field-free for a period of time, allowing the ions to spread out according to their velocities. Ions with higher velocities in the time-of-flight direction (z) will travel further towards the detector. After a certain amount of time, the accelerating potentials are turned on, and depending on their positions at that time, the ions will experience different potentials. Initially slower ions further from the detector will experience a higher field, while faster ions have a lower field. These will therefore achieve different final velocities, and a carefully-chosen time delay will result in a time focus plane that coincides with the detector plane, producing a good time and hence mass resolution for each mass.

Additional ion optical elements can also be used to compensate for the velocity spread of ions. A reflectron, first described by Mamyrin *et al.* [33], is in its simplest form a set of electrodes creating a strongly decelerating uniform field. This is used to turn the ions back on themselves in a time-of-flight spectrometer. As higher-velocity particles take a longer time to turn around, a correctly-tuned reflectron will return ions onto a time-focus plane on the detector. Reflectrons have been successfully used in conjunction with velocity-mapped imaging [34], and by extension may potentially be applied to spatial-mapped imaging.

Time focussing in TOF-MS can also be improved by the introduction of electrostatic and magnetic sector analysers. Careful construction of these systems provide not only time focussing but also compensate for aberrations in spatial imaging, such as in the stigmatic imaging system developed by Schueler [35]. More recently, a mass resolution, $m/\Delta m$, of 350,000 was reported by Toyoda *et al.* with a multi-pass sector loop [36, 37]. However, this came at the cost of the quality of the spatial image (see Sec. 1.3), with increasing distortions for each additional pass of the loop [38].

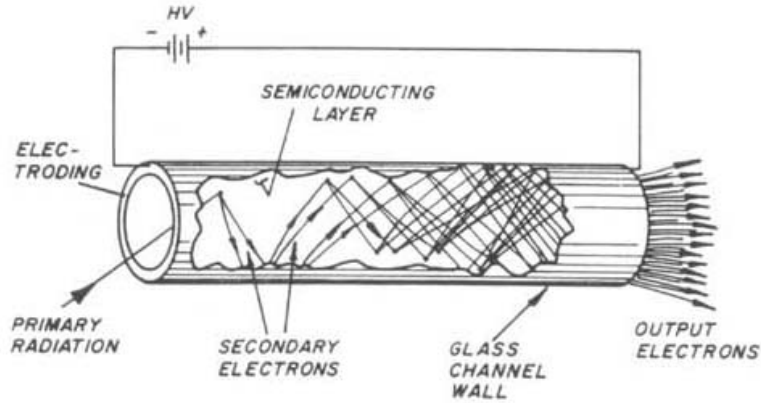


Figure 1.6: Illustration of the principles of microchannel plates. One channel is shown. An impinging ion or radiation releases secondary electrons from the semiconducting layer in the channel. The electron is drawn further into the channel by the bias and causes further secondary electrons. The electron cascade continues through the tube. Adapted from Ref. [39]

1.1.3 Ion Detection

After the masses have been separated, their arrival times and/or positions must be detected and recorded. Due to the small charge of each individual ion, some form of electron multiplying method must be utilised to convert the impact into an electrical signal that can be manipulated. In application to mass spectrometry and time-of-flight ion imaging, the most common approach is the use of microchannel plates (MCPs).

Microchannel plates are essentially plates of glass made up of numerous aligned tubes (channels). These usually have diameters in the range of $2\text{--}10\ \mu\text{m}$, and are angled at about $10^\circ\text{--}20^\circ$ to the normal. Close packing of the channels leaves approximately 60% open area on the surface. When a bias is applied across the plate ($\sim 1\text{ kV}$), an ion impacting inside of one of these tubes may eject secondary electrons (Fig. 1.6). These are drawn further down the tube by the bias and create more secondary electrons. This creates a cascade of $\sim 10^3$ electrons. Additional plates may be added to further amplify the number of electrons; typically these are arranged with the channels angled in opposite directions, leading to two-layer “chevron” and

three-layer “Z” MCP stacks. Each further plate gives an additional gain of around $\times 10^3$ electrons. MCP systems have high response times, in the order of 1 ns, but these may be compromised by detector saturation [40]. Hence, signal (and noise) levels must be carefully controlled, particularly in the case of MALDI MS in which a large amount of matrix signal is observed. Generally, the laser flux is best held at just above the desorption threshold and the signal integrated over multiple shots.

If the main information of interest is the time of arrival, the electrons produced from the MCPs may be collected by an anode, producing a signal integrated over the whole detection area of the MCP. This can then be amplified and recorded. If information on the position of the ion impact is desired, a two-dimensional detection system is required. The electrons emitted from the back of the MCPs may be detected directly [41], or, more commonly in ion imaging systems, the MCPs coupled to a phosphor screen. The screen flashes when struck by the high-energy electrons, and these flashes may then be captured with a camera. The time resolution is then dependent on the capabilities of the camera, as well as the decay times of the phosphor. The majority of detection systems are, in this application, effectively mass selective: a period of the time-of-flight is selected, by gating of the MCPs or camera, which produces an integrated image of a certain mass window [42].

1.2 Ion Imaging

The behaviour of charged particles through an aperture of a charged electrode was first described mathematically by Davisson and Calbick in 1931 [43]. It was found that the interaction of the electrodes with the ions was similar to that of simple geometric optics: a slit behaved as a cylindrical lens, while a round aperture acted as a spherical lens. The focal length was determined by the potential of the electrode and the strength of the fields on either side of the electrode. The lens acted to form real and virtual images depending on its convergent or divergent nature.

In 1960, Castaing *et al.* [44] described the use of these ion optics in conjunction with SIMS. The authors identified the masses of isotopes of metals such as Al^+ and

Mg^+ as well as their hydroxide ions $(\text{MgOH}_2)^+$ and $(\text{AlOH}_2)^+$. The authors later combined this with a magnetic sector and reflectron TOF-MS to acquire spatially-resolved distributions of ^{24}Mg , ^{27}Al , and ^{28}Si from a sample alloy of the three metals [1]. The magnetic sector served to remove chromatic aberrations, which together with a stigmatic corrector produced achromatic and stigmatic images. A fluorescent screen was used to detect the high-energy ions ejected from the sample, and the image captured through a side window *via* a mirror.

In 1978, Slodzian [45] described the characteristics of the backfocal plane (pupil plane) in electrostatic optics. It was noted that the dimensions of the pupil depended on the initial energies and directions of the ions. Thus, ions with the same magnitude of velocity in the sample plane, for example, would be projected on a circle in the pupil plane. In the case of microscopy, this plane is used as the position for an aperture stop, reducing high transverse velocity and particles and non-paraxial rays, reducing aberrations in the resulting image.

Separately, in 1955, Wiley and McLaren [32] developed methods, using uniform accelerating potentials applied to grids, to direct the products of fragmenting molecules. This was adopted by Chandler and Houston [46] in order to investigate the angular distribution of products from fragmenting molecules. The entire three-dimensional distribution of the photofragments were thus projected onto a two-dimensional detector consisting of a MCP/phosphor array with either polaroid film or reticon array. Eppink and Parker [47] improved on the resolution of this technique by the removal of the grids from the electrodes, effectively using an electrostatic aperture lens to project the backfocal plane onto the detector. This ensured that, to first approximation, the arrival positions on the detector were dependent only on their initial velocities and not on their point of origin. This compensated for the finite interaction region of the lasers with the molecular beams.

This “velocity-mapping” technique is used by the majority of apparatus in the investigation of molecular dynamics to date, with the recoil velocities of photofragments giving valuable information on angular momentum polarisation and reaction

mechanisms [46–48]. The initial and final quantum states of the reactants may be fully defined by careful preparation of the molecular beam and methods such as resonantly-enhanced multi-photon ionisation (REMPI) [49]. Images of the angular distribution of each fragment allow the angular momentum polarisation parameters to be extracted, shedding light on the nature of the potential energy surfaces involved and the mechanism of reaction. The addition of femtosecond laser pump-probe techniques led to the ability to study coherent excitations and directly image the nuclear wavefunctions through the course of the reaction [50]. An example of an analysis to extract angular momentum polarisation information is discussed in Sec. 3.3.

1.3 Spatial Imaging Mass Spectrometry

1.3.1 Advantages

There are a number of advantages in the use of spatial imaging mass spectrometry as an analytical method. The high detection efficiency of the microchannel plates allows the detection of potentially very low concentrations of the analytes of interest. Concentrations that would not be detectable to the eye in optical staining or marking can be picked up with sensitive MCPs. The mass separation improves the signal-to-noise ratio in the region of the mass of interest: large amounts of another analyte which could potentially swamp the signal are removed from the data by mass selection. The number of ion impacts in the spatial distribution provides a measure of the concentrations of the particular analyte in an area of the sample, allowing easy identification of correlations between analytes and local features.

Mass spectrometric imaging is also label-free in nature. Most forms of biological imaging, such as in staining, or fluorescence and confocal imaging [51] require the introduction of markers to make specific areas of interest visible. This may be dyes specific to certain cell areas (*e.g.*, H & E staining) or cell types (*e.g.*, Gram staining). The identification of more specific targets, such as particular proteins,

requires synthesis of custom antibodies which contain fluorescent of visible pigments. These antibody markers attach to the target analyte and indicate the presence of the target. In contrast, mass spectrometric imaging in principle requires no such markers, with the identity of the analytes determined from their masses.

Finally, the lack of a need for marker preparation or analyte precognition allows for automation of the sample analysis: a sample can be mounted, placed in the apparatus, and the spatial distributions of its composite masses determined automatically by the apparatus.

1.3.2 Approaches

There are two general approaches for spatial-mapped imaging mass spectrometry that are commonly used, namely microprobe mode and microscope mode IMS [52]. The general principles of each of these methods will be described here together with their particular advantages and disadvantages.

Microprobe Mode

In microprobe mode, a small area of a sample is analysed at a time. This area of the sample may be ionised by, for example, a focussed laser or ion beam. All the ions produced from the desorption are then passed to a mass analyser, and a full mass composition of the sample corresponding to that point acquired. The point of desorption is then altered, by the movement of either the sample or the ionising beam. Another sample is then acquired for the new area of the sample. By rastering the ionising area over the entire sample, a collection of m/z compositions for each x and y coordinate may be acquired. By treating each ionisation point as a pixel, spatial distributions of each mass may be reconstructed, giving a complete description of the sample. A schematic of the process is shown in Fig. 1.7.

From the principles of the method, it can be seen that the spatial information for the acquired data is based on the position of the ionising area on the sample. Thus, the resolution will be limited either by the distance moved by the raster or

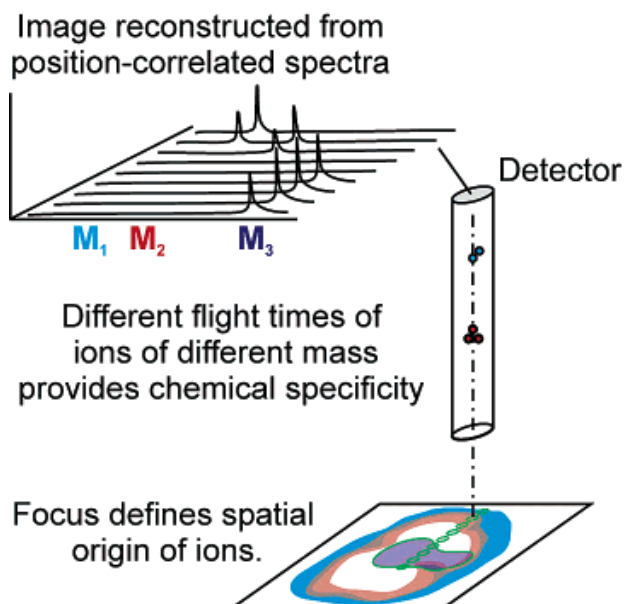


Figure 1.7: A schematic showing the principles of microprobe-mode imaging mass spectrometry. The mass spectrum of each spot is collected separately, and the full $x, y, m/z$ distribution is reconstructed with each mass spectrum as a pixel on the final image. Adapted from Ref. [52].

the ionising area of the sample, whichever is the greater. A large movement of the ionising beam analyses a well-removed part of the sample, and may potentially miss information between the two points. If the ionisation area is large, spatial details will not be resolved as all ions produced in the ionisation region will be passed to the mass analyser with the same spatial information. A consequence of this is that attaining resolution below the diffraction limit of light is a significant challenge. Methods involving oversampling, in which the ionisation areas of sequential ionisations overlap, have been developed in an attempt to circumvent this limitation [53, 54].

The number of experimental cycles required to characterise a sample with microprobe mode is also closely tied to the resolution desired. A mass spectrum is required for each “pixel” of the spatial distribution, and a larger number of these pixels requires a greater amount of acquisitions. For a given pixel pitch on the sample, then, the number of acquisitions and hence collection time will scale with the

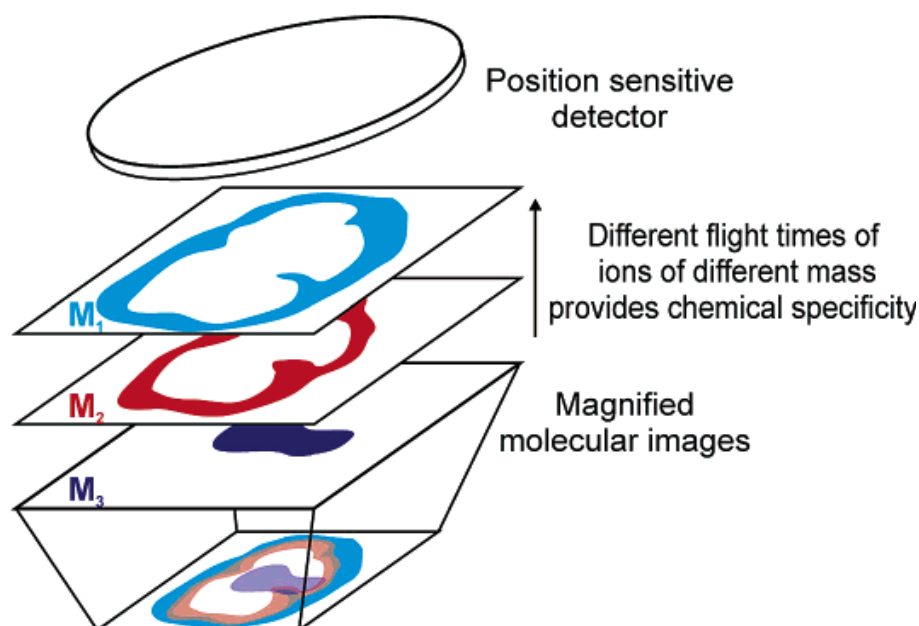


Figure 1.8: A schematic showing the principles of microscope-mode imaging mass spectrometry. A large area is irradiated with a defocussed ionisation beam. The desorbed ions are separated by mass and projected on to a two-dimensional detector retaining their x/y spatial information. Adapted from Ref. [52]

square of the field-of-view. Typical acquisition times for a sample of 6×6 mm range from 40 minutes to 4 hours [55, 56].

An advantage of microprobe mode is its compatibility with any method of mass analysis, and consequently has mass resolution limited by the state-of-the-art of mass spectrometry. The ability to adapt high-resolution mass spectrometry to conduct spatial imaging has lead to microprobe-mode being the dominant analysis method for biological samples, in which a large number of mass peaks may be present and the ability to collect all of them simultaneously is advantageous.

Microscope Mode

Microscope-mode imaging mass spectrometry operates the apparatus as an optical microscope. A large area of the sample is irradiated by a defocussed laser or ion beam. The irradiated area may cover the entire field of view. In the cases where the area is smaller than the field of view, several cycles may be used to image the entire

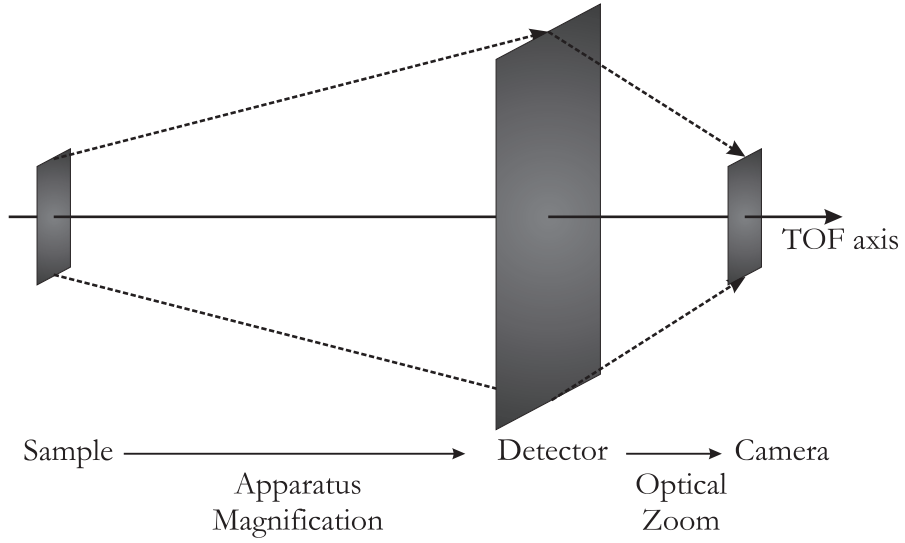


Figure 1.9: Diagram illustrating effect of magnification, pixel resolution and field-of-view on the spatial resolution of microscope-mode imaging. The sample distribution is projected onto the detector with magnification determined by the design of the apparatus. The detector then bins the signal within the field of view into a grid depending on the number of pixels available. The size of the area covered by each pixel is dependent on the magnification of the apparatus and, in the case of phosphor/camera arrangement, the optical zoom of the camera.

sample, and post-analysis is required to join the images together (“stitching”) [57]. The entire irradiated area produces ions from the surface of the sample. The ions thus released from the surface are steered through ion optics designed to project them onto a two-dimensional detector. Ideally, the arrival positions of these ions on the detectors, upon passing through these optics, are determined solely by their point of origin of the sample. In the course of passing through the ion optics, the ions are also separated, usually in time, by their mass. By selecting the time of acquisition, the x/y distributions for ions of particular m/z may be selected. A schematic of the microscope-mode method is shown in Fig. 1.8.

A major advantage of microscope-mode imaging is the potential for the spatial resolution to be lower than that determined by the diffusion limit, as the determination of the x/y coordinates does not rely on the focus of the laser beam. In this case, the limitations are the pixel resolution of the detector and the aberrations introduced by the ion optics. A two dimensional detector will have a set number of

pixels into which it may resolve the image. Even in the case of non-pixel-based detectors (*e.g.*, delay lines), there will be a limit to the resolving power of the detector itself. Thus, the detectors will effectively bin the signal they acquire within their field of view into a grid, the coarseness of which corresponds to its pixel resolution. The area of the sample each pixel corresponds to then depends on the magnification of the apparatus (Fig. 1.9). On top of this, ion optics design may limit the ability of the apparatus to map the ions accurately to the detector, due to differing initial recoil velocities and energy. These effects are further discussed in Sec. 2.1.6. However, these limitations notwithstanding, spatial resolution to below the airy radius of the ionising laser has been demonstrated by Luxembourg *et al.* [58].

Due to the required retention of spatial information of the ions as they pass from the sample to the detector, the mass analysis techniques that are compatible with microscope mode are limited. Generally, techniques requiring RF fields will spoil the trajectories as the ions' paths lose their spatial relation as they oscillate within the apparatus. Thus, many signal- and resolution-improving techniques such as ion traps and (Fourier transform) ion cyclotron resonance are precluded from microscope-mode imaging. Time-of-flight mass separation remains as the typical mass separation method, together with electrostatic or magnetic sector analysers to reduce the effects of aberrations [35, 44, 59].

A limitation of microscope-mode imaging experiments is that the majority of two-dimensional detectors used are mass selective [42], *i.e.*, the detectors will collect information over a certain mass window, while discarding the rest of the signal. With the signal collected by a camera following the phosphor screen, the detectors integrate the signal received over a set period of time. This period is determined by the gating of the camera, the MCPs, or the ions. In the case of electrostatic or magnetic sectors, one particular mass may be passed through the analyser to be imaged at a time. In effect, this has so far limited the acquisition to one particular mass peak per experimental cycle. In order to acquire more than one peak with mass selective detectors would mean widening the mass selection window, giving the

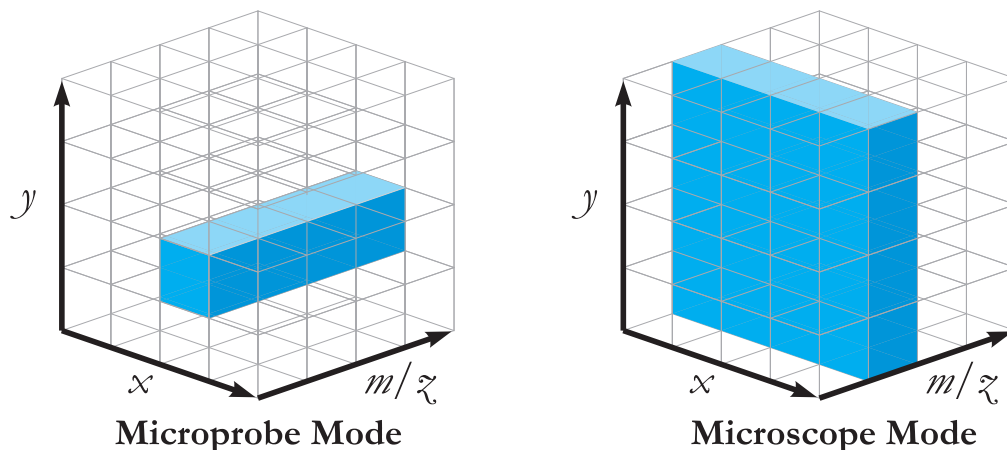


Figure 1.10: Diagram illustrating data collection of microprobe mode and microscope mode imaging mass spectrometry. Blue voxels represents data collected with information x , y , and m/z . In microprobe mode mass spectrometry, all m/z data for a given x, y are collected in each experimental cycle. In conventional microscope mode, all (x, y) data are collected for an m/z window.

overlaid distributions of the two, without any way of separating the distributions by mass. Thus, the time required to collect a complete set of data for a sample then scales with the number of mass peaks of interest. Additionally, the mass to be collected must be identified and selected beforehand. Finally, in cases where there are a large number of closely-spaced mass peaks within a small mass window, the separation of these becomes a significant challenge.

An illustration of the principles of microprobe-mode and microscope-mode mass spectrometry is shown in Fig. 1.10. Effectively, microprobe-mode imaging mass spectrometry in conventional form collects intensities for every m/z for one x and y coordinate at a time, while microscope-mode collects intensities for all x and y for one m/z at a time.

1.3.3 Applications

The field of spatial imaging mass spectrometry has been subject to much attention in the past fifteen years. In 1997, Caprioli *et al.* [2] applied MALDI as a method of ion generation in imaging mass spectrometry. The suitability of MALDI to the

desorption of high-mass proteins of over 50 kDa with little or no fragmentation made this of great interest in biologically-related fields. The ability of imaging mass spectrometry to visualise the distribution of a large variety of analytes makes it a versatile tool [56, 60–62]. Much investigation has been performed in the fields of disease pathology [63, 64], proteomics [65], oncology [66], pharmacokinetics [67], and metabolomics [68]. The applications are too numerous to describe in detail in this thesis, and the reader is directed to the reviews above for full details on previous work. However, a few examples will serve as a demonstration of the scope of the technique.

In 1998, Braun *et al.* [69] presented a microscope-mode chemical imaging time-of-flight spectrometer. The unique features of this apparatus lay in its wide field-of-view (~ 2 mm diameter), and its cryogenic sample handling capability. The cryogenic stage was designed to overcome the room-temperature requirement of many organic samples. This apparatus utilised a liquid metal ion gun (LMIG) as an ionisation and desorption mechanism. The diameter of this beam was insufficient to desorb secondary ions from the entire field of view simultaneously, and so was rastered over the sample. Ion optics mapped the desorbed ions onto a two-dimensional MCP detector. A variety of low-mass analytes were investigated with this apparatus, such as Cu^+ ions from grids, and stearamide from polystyrene beads. A mass resolution of $m/\Delta m = 900$ was reported at a mass ~ 280 Da, and $m/\Delta m = 750$ at 28 Da. The authors described a trade-off between the field-of-view and mass resolution. The apparatus was also suitable for post-ionisation studies, with laser irradiation being introduced to overlap the desorption plume from the surface. The spatial distribution of the ion depletion could be successfully imaged.

Fletcher *et al.* [70] used buckminsterfullerene C_{60} as a primary ion source for the “3D” imaging of single-cells, oocytes, in 2007. The use of C_{60} provided a primary ion source capable of generating large degrees of secondary ions. Using microprobe-mode SIMS on a single cell, a beam of C_{60} was focussed to a spot size of $5\text{ }\mu\text{m}$. The beam was used to simultaneously desorb analyte from, and to etch, the sample.

The mass distributions for each layer could then be reconstructed into a three-dimensional data volume. Thus, the molecular distribution throughout the sample could be characterised. However, the authors described difficulties in desorbing intact proteins due to the SIMS method, as well as the disturbances of distributions by sample preparation and matrix effects. Finally, much longer analysis times were required by this method, with large amounts of data generated.

Finally, in 2011, Römpp *et al.* [71] applied imaging mass spectrometry to determine the drug distribution in tissue sections. Using “high mass resolution and high spatial resolution”, or “HR²”, the spatial distribution of anti-cancer drugs in mouse organs was determined. The “HR²” refers to the combination of sub-ppm mass resolution and spatial resolution of 5 μm . These high resolutions allowed the histological analysis of the organs. The positions of the anti-cancer drugs imatinib and ifosfamide were localised in mouse kidney, and the distributions confirmed with MS/MS and post-analysis histological staining.

1.4 Fast Detectors in Imaging Mass Spectrometry

A major limitation of microscope-mode imaging has been the mass-selective (as opposed to mass-determining) nature of the detectors. This has resulted in, effectively, the image of only one mass to be imaged per duty cycle. A two-dimensional detector fast enough to record multiple mass arrivals within the time-of-flight period while retaining m/z or time information would provide a number of advantages.

The most immediate advantage of such a “fast detector” would be the reduction in collection times. While current two-dimensional detectors require a separate acquisition run for each mass peak of interest, a fast detector would improve the analysis time by a factor equalling the number of masses imaged, as each of these images may be collected within the same sample. This time saving has a number of beneficial consequences in addition to improved convenience. Many biological samples are temperature sensitive, degrading quickly at the room temperature conditions required to run mass spectrometers [65]. The faster acquisition would therefore

reduce this damage to such delicate samples. The reduction in acquisition cycles also decreases the number of ionisation events required to analyse the sample. Both LDI and SIMS result in degradation of the sample, and reduction of the number of ionisation events required for a full characterisation gives less damage to the sample. The characterisation thus has a greater chance of completion before significant degradation occurs. This may be useful in sequential analysis by multiple techniques such as those described by van Hove *et al.* [72]. In this multi-modal analysis, the same sample is used multiple times for different techniques, and is washed and re-prepared between each analysis. Evidently, the less damage incurred at each step of the process, the more accurate the analysis of the sample.

In addition to the decreased acquisition time, there are a number of other advantages to the application of fast detectors to ion imaging systems. Fast detectors collect images in “real time”, *id est* without having to collect the data multiple times to undergo reconstruction post-processing. This complements the label-free nature of spatial imaging mass spectrometry: a fast detector could potentially begin acquisition upon irradiation of the sample, and image every mass distribution produced from the sample. By using such a collection method, no prior knowledge of the masses at which to collect the images is required. To date, although cameras with high frame rates have been used in conjunction with microscope mode TOF-MS [52], these have not been fast enough to obtain acquisitions of multiple masses within the same time-of-flight window.

Fast-Framing Cameras

Improvements in cameras have recently produced cameras with extremely high frame rates, of up to a hundred megaframes per second (100 Mfps). This corresponds to a time resolution of 10 ns, short enough to acquire multiple frames over a time-of-flight mass spectrum, typically lasting in the order of 10^{-5} s. The state of the art of fast-framing cameras has been recently reviewed by Son *et al.* [73], and is discussed in Sec. 6.1.2. The use of these cameras is one possible method of combining fast detec-

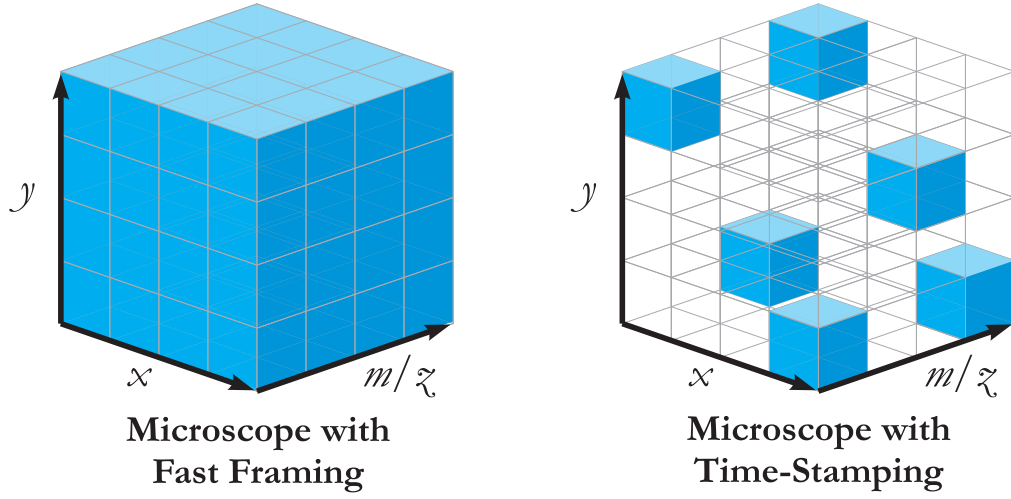


Figure 1.11: Diagram illustrating data collection with microscope mode using fast-framing and time-stamping detectors. Fast-framing cameras (ideally) record all data for x , y and m/z , creating large amounts of (mainly null) data for analysis. Time-stamping detectors record points across the whole of the data space, but only for the non-null data.

tors with microscope-mode imaging. The detectors could collect sequential frames over the time-of-flight of the different masses and record images at each time-step. This is represented schematically by “Microscope with Fast Framing” in Fig. 1.11, in contrast to both “Microprobe Mode” and “Microscope Mode” in Fig. 1.10, in which either a position or a mass had to be chosen beforehand. A point to note is that the $\Delta x, \Delta y$ voxel size in this case is determined by the pixel resolution of the detector and the aberrations introduced by the ion optics, while $\Delta m/z$ is determined by the framerate of the fast camera.

In practice, there are a number of challenges faced by the utilisation of fast-framing detectors. The majority of these detectors which may be used in conjunction with microscope-mode imaging are based on charge integration, transfer, and storage on-chip. Limitations in storage space in turn restrict the number of frames which may be stored on-chip before the lengthy read-out process. Currently, the number of frames that may be stored on-chip is limited to approximately 100 [74–76]. Each of these frames represents a period of integration time during the acquisition cycle, with the acquisition duration the total of the frames’ individual durations. Thus, the

time resolution and total acquisition window must be traded off against each other: a longer time for each frame would increase the acquisition window but reduce time resolution, and *vice versa*.

A possible solution to this was presented by Brouard *et al.* [77], in which short acquisition times of frames have been programmed around each mass peak of interest while using longer sacrificial frames between peaks to remove noise and extend acquisition time. However, this then means that extensive knowledge of the time-of-flight spectrum is required before the image acquisition step.

Finally, acquisition over the whole time-of-flight period generates large volumes of data. This is challenging in terms of data read-out from the chip. Limits to data transfer out of the chip to a controller for storage and processing results in a major bottleneck to the speed of acquisition. Additionally, these large amounts of data become difficult to store and analyse.

Time-Stamping Detectors

Considering the relatively low proportion of the area illuminated within each image acquired during an ion imaging experiment, a large proportion of this is null data, which is stored needlessly. In this case, a different approach to image acquisition may be used to solve the above-mentioned problems. Time-stamping detectors compare the signal received in their active area over the entire acquisition period. Upon detection of signal above a pre-set threshold, a “hit” is registered. The spatial x/y position of the detected signal on the screen is recorded, together with its time of arrival (t). The mass accuracy of the system is determined by the detector’s clock. This is represented by “Microscope with Time-Stamping” in Fig. 1.11. In comparison to “fast-framing”, whilst the $\Delta x, \Delta y$ resolution of the voxel is also dependant on the pixel resolution and aberrations of the apparatus, $\Delta m/z$ is now dependent on the internal clock of the timestamping detector.

The advantage to this approach is that data is only recorded when signal is observed and so the recording of null data is avoided, reducing the data load. This

reduction in the amount of information that has to be stored both on-chip and transferred through its readouts greatly extends the available acquisition window. However, as for the fast-framing detectors, the information must be stored on-chip before readout. The state-of-the art of time-stamping detectors and comparisons between them are discussed in detail in Sec. 6.1.2.

1.5 Summary and Outline of Thesis

In this thesis, proof-of-concept experiments determining the feasibility of the combination of fast detectors with time-of-flight mass spectrometry described in this chapter. These have been performed on an adapted conventional velocity-mapped time-of-flight ion imaging apparatus. Laser desorption methods have been chosen for ion production from the sample due to their ability to irradiate large areas. TOF-MS methods have been used for mass analysis, with their potentially unlimited range and compatibility with imaging mass spectrometry. Chapter 2 describes calculations and simulations used to determine the suitability of the apparatus to spatial imaging and the trajectories of ions during operation. The information is used to determine the theoretical limits to resolution of the apparatus. Chapter 3 describes the experimental set-up, including characterisation of the detector system and methods for data analysis. An analysis of a typical example of velocity-mapped imaging experimental data for which the apparatus was initially constructed to collect is presented. Chapter 4 describes initial experiments testing the accuracy of the simulations in Chapter 2. Chapter 5 presents experiments using matrix-assisted laser desorption/ionisation (MALDI) designed to determine the resolution of the experiment, and compares those with the results described in Chapter 2. Chapter 6 demonstrates the application of fast detectors to imaging mass spectrometry and its advantages. Finally, Chapter 7 presents preliminary experiments in the imaging of biological samples as an example of a field in which the combination of fast detectors and microscope-mode imaging mass spectrometry may prove beneficial.

Chapter 2

Simulation of Ion Trajectories

There are two main methods which may be used to theoretically determine the paths of ions taken through a set of electrostatic lenses and the imaging apparatus: transfer matrices and field simulation methods. There are a number of advantages to both of these methods.

Transfer matrices are used to describe the position and direction of motion of charged particles as they move through the fields created by elements of the ion optics. Each element of a set of ion optics, such as the aperture, drift space, and electric field may each be described by a single matrix. The behaviour of a combination of these elements is easily described by matrix multiplication of the respective elements. These are therefore very useful in conducting quick calculations, as well as in designing a set of ion optics. However, in order for the transfer matrix method to be tractable, a number of assumptions usually have to be made. These result in the method producing only first-order predictions for the trajectory: it is unable to determine the extent of the higher order effects which lead to chromatic and spherical aberrations. Additionally, the calculations are for the trajectories, the “rays” of the particles: it is not time dependent and provides no information on the times taken for each trajectory or any changes in the field over the flight period.

Field simulation programs create a three-dimensional model of the electrostatic fields that would be produced by the ion optics and apparatus. By inputting the dimensions of the elements of the ion optics and their potentials, their effect on the

field within the experiment may be determined. By setting initial positions and velocities of ions, their acceleration due to the field may be calculated. The ions are allowed to progress for a small time step, and their velocities again recalculated at their new position. This method of determining the trajectories, unlike the transfer matrix method, can take into account the higher order effects leading to aberrations. Additionally, the time-dependent positions are determined and so the temporal resolution of the apparatus can also be determined. The effect of a changing potential can also be modelled. The disadvantages of field simulation methods are that many more calculations are required for each time step and as such the simulations are limited by both the calculation speed and processing power of the computer: larger apparatus or more finely-grained modelling require much greater processing power and time. Additionally, its modelling nature means that the method may only be used to predict the trajectories for a given path, but is not particularly useful for determining the dimensions required for a new set of ion optics.

In this chapter, the transfer matrix method described by Liebl [78] will be applied to the apparatus used in this work, and the required potentials for both velocity-mapped and spatial-mapped imaging calculated. The system is then modelled on SIMION 8.0 [79] to determine the impact of the higher-order effects on the trajectories, and to determine the theoretically attainable resolution of the apparatus. These can then be compared with the results obtained in the later experimental chapters.

2.1 Transfer Matrix Methods

A particle's trajectory in a cylindrically symmetric system (such as in a linear time-of-flight mass spectrometer) may be expressed with two values, the distance from the optical axis, r_n , and either its slope, $r'_n = \frac{dr}{dz}$ or its angle from the optical axis, α_n (see Fig. 2.1). In this case, the optical axis z will be taken as parallel to the time-of-flight axis. Note that by construction, $\tan \alpha_n = \frac{dr}{dz} = r'_n$. The indices n for the values r , r' , and α correspond to the initial (i) and final (f) values before and after the

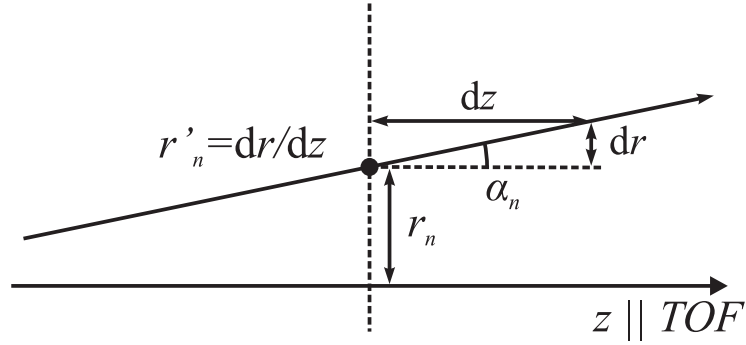


Figure 2.1: Diagram showing the parameters describing a particle's (●) trajectory with respect to the time-of-flight (z) axis. r_n is the distance from the axis, $r'_n = \frac{dr}{dz}$, the slope of the trajectory at that point, and α_n the angle of the trajectory from the z axis.

transformation. In the case where there are multiple ion optics elements, the indices may be numbered to indicate the point along the trajectory they are at, with $n = 1$ corresponding to the initial position, $n = 2$ for after one matrix transformation and so forth. The position and direction of the ions may be described by a column vector $\begin{pmatrix} r \\ r' \end{pmatrix}_n$ or $\begin{pmatrix} r \\ \alpha \end{pmatrix}_n$. The effect of each element of the ion optics on the trajectory can then be concisely described by a matrix:

$$\begin{pmatrix} r \\ r' \end{pmatrix}_f = \begin{pmatrix} m_{11} & m_{12} \\ m_{21} & m_{22} \end{pmatrix} \begin{pmatrix} r \\ r' \end{pmatrix}_i, \quad (2.1)$$

where m_{jk} indicates each matrix element. The values of m_{jk} will be dependent on the nature of the ion optics modelled. There are a few transfer matrices that are of relation to the apparatus used in this work, the matrix of field-free drift space, the uniform electrostatic field, and the aperture lens.

2.1.1 Field-Free Drift Space

In field-free drift space, a particle with non-zero velocity at position 1 (see Fig. 2.2) will travel to position 2 (indicated by the dashed lines) over a distance L along the z axis. It can be seen from construction that the slope, r'_n , and angle, α_n , will remain

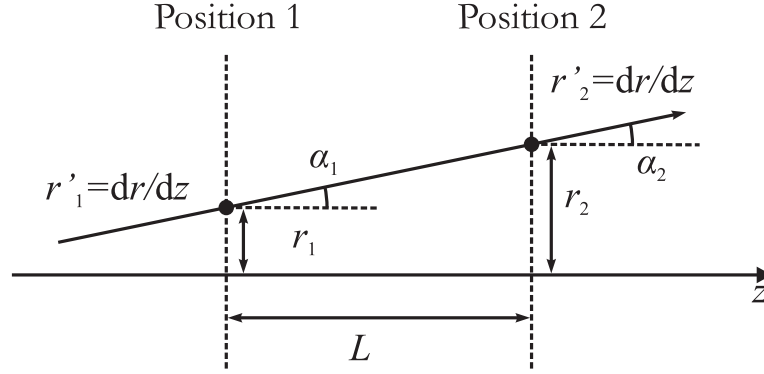


Figure 2.2: The particle's trajectory in field-free space. A particle (●) with a non-zero velocity at Position 1 will, after some time, have travelled to Position 2, a distance L along the z axis. The particle experiences no acceleration and so the slope and angle will be unchanged but the distance from the z axis may be different.

unchanged over the duration of the travel. However, the distance from the axis, r_2 , will increase by the distance travelled according to the slope, r'_1 . This is summarised by the two equations

$$r_2 = r_1 + Lr'_1 \quad (2.2)$$

$$r'_2 = r'_1, \quad (2.3)$$

The equivalent expression to these two equations in matrix form, and thus the transfer matrix for field-free drift space, is given by

$$\begin{pmatrix} r \\ r' \end{pmatrix}_2 = \mathbf{M}_{\text{drift}} \begin{pmatrix} r \\ r' \end{pmatrix}_1 = \begin{pmatrix} 1 & L \\ 0 & 1 \end{pmatrix} \begin{pmatrix} r \\ r' \end{pmatrix}_1 \quad (2.4)$$

2.1.2 Uniform Electric Field

In empty space bounded by planes P_1 and P_2 between two areas of potential V_1 and V_2 (see Fig. 2.3), there will exist a uniform field along the z axis $\mathbf{E} = (V_2 - V_1)/\mathbf{L}$. Particles with an initial energy $eV_i = \frac{1}{2}mv_i^2$, and angle α_i from the optical axis, z , will experience an acceleration in the z direction of eE . E is the component of $\mathbf{E}$ along the z axis. Since the field is uniform and directed along the z axis, $E = |\mathbf{E}|$.

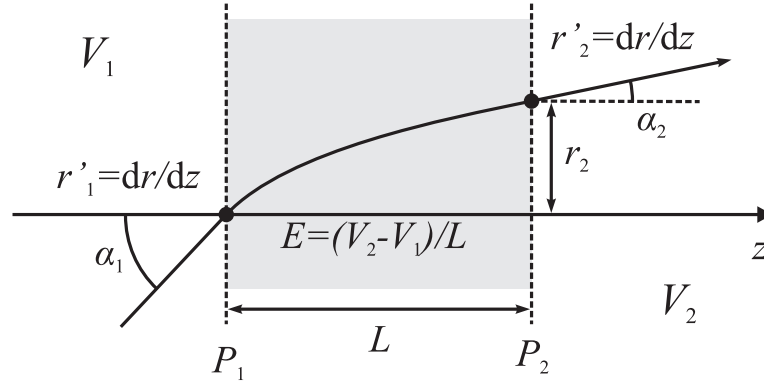


Figure 2.3: Trajectory of particles in uniform electric field.

The position and slope of the trajectory after the field is given by [78]:

$$r_2 = \frac{2L \sin \alpha_1}{\frac{V_2}{V_1} - 1} \left(\left(\frac{V_2}{V_1} - \sin^2 \alpha_1 \right)^{\frac{1}{2}} - \cos \alpha_1 \right) \quad (2.5)$$

$$r'_2 = \sin \alpha_1 \left(\frac{V_2}{V_1} - \sin^2 \alpha_1 \right)^{-\frac{1}{2}} \quad (2.6)$$

The potentials V_n are the energy with reference to the particle at rest, *i.e.* at $V = 0$, the particles would have no kinetic energy. The assumption of the paraxial case is used to simplify the system. Paraxial trajectories are close to the optical axis, meaning $\alpha_i \ll 1$. This is the case if the accelerating voltages are much higher than the initial velocities. Since by definition $r' = \frac{dr}{dz} = \tan \alpha$ and $\tan \alpha \approx \alpha$ for small α , in this limit $r' \rightarrow \alpha$. The resulting transfer matrix is

$$\begin{pmatrix} r \\ \alpha \end{pmatrix}_2 = \mathbf{M}_{\text{field}} \begin{pmatrix} r \\ \alpha \end{pmatrix}_1 = \begin{pmatrix} 1 & \frac{2L}{\left(\frac{V_2}{V_1}\right)^{\frac{1}{2}} + 1} \\ 0 & \left(\frac{V_1}{V_2}\right)^{\frac{1}{2}} \end{pmatrix} \begin{pmatrix} r \\ \alpha \end{pmatrix}_1 \quad (2.7)$$

2.1.3 Round-Aperture Electrodes

The aperture electrodes used in this work all have round apertures, and it has been shown by Davisson and Calbick [43, 80] that the passage of charged particles through such an aperture results in behaviour similar to a spherical lens. The key

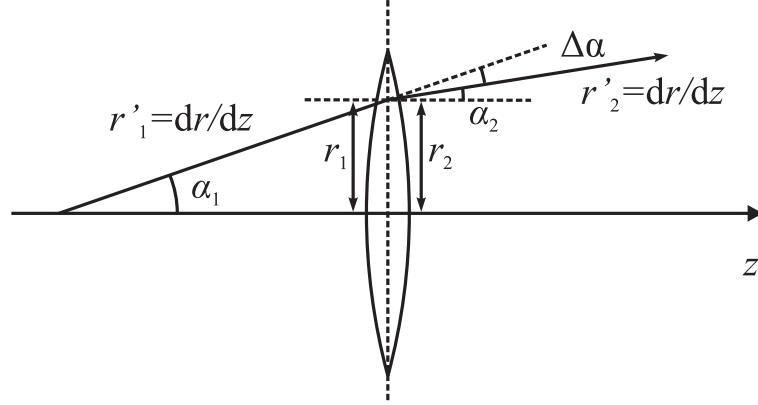


Figure 2.4: Trajectories through a lens. The distance from the axis, r , is the same entering and leaving the lens, but the trajectory undergoes a deflection $\Delta\alpha$.

property of a lens in this case is that the deflection of a trajectory (ray) passing through it, $\Delta\alpha$, (see Fig. 2.4) is proportional to its distance from the optical axis. The degree of deflection is inversely proportional to its focal length, f , which for aperture electrodes is given by:

$$f = \frac{4V_{\text{ap}}}{E_2 - E_1}. \quad (2.8)$$

V_{ap} is the energy of the charged particle, which in this notation is equal to the potential of the aperture. E_1 and E_2 are the strengths of the electric fields before and after the aperture, respectively. Aperture electrode lenses are symmetrical in the sense that the front and back focal lengths are the same. The transfer matrix equation for the aperture electrode lenses is thus

$$\begin{pmatrix} r \\ \alpha \end{pmatrix}_2 = \mathbf{M}_{\text{aperture}} \begin{pmatrix} r \\ \alpha \end{pmatrix}_1 = \begin{pmatrix} 1 & 0 \\ -\frac{1}{f} & 0 \end{pmatrix} \begin{pmatrix} r \\ \alpha \end{pmatrix}_1. \quad (2.9)$$

2.1.4 Combination of Transfer Matrices

These individual matrices representing the elements of the ion optics may be combined to represent the entire system of interest by matrix multiplication. From Fig. 2.5, the apparatus can be identified as consisting of a uniform accelerating field

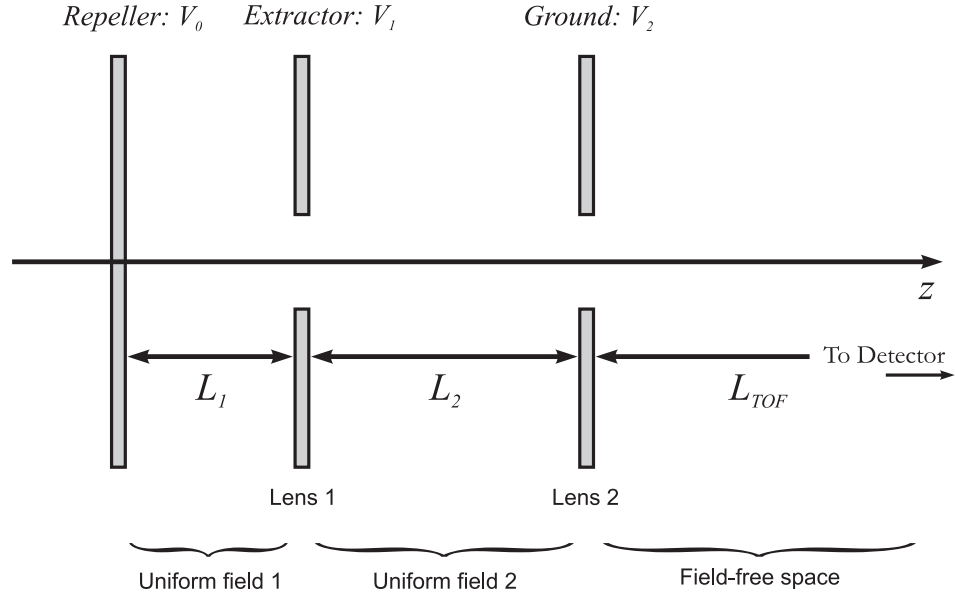


Figure 2.5: Schematic of the entire ion optics arrangement in the apparatus.

of length L_1 , round aperture at potential V_1 with focus length f_1 , uniform field of length L_2 , round aperture at potential V_2 with focus length f_2 , and finally drift space of length L_{TOF} . The representation in transfer matrix form would then be, with V_i as the initial (kinetic) energy of the particle:

$$\begin{pmatrix} r \\ \alpha \end{pmatrix}_2 = \mathbf{M}_{\text{drift}} \mathbf{M}_{\text{aperture}} \mathbf{M}_{\text{field}} \mathbf{M}_{\text{aperture}} \mathbf{M}_{\text{field}} \begin{pmatrix} r \\ \alpha \end{pmatrix}_1 \quad (2.10)$$

$$\begin{aligned} &= \begin{pmatrix} 1 & L_{TOF} \\ 0 & 1 \end{pmatrix} \\ &\times \begin{pmatrix} 1 & 0 \\ -\frac{1}{f_2} & 1 \end{pmatrix} \begin{pmatrix} 1 & \frac{2L_2}{\left(\frac{V_2}{V_1}\right)^{\frac{1}{2}} + 1} \\ 0 & \left(\frac{V_1}{V_2}\right)^{\frac{1}{2}} \end{pmatrix} \\ &\times \begin{pmatrix} 1 & 0 \\ -\frac{1}{f_1} & 1 \end{pmatrix} \begin{pmatrix} 1 & \frac{2L_1}{\left(\frac{V_1}{V_i}\right)^{\frac{1}{2}} + 1} \\ 0 & \left(\frac{V_i}{V_1}\right)^{\frac{1}{2}} \end{pmatrix} \end{aligned} \quad (2.11)$$

The problem of $\mathbf{M}_{\text{aperture}}\mathbf{M}_{\text{field}}\mathbf{M}_{\text{aperture}}\mathbf{M}_{\text{field}}$ can be identified as an emission lens [78]. Expansion of the matrices, using the assumption that $V_i \ll V_a, V_a - V_1$, gives the solution

$$\mathbf{M}_{\text{emission}} = \begin{pmatrix} m_{11} & m_{12} \\ m_{21} & m_{22} \end{pmatrix} \quad (2.12)$$

$$m_{11} = 1 - \frac{1}{f_1} \frac{2L_2}{\left(\frac{V_2}{V_1}\right)^{\frac{1}{2}} + 1} \quad (2.13)$$

$$m_{12} = \frac{2L_2}{\left(\frac{V_2}{V_1}\right)^{\frac{1}{2}} + 1} \quad (2.14)$$

$$m_{21} = -\frac{1}{f_2} - \frac{1}{f_1} \left(\left(\frac{V_1}{V_2}\right)^{\frac{1}{2}} - \frac{1}{f_2} \frac{2L_2}{\left(\frac{V_2}{V_1}\right)^{\frac{1}{2}} + 1} \right) \quad (2.15)$$

$$m_{22} = \left(\frac{V_1}{V_2}\right)^{\frac{1}{2}} - \frac{1}{f_2} \frac{2L_2}{\left(\frac{V_2}{V_1}\right)^{\frac{1}{2}} + 1} \quad (2.16)$$

The focal lengths, f_1 and f_2 , are given by Eqn 2.8:

$$\begin{aligned} -\frac{1}{f_1} &= \frac{V_1}{4L_1V_1} - \frac{V_2 - V_1}{4L_2V_1} \\ &= \frac{1}{4L_1} - \frac{1}{4L_2} \left(\frac{V_2}{V_1} - 1 \right) \end{aligned} \quad (2.17)$$

$$\begin{aligned} -\frac{1}{f_2} &= \frac{V_2 - V_1}{4L_2V_2} \\ &= \frac{1}{4L_2} \left(1 - \frac{V_1}{V_2} \right) \end{aligned} \quad (2.18)$$

2.1.5 Emission Microscopy

For emission microscopy, the projection of an ion image from the sample onto a plane with some magnification is desired. The distance between the end of the ion optics and the detector, L_{TOF} , is much greater than either L_1 or L_2 , and the system can then be treated as projecting an image to infinity. The condition for such an image, then, is that all ions originating from the same spatial position have the same

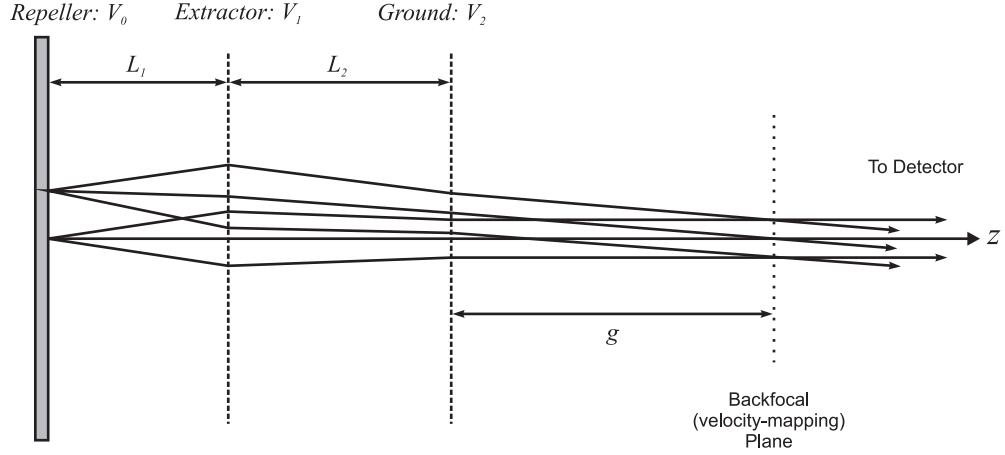


Figure 2.6: Trajectories of ions for emission microscopy

final direction (α_f) (Fig. 2.6). For this emission lens system, the virtual origin of the particle is from a distance L_1 behind the surface [78]. Hence, $r_1 = 2L_1 r'_1$ and,

$$\begin{aligned} (r'_2)_{r_i=0} &= m_{21}r_1 + m_{22}r'_1 \\ &= 2L_1 m_{21}r'_1 + m_{22}r'_1 = 0 \end{aligned} \quad (2.19)$$

$$\Rightarrow 2L_1 m_{21} + m_{22} = 0 \quad (2.20)$$

The values of m_{jk} are the same as those used in Eqns. 2.12-2.16. The numerical values of the distances between the ion optics in the experiment (Sec. 3.1.3) can now be input, with $L_1 = 15$ mm and $L_2 = 21$ mm. Evaluation of Eqn. 2.20 gives two values for $V_1 : V_2$ of 0.145 and 5.150. The first ratio is employed in order to reduce the chances of voltage breakdown in the apparatus. For example, in the case where the total acceleration potential, corresponding to V_2 , is -2 kV, V_1 would have a potential of $-2000 \text{ V} \times 0.145 = -289 \text{ V}$. In reality, it is the drift tube and hence V_2 that is held at ground, and the repeller (V_0) and extractor (V_1) that have their potentials raised. This may easily be achieved by raising the potentials of the whole calculated system by the acceleration voltage, leading to $V_0 = 2$ kV, $V_1 = 1.711$ kV, and $V_2 = 0$ V. This $V_0 : V_1$ ratio of 0.855 is quite close to the 2 kV/1.932 kV potentials that were used experimentally in this work (Sec. 3.1.3) but still differs by about 0.111. This

disparity is expected, in that the value V_1 will have to be raised in order to bring the focus point for the image plane in from infinity onto the detector, which is reflected in the slightly higher value of the potentials used experimentally. Additionally, the model also assumes that the size of the apertures are small in comparison to the distance between the plates, which in this case is not valid: the distances are of the same order of magnitude.

The backfocal plane, the velocity mapping plane in charge particle imaging, is also indicated in Fig. 2.6 by the dotted line. The distance of this from the ground plate, g , can be found by setting $r_1 = r_0$ and $r'_1 = r'_0 = 0$, and using the slope of r'_2 :

$$g = \left(\frac{r_2}{-r'_2} \right)_{r'_0=0} = \frac{m_{11}r_0}{-m_{21}r_0} = -\frac{m_{11}}{m_{21}} \quad (2.21)$$

With a value of $g = 583 - (L_1 + L_2) = 545$ mm, the value of $\frac{V_1}{V_2}$ is found to be 3.494 or 0.269. At 2 kV acceleration voltage, this gives 2 kV/1.462 kV and the ratio 0.731, in excellent agreement with the ratio of 0.738 used in the velocity-mapped imaging experiments.

2.1.6 Effect of Aberrations

In reality, the assumptions of paraxial trajectories and small initial velocities may not hold, and are not accounted for in the first-order calculations of the transfer matrices. The consequence of these higher-order effects manifest themselves as aberrations, leading to a variety of effects and decreasing the sharpness of focus. The two main aberrations concerning the ion optics are spherical and chromatic aberrations, and these are expected to be the main sources of theoretical limitations of resolution in the apparatus.

Spherical Aberrations

The refraction of a trajectory in a spherical lens is slightly more than proportional to its distance from the optical axis, r_a (Fig. 2.7). This difference from proportionality

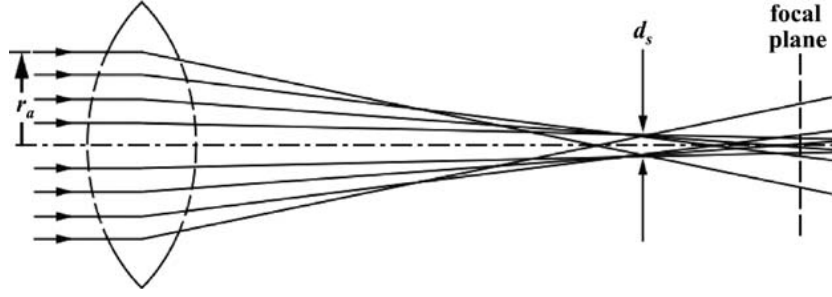


Figure 2.7: Diagram illustrating spherical aberrations in a lens. Adapted from ref. [78].

is negligible at small r_a (paraxial trajectories), but at higher r_a , the difference in refraction leads to different focal lengths, leading to imperfect focussing. The smallest diameter that a point may be focussed to, then, is the “circle of least confusion”, shown as d_s in Fig. 2.7. This is given by [78]

$$d_s = \frac{K_s r_a^3}{D^2}, \quad (2.22)$$

where K_s is a lens-specific factor, while D is a scaling parameter related to the extent of the lens field along the optical axis. The effect of the spherical aberrations may be limited by the use of high acceleration potentials (V_2) in the ion optics. This would increase the axial velocity in comparison to the transverse velocity, producing more paraxial rays.

Chromatic Aberrations

The deflection of charged particles are also dependent on their relative energy, with lower energy particles deflected further. The effect of this is shown in Fig. 2.8. The circle of least confusion, in this case, is given by:

$$d_c = K_c r_a \frac{\Delta V}{V_0}, \quad (2.23)$$

with K_c another lens-specific factor and $\frac{\Delta V}{V_0}$ the relative energy spread of the particles. The effect of these chromatic aberrations can also be reduced by using high

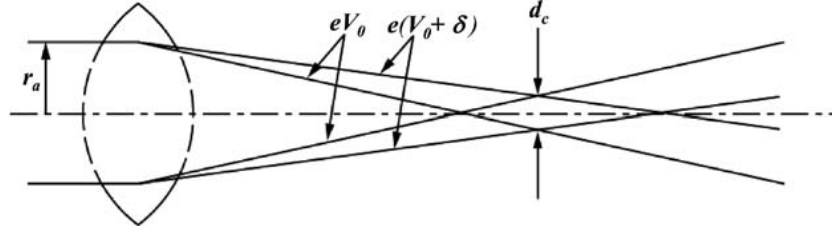


Figure 2.8: Diagram illustrating chromatic aberrations in a lens. Adapted from ref. [78]

acceleration potentials. In this case, the absolute energies of the particles as they pass through the optics would be increased. This results in a lower relative spread of energies, thus reducing the aberrations.

2.1.7 Summary

In this section, the transfer matrix method of modelling ion optics has been described and matrices for ion optics elements applicable to the experimental system used in this work presented. The apparatus may be well-described as an emission lens, and values for potentials to be used on the ion optics for both spatial-mapped and velocity-mapped have been calculated. These values are in good agreement with the actual values used experimentally, verifying the applicability of transfer matrix methods as a predictive tool. The calculated values for the potentials serve as a reference point for the field simulation programs which will be discussed in the next section, which will allow a more accurate determination of the aberrations described here.

2.2 Field Simulation Programs

Field simulation programs such as SIMION calculate the trajectories of charged particles by “flying” them through a potential surface, similar to trajectories in dynamical calculations. In SIMION, a potential array is created by the program, consisting of a two- or three-dimensional grid of points. This represents the virtual

space in which the system will be modelled. The greater the number of points, the more computational power required, and the higher the density of points representing a system, the more accurate the simulation.

The electrodes are defined by the user, specifying dimensions and potentials for each electrode in the system. The effect of these electrodes on the space between them are then calculated by SIMION by solving the Laplace equation using the electrodes as boundary conditions:

$$\nabla^2 V = \nabla \cdot \nabla V = 0 \quad (2.24)$$

$$\nabla V = \left(\frac{\partial V}{\partial x}\right) i + \left(\frac{\partial V}{\partial y}\right) j + \left(\frac{\partial V}{\partial z}\right) k = E \quad (2.25)$$

$$\nabla^2 V = \nabla \cdot E = \frac{\partial E_x}{\partial x} + \frac{\partial E_y}{\partial y} + \frac{\partial E_z}{\partial z} \quad (2.26)$$

The potential at each of these points not defined as an electrode is then assigned to create a potential energy surface. The charged particles are created within this space with a position, charge, mass and velocity. The acceleration $\mathbf{a}$ at each time step is calculated from

$$\mathbf{a} = \frac{d\mathbf{v}}{dt} = \frac{-(e\mathbf{E})}{m} \quad (2.27)$$

and the space charge effects are estimated by Coulombic charge repulsion. Hence, by creating a model of the apparatus within SIMION, and setting realistic initial conditions for the nascent ions, the resulting trajectories may be predicted. These may be used to discover the effect of the aberrations and the theoretical limits to time and spatial resolution.

2.2.1 Simulations of ion optics

An image of the apparatus simulated in SIMION is shown in Fig. 2.9. The dimensions of the chamber are reproduced in the potential array, and the potentials of the individual elements were varied between limits that were obtainable with the experimental apparatus. The back face and top of the chamber have been removed for the illustration in order to show the ion optics inside. Note the slightly different axes

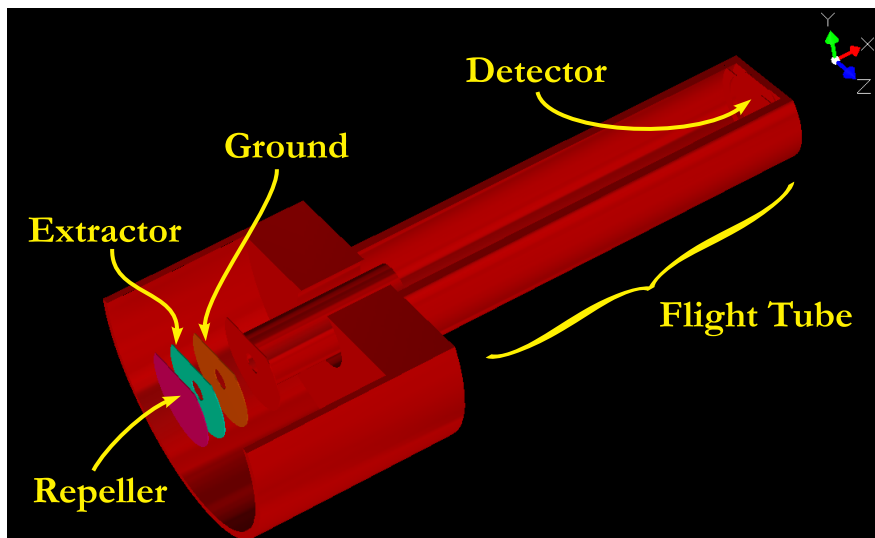


Figure 2.9: An image of the chamber as simulated by SIMION.

imposed by the simulation program, in which x is defined as the axis of cylindrical symmetry. This cylindrical symmetry, which serves as a good approximation for the relevant aspects of the apparatus, has been used to reduce the required memory and processing power for the simulation: the calculation of the potentials may be carried out in a two-dimensional array which is then expanded by revolution around the x axis.

The initial conditions set for the ions were position ($x/y/z$), charge (q), mass (m), and velocity (v). The position coordinates were set such that the ions were created on the surface of the repeller, as may be expected for ions created by irradiation of a sample. The charges were set to 1+, assuming imaging of mainly singly-charged particles. The mass was set to 443.24 Da, which was found to be a major mass peak in the red pigment used in fast imaging experiments in this work (Sec. 6.2, Fig. 6.3). Conveniently, this was also within the band of peaks for the polyethylene glycol used in the mass resolution determination in this work (Sec. 5.3.4).

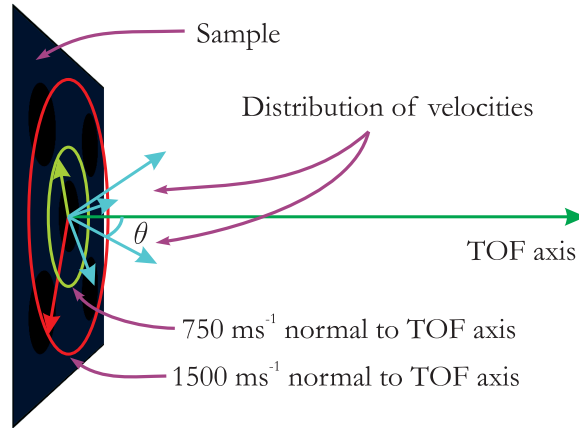


Figure 2.10: Initial recoil velocities of ions used in SIMION simulations. The angle from the time-of-flight axis is defined together with its speed. For determination of potentials in spatial-mapped imaging, selected recoil velocities of 0 ms^{-1} , 750 ms^{-1} , and 1500 ms^{-1} were used at $\theta = 90^\circ$.

2.2.2 Spatial-Mapping Potentials

In order to determine the relevant potentials for spatially-mapped imaging, a small number of initial velocities were used in order to enable the easy identification of the focal planes by inspection. Fig. 2.10 shows the scheme in which the velocity distributions are defined. The ions are taken to be emitted from the surface of the sample with a distribution of velocities, at angle θ from the time-of-flight axis. In a similar manner to ray-tracing in optics, the limiting cases of zero and maximum speeds were used. A speed of 1500 ms^{-1} was used as a reasonable upper limit to the speed normal to the time-of-flight axis as the vast majority of reported recoil velocities in the literature lie below 1000 ms^{-1} (Sec. 5.1.3). The limiting value of 90° from the time-of-flight was also used for maximum deviation from the axis. Additionally, particles at 750 ms^{-1} were used to better indicate if the imaging was recoil velocity dependent. The ions were created originating from three points along the y axis, at $y = -1, 0, 1$. Each point is the origin for five particles, two at 1500 ms^{-1} with velocities going up and down the y axis, two at 750 ms^{-1} , also up and down the y axis, and one with no initial velocity.

The potentials were varied within the program until the focal plane, at which

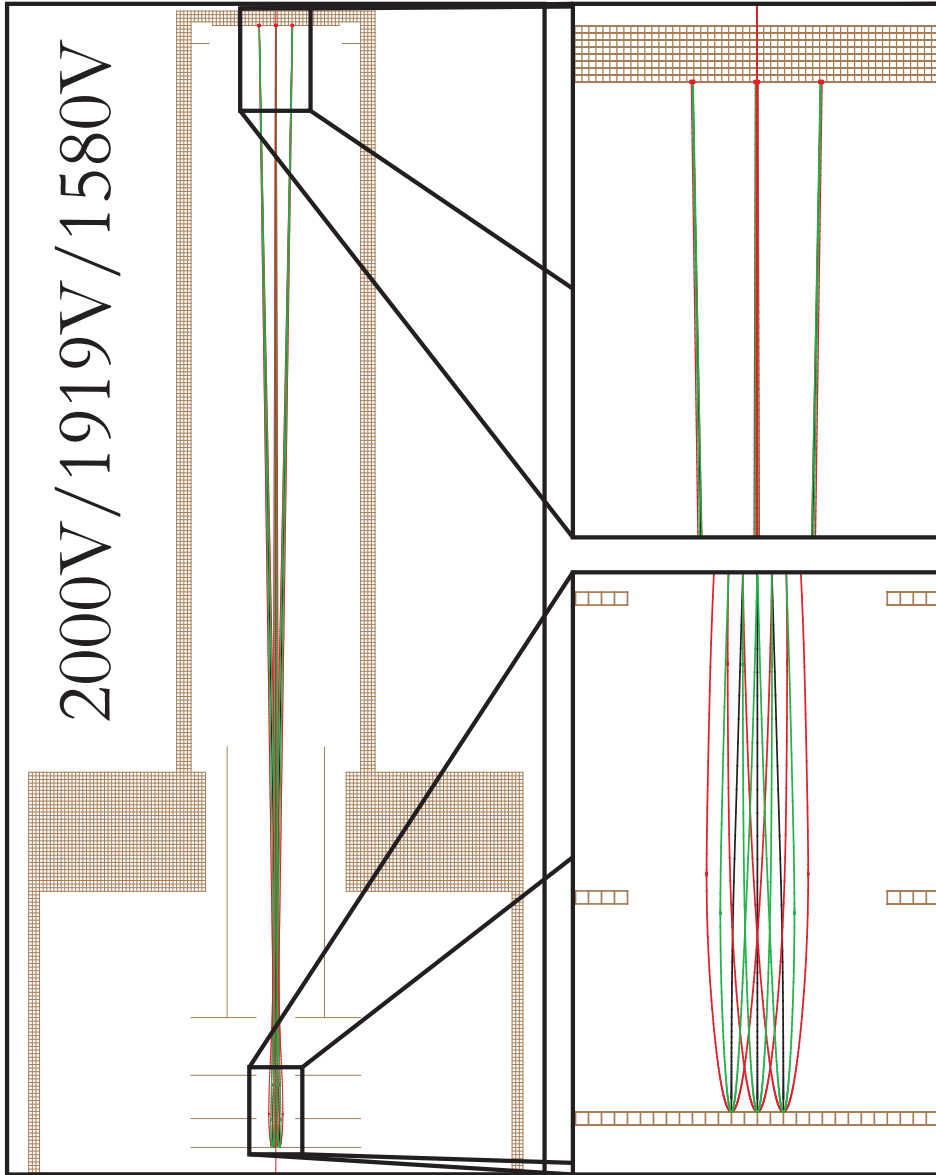


Figure 2.11: Trajectories in a simulation in which the image has been focussed using three electrodes. The three ion source points on the sample can be seen to map to three points on the detector, determined solely by their point of origin and independent of the recoil velocities. The voltages indicated on the top right correspond to potentials applied to the repeller, extractor and third plate respectively.

would be projected a real image, could be seen to coincide with the detector plane. It was found that a repeller voltage of 2000 V and an extractor voltage of approximately 1930 V would produce this result. This is in excellent agreement with the experimental values for the repeller and extractor of 2 kV/1.932 kV respectively. It was also found that additional utilisation of the third electrode on the system could also produce the same result, albeit with slightly different voltages. One of these solutions is shown in Fig. 2.11. Using all three electrodes suggested a useful approach for the experimental procedure: the distance of the focus plane was found to be very sensitive to the potential of the extractor, but not as sensitive to the potential on the third electrode. Thus, when performing the experiment, one could set the extractor close to the required voltage and fine-tune the focus with the third plate.

Examination of the calculated potential energy surfaces of the chamber also revealed that the body of the chamber was not necessary for accurate trajectory simulation. The chamber around the ion optics was far enough away that the potential in the interaction region was unaffected, while within the field-free region the field was maintained at ground by the later electrodes. The removal of the body of the chamber in later simulations allowed smaller potential array volumes with higher grid density. Thus, only the ion optics and the final ground plate ensuring the field-free flight were included in the simulation, leading to faster and more accurate calculations. An example of these simulations, using the two-electrode system (repeller/extractor), is shown in Fig. 2.12.

2.2.3 Initial Ion Distribution

In order to investigate the effect of aberrations on the theoretical limit to the resolution of the apparatus, a much larger number of ions were required, with a more realistic velocity distribution, was used in simulations conducted within the group [81]. A Fortran program was written to create in the order of 400 particles with a defined velocity distribution in the simulations. This was an upper bound to the number of ions typically observed in velocity- and spatial-mapping ion imaging

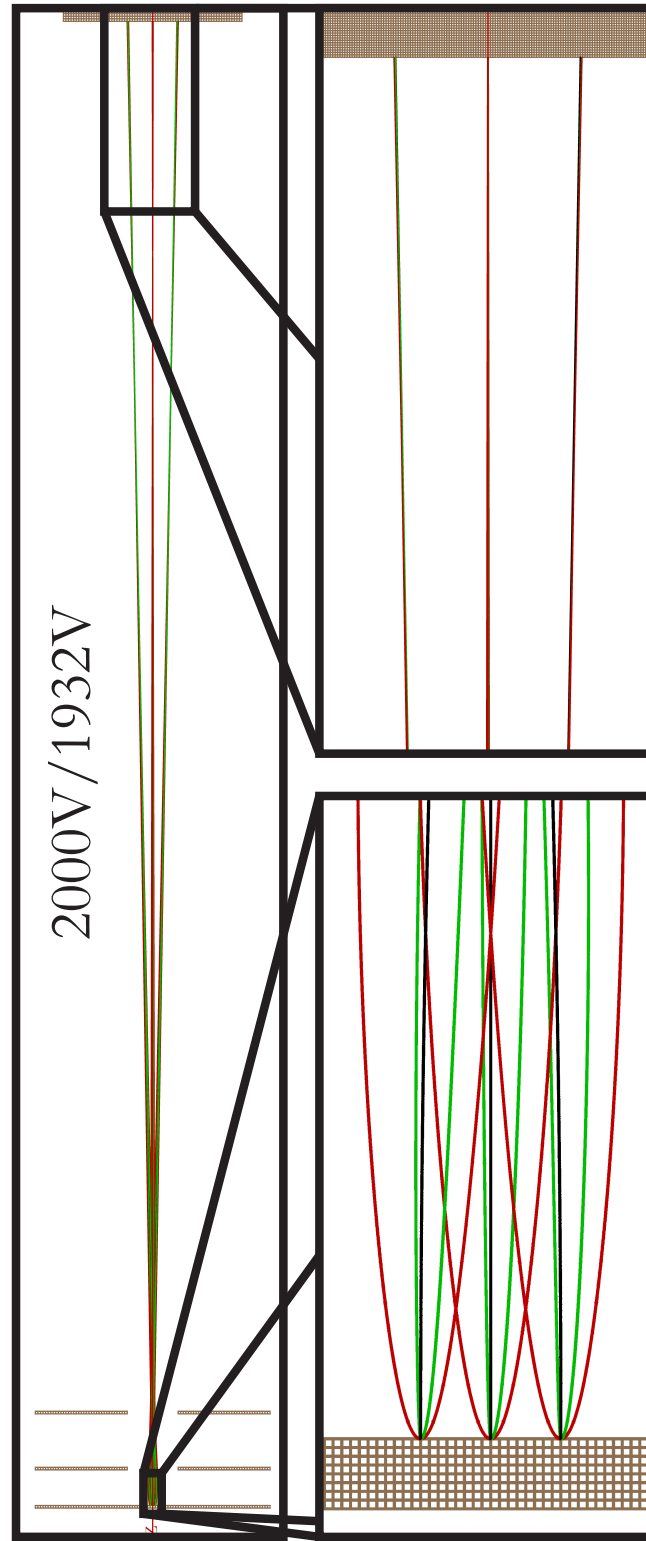


Figure 2.12: Trajectories in a simulation in which the chamber has been omitted. The ions have been focussed using the repeller and extractor only, with voltages of 2 kV and 1.932 kV respectively. The simplified model reduces calculation time without significant difference in results from when the chamber was included.

experiments. These ions were given a velocity distribution of separable form:

$$f(\mathbf{v}) = g(v)h(\theta), \quad (2.28)$$

where $f(\mathbf{v})$ is the distribution of velocity $\mathbf{v}$, $g(v)$ the distribution of speed v , and $h(\theta)$ the distribution about θ , the angle from the normal. For ions of masses 1-15 kDa, Beavis and Chait [82] observed a Gaussian speed distribution with $\sigma = 150 \text{ ms}^{-1}$ centred around $v = 750 \text{ ms}^{-1}$. The plume expansion can be taken to have a bias normal to the surface according to [83]

$$h(\theta) = (n + 1) \cos^n \theta, \quad (2.29)$$

with values of n all the way up to 28, where $(n + 1)$ is a normalisation factor. As aberrations are caused primarily by non-paraxial trajectories, in order to determine the lower limit of resolution for the apparatus while retaining the $\cos^n \theta$ character, a value of $n = 2$ has been chosen for the simulations in this work. Four origin points on the repeller plate were used, spaced 1 mm apart on a line originating from the centre of the plate. 100 ions were created at each point with velocity distributions as described above. The particles were allowed to fly through the simulated apparatus, and the arrival times and positions of each individual ion at the detector recorded. Due to the magnification of the system, this results in a line of spots from the centre to the edge of the detector, and so gives an indication of the resolution across the field of view.

2.2.4 Spatial Resolution

The aberrations caused by the spread of transverse velocities from each spot would result in a imperfect focus of the spot on the detector, as described in Sec. 2.1.6. As each ion in the simulation is individually numbered, it is possible to determine which group of ions (from a particular point of origin) it came from. Hence, the spread of ions from each point of origin can be determined separately. Additionally,

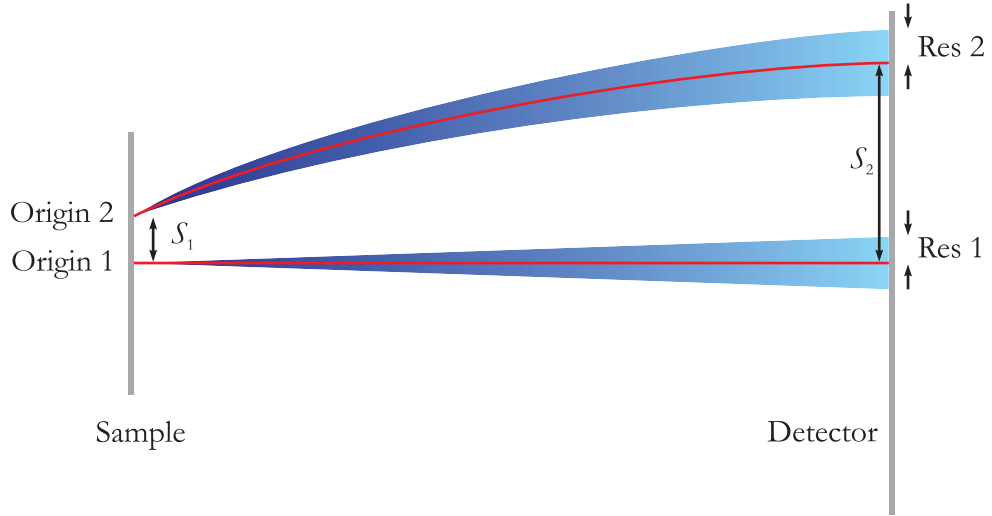


Figure 2.13: Determination of spatial resolution from a series of origins. A series of origins (Origin 1, Origin 2) from the centre of the sample are simulated. Only two are shown here. Ions (blue) from each origin map to an area on the detector. The ions with no initial axial velocity (red lines) report the centre of the spot. The resolution (Res 1, Res 2) is taken to be the radius from the centre of the spot which encompasses 2σ of the ions of that spot. The ratio of $S_2 : S_1$ gives the linear magnification of the apparatus.

each group of ions has one ion with no transverse velocity, allowing the centre of the spot to be determined. The resolution of the spot is taken as the radius from the centre of this spot encompassing two standard deviations (2σ) of the ions. An illustration of this is shown in Fig. 2.13. Note that this illustration has been greatly simplified for clarity: the actual trajectories of the ions cross over at the backfocal plane and result in an inverted image.

The resolution that is directly measured is the resolution on the detector, while it is the resolution on the sample that is the value of interest. This may be converted by taking into account the magnification and dividing the resolution on the detector by it. This value for magnification was found from the distance between the central ion of each group (S_2): as the distance between the spots on the sample is well known (S_1), comparison of the two easily gives the magnification factor of the system.

As discussed in Sec. 2.1, the higher-order effects leading to aberrations are caused by deviation from the paraxial system in which the initial kinetic energy of the

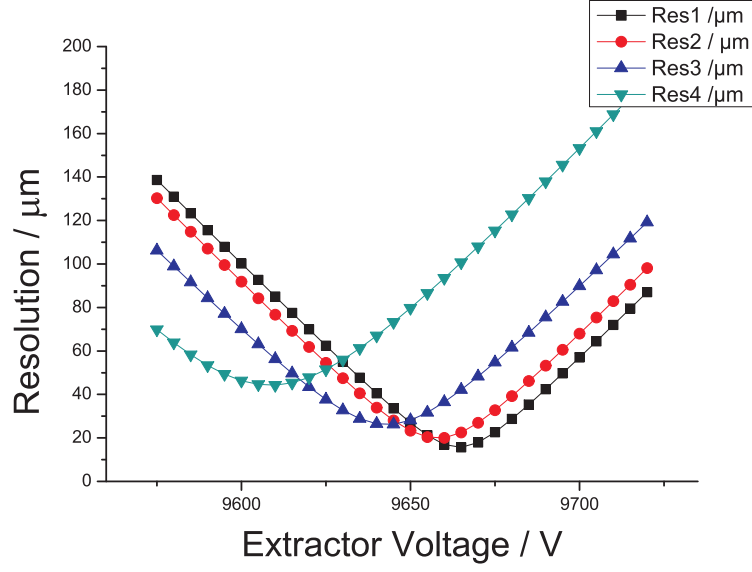


Figure 2.14: Dependence of the resolution of the four simulated spots on the extraction voltage applied. “Res 1”- “Res 4” correspond to spots created 0-3 mm from the centre of the sample plate respectively.

ions is negligible in comparison with the acceleration potentials. Hence, if one were to attempt to minimise these effects, the highest acceleration potential should be applied. With a higher accelerating potential, the rays become more paraxial as the ratio of the transverse to axial velocity is decreased: spherical aberrations are decreased. The relative energy spread is also decreased, reducing chromatic aberrations. In the case of our experiment, this is limited to 10 kV. Simulations of spatial and temporal resolution are therefore calculated with this value on the repeller.

A plot of the spatial resolution of each spot against extractor potential is shown in Fig. 2.14. Each curve on the plot represents the resolution of each spot, with “Res 1” corresponding to the spot at the centre of the repeller, “Res 2” the next one out from the centre, and so forth up to “Res 4”. As can be seen, spatial resolution of the focus is strongly dependent on the ratio of the extractor and repeller voltages, corresponding to V_1/V_2 in the transfer matrix method. Each curve exhibits a minimum as the point comes into and out of focus with the varying strength of the lens.

An important point to note is that the optimal focus voltage is different for each spot, with the resolution minimum occurring at decreasing extractor voltage as the origin of the spot is further out from the centre. This is expected as the trajectories pass through the electrodes further and further from the optical axis: the approximation that the rays are paraxial begins to fail and the effect of positive spherical aberrations can be seen. These aberrations mean that the deflection of the trajectories increases with r_a , and so the outer trajectories will be focussed at a shorter distance. Therefore, a less converging lens (*i.e.*, lower extractor voltage) would be required to place the focus point for that spot on the detector. This can be seen in the decreasing value of the extractor voltage at the curve minima as one moves the origin of each spot further from the optical axis. This problem is compounded further by Petzval field curvature [84], which causes a flat surface passing through a lens to be focussed on a curved surface in space. This prevents the imaging of a flat surface onto a flat detector.

Evidently, it will not be possible to perfectly focus all of the points onto the detector, and a compromise between the focal lengths will be required. However, it can be seen that the curve corresponding to “Res4”, the point furthest from the centre of the repeller, has its minimum far from the other three curves, as well as having a poorer minimum resolution. Considering the ~ 7 mm field of view obtained experimentally (Sec. 5.3.4), corresponding to ± 3.5 mm from the centre of the repeller, the outermost point in most cases would not be observed. The optimum extractor potential was hence taken as the voltage at which the total resolution of only the centre three points is the lowest. For example, in this case, the optimum extraction voltage was 9.65 kV. Further simulations employed this method of determining the theoretical resolution. With these potentials, the simulations produced a magnification of $\times 9.7$. Taking into consideration a detector size of 70 mm, the field-of-view predicted by the simulations is $70 \text{ mm}/9.7 = 7.2 \text{ mm}$, in excellent agreement with the experimentally observed values. The magnification did not change significantly with the absolute acceleration potentials as long as the

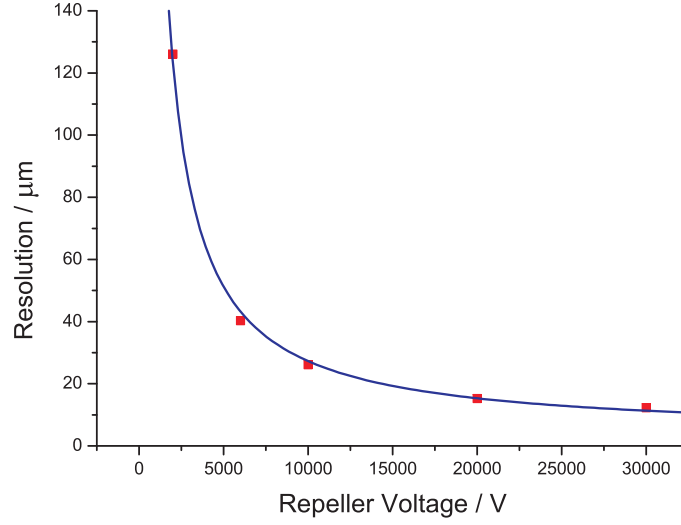


Figure 2.15: Plot of theoretically-attainable spatial resolution against accelerating potential.

ratios between the extractor and repeller were maintained.

A plot of the dependence of the theoretically obtainable resolution on the accelerating potential is shown in Fig. 2.15. As expected, it exhibits a strong dependence on the accelerating potential, with an almost inverse exponential curve. The theoretical resolution attainable at 10 kV with the current apparatus and this velocity distribution of ions was found to be $26 \mu\text{m}$.

In summary, the field simulations of the apparatus indicate that the spatial resolution attainable with a single emission lens set of ion optics is limited by the necessity of a compromise in quality of focus between different areas of the field of view. Areas of different distances from the optical axis require different lens strengths in order to focus onto the (flat) detector plane. The source of this restriction is the spherical aberrations and Petzval field curvature. Increasing extraction voltages reduces these detrimental effects, as predicted in Sec. 2.1.6.

2.2.5 Time Resolution

In addition to the ability to separate the origins of points in space on the sample, the ability to separate ions of different masses is obviously of great importance in

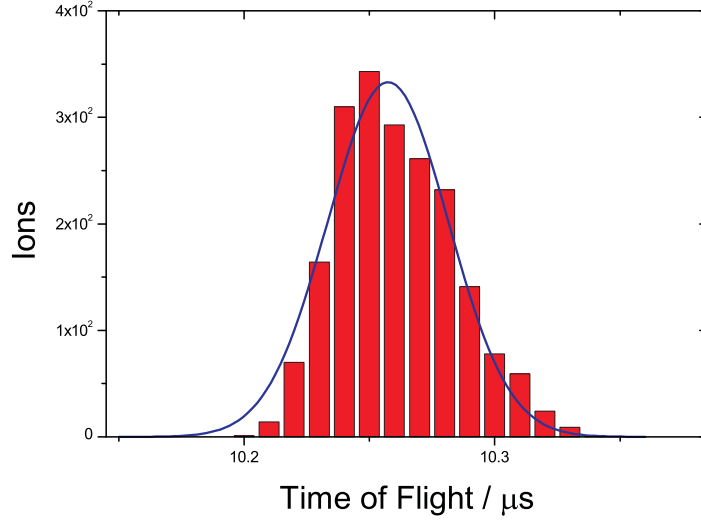


Figure 2.16: Histogram of arrival times at the detector of ions from a single point source. The blue line is a Gaussian fitted to the curve, with a FWHM of 57 ns, equivalent to an $m/\Delta m$ of 93.

imaging mass spectrometry. In the case of time-of-flight mass spectrometry, this m/z separation is directly related to the ability to separate the ions in time, t . An advantage of the field simulation program over the transfer matrix methods was that trajectory calculations are time-dependent, and so the time of flight and time of arrival at the detector for each ion was also determined. The spread of these times could be used to determine the theoretical time resolution of the apparatus.

The simulations described in Sec. 2.2.4 were also used to provide information on the time resolution of the apparatus. The times of arrival of the ions at the detector from each spot were recorded, and their spread used to determine the theoretically attainable time resolution. The arrival times of ions from a single spot are plotted in a histogram in Fig. 2.16. The ability to separate two peaks which are close to each other in the time-of-flight spectrum may be taken to be the full width half maximum (FWHM). Fitting this histogram with a Gaussian function gives a FWHM of 57 ns. At this mass, this corresponds to an $m/\Delta m$ of 93.

Possible sources of this time spread may be different from those limiting the spatial resolution. The differences in focus lengths caused by spherical and chromatic aberrations cause blurring in the x, y plane, damaging spatial resolution. However,

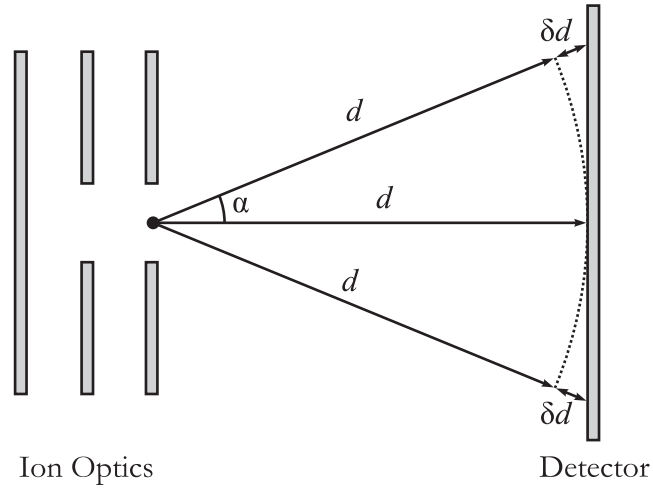


Figure 2.17: Effect of different trajectory angles (α) in the drift tube on time resolution. Off-axis trajectories have the same kinetic energy and hence speed. After a certain time, when the on-axis trajectories arrive at the detector, the off-axis trajectories have travelled the same distance d to an arc ($\cdots$). The ions have an additional distance δd to travel, increasing the spread in the time of arrival.

an effect similar to (but not) Petzval field curvature remains a problem. Upon leaving the acceleration region, all particles will have been imparted with the same amount of kinetic energy from the acceleration potentials. These will have slightly different trajectories with differing angles from the optical axis (see Fig. 2.17). These ions spread out along an arc at the same velocity from their origin. When the ions flying along the time-of-flight axis have arrived at the detector, all the other trajectories will be at a point in space indicated by the dotted line. These ions have an additional distance δd to travel, increasing the time spread. The impact of this effect on the absolute arrival time resolution may be reduced by taking the first arrival of the ion as the time-of-flight. However, this problem is not separable from the additional effect of the spread of initial kinetic energies of the ions upon desorption from the sample. Differing initial velocities in the z direction will mean that final kinetic energies of the equal m/z ions travelling along the flight tube may be different, another factor in the spread in arrival times.

Another cause of differing arrival times is the difference in curvature at different parts of the accelerating region. Fig. 2.18(a) shows a plot of the potential energy

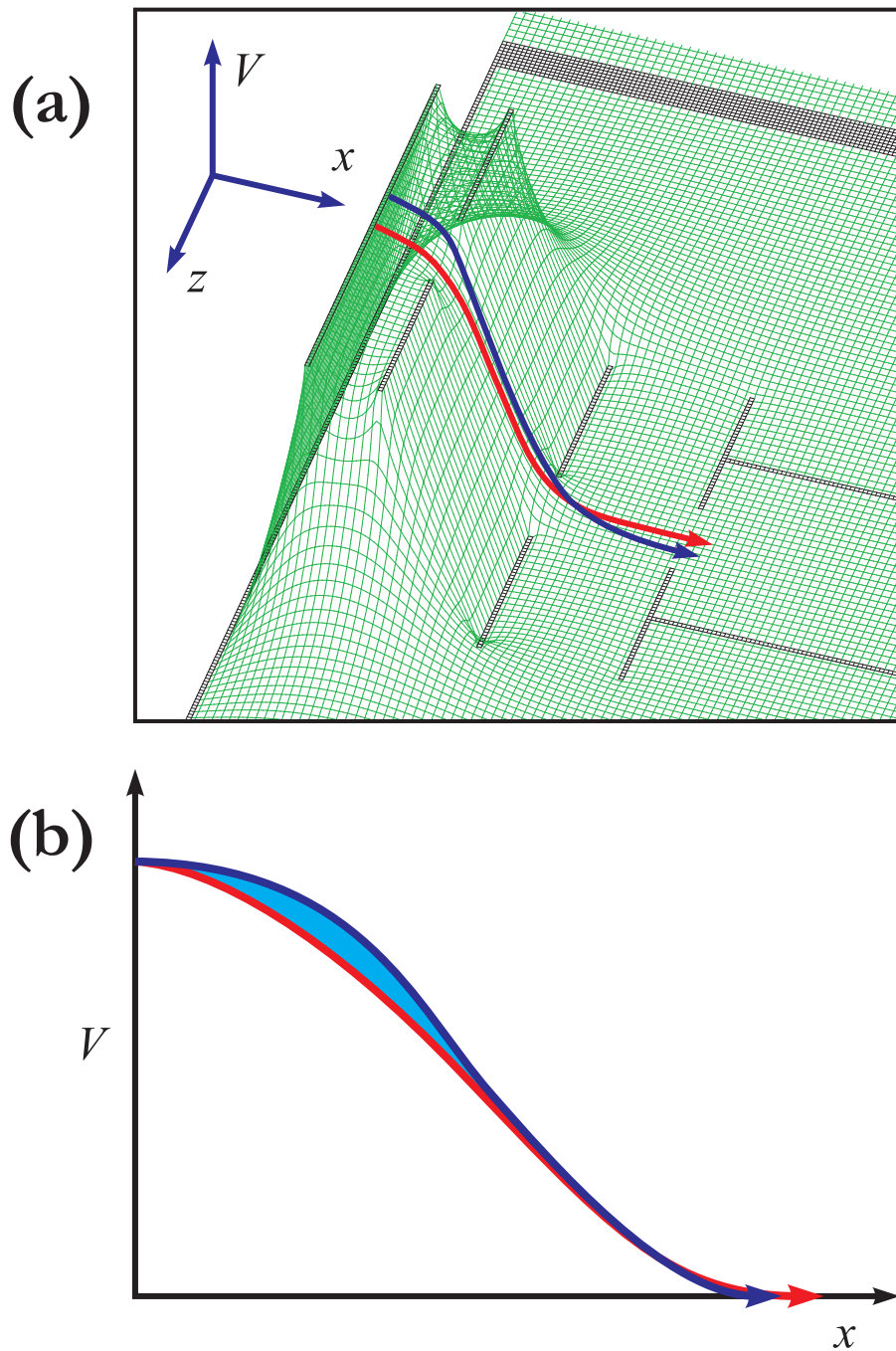


Figure 2.18: Diagram showing different potentials within the accelerating field. (a) shows the potential surface of the ion optics used. Note again the slightly different coordinates used in SIMION: x is taken as the time-of-flight axis. An ion originating from the centre of the sample will follow the red trajectory shown, while one from off-centre may follow the blue trajectory. (b) plots the potentials along the time-of-flight axis. In the blue-coloured region, the ion following the red trajectory will have a higher kinetic energy.

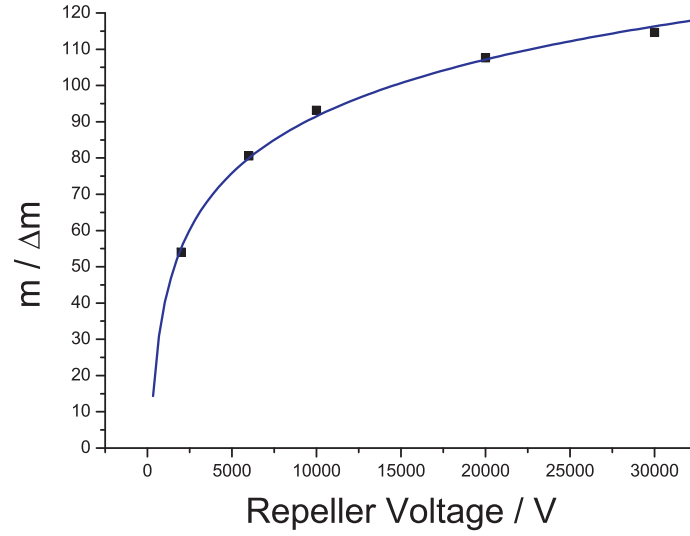


Figure 2.19: Plot of time resolution dependence on accelerating potential

surface at a cut through the ion optics. Note again the different coordinates used in SIMION, in which the x is the axis of rotational symmetry and hence used as the time-of-flight axis (Sec. 2.2.1). The higher above the x/z plane, the higher the potential energy, V , at that point. Two example trajectories which would be followed by ions originating on- and off-centre from the surface are shown with the red and blue arrows respectively. As the potentials distort in a similar way to a rubber sheet, the potentials nearer the centre of the extractor aperture are lower than at the edge of the hole. A plot of the potential experienced by the ions along the red and blue trajectories is shown in Fig. 2.18(b). It can be seen that although the initial and final potentials for the two trajectories are equal, the potentials experienced during the course of the paths are different. The red trajectory will have a higher kinetic energy than the blue equal to the difference in potential (turquoise). This means that in the acceleration region, the ions from the centre of the sample will have a higher velocity and separate out slightly from off-centre ions. The effect is greater the further out from the centre. Although the trajectories do cross over and the blue path does for a short time have higher kinetic energy, the area is much smaller and the outer ions do not catch up within this time.

It may be expected that the time resolution, like the spatial resolution, would

also be affected by the strength of the accelerating field. The dependence of the time resolution on the accelerating potential was also investigated with the simulations. A plot of $m/\Delta m$ against the voltage applied to the repeller is shown in Fig. 2.19. As expected, the mass resolution was also found to be strongly dependent on the accelerating potential. The relative spread in initial velocities, in a similar manner to the chromatic aberrations, becomes smaller when an increased extraction voltage is used. This reduces their effect on the time resolution. The effect of the differing potentials for different trajectories through the acceleration region, however, is not improved as much. Although the ions spend less time in the accelerating field, the difference in potentials is also increased. This may be thought of as stretching the potential energy curves in Fig. 2.18(b) along V : the area between the two curves increases. The effect of this is that the time separation between the two ions entering the drift space is decreased, but by approximately the same factor as the reduced time-of-flight caused by the increased voltages. Thus, although the spread of ions may be less, the separation of the peaks will also decrease by the same amount. Finally, the differences in trajectory lengths in the field-free region is entirely geometrical in origin, and is also unaltered by increasing the accelerating potential. A possible method of reducing this effect is the extension of the flight tube. This would result in a larger magnification, with a smaller arc and enclosed angle α . This then gives a smaller distance δd and thus a smaller time difference.

2.2.6 Mass Dependence of Resolution

Finally, the dependence of the spatial and mass resolutions on the ion mass were also investigated, as an indication of the ability of the apparatus to measure a range of masses. The masses of the ions used in the simulations were varied, and the spatial and time resolutions determined in the same way as described in the previous sections. The results are shown in Fig. 2.20.

Fig. 2.20(a) shows the change of spatial resolution with the mass of the ion. The radius of each spot on the detector increases with mass, indicating that resolution

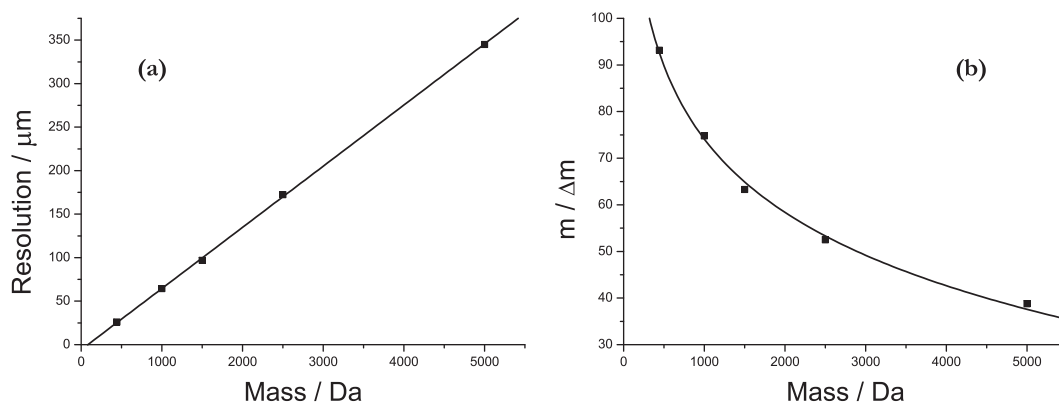


Figure 2.20: Plots showing the mass dependence of (a) spatial resolution and (b) mass resolution. The calculations were done using the same methods as described in Sec. 2.2.4 and 2.2.5.

becomes poorer with mass. As the kinetic energy of an ion of a given speed increases linearly with mass, and the radius of the disc of least confusion for chromatic aberrations (Eqn. 2.23) is also linearly dependent on the kinetic energy spread of the ions, it is possible that the main cause of this loss of resolution is caused by the chromatic aberrations of the apparatus. It is worth noting, however, that a greater directionality for higher mass ions created with MALDI has been observed [83]. This means a greater value of n in Eqn. 2.29, and so the ions are directed more along the time-of-flight axis. Thus, the experimental spatial resolution may not decrease in quality as sharply as predicted by this lower-limit simulation.

From Fig. 2.20(b), the mass resolution can also be seen to decrease with increasing mass. The relationship between the resolution and the mass is not as easy to elucidate, with a non-trivial relation between the energy spreads, increasing mass, and focus strengths. Clearly, from plot (b), Δm is increasing at a higher rate than m . A possible contribution may be from the chromatic aberrations. The increasing $\Delta V/V_0$ term in Eqn. 2.23 will produce a larger spread of focus and trajectory lengths with the increasing energy spreads. The larger energy spreads from the higher masses at the same velocity will of course also contribute to the differences in velocities in the time-of-flight axis.

2.3 Summary

Two methods of theoretically determining the trajectories of charged particles in an ion imaging system have been described in this chapter. Transfer matrices provide a quick method of approximating required dimensions and potentials, and are hence useful in designing systems. This method was used to calculate a rough value for the required potentials for emission microscopy in the current apparatus and indicated that such microscopy was possible for any experiments constructed for velocity-mapped imaging. Additionally, the transfer matrix method also sheds light on the origins of aberrations in ion imaging.

Field simulation programs were used for more detailed simulations in order to calculate the effects of these aberrations, and the resulting limits to resolution attainable by the apparatus. The effect of accelerating potential on the attainable resolutions was also investigated. For an accelerating potential of 10 kV, an expected spatial resolution of $26\text{ }\mu\text{m}$ and mass resolution $m/\Delta m$ of 93. These values serve as a reference point in order to shed light on the factors limiting the experimentally observed resolution in later chapters.

From the simulations presented in this chapter, microscope-mode imaging mass spectrometry conducted with the available apparatus will give a wide field-of-view ideally suited to certain biological samples (see Chapter 7). Considering the size of the field of view, the apparatus will produce a good relative spatial resolution. However, the mass resolution predicted is not especially good, and methods for the improvement of this are currently under investigation [85, 86]. Potential approaches include the introduction of an aperture, and differential post-acceleration techniques.

As most losses in resolution are caused by the deviation of the trajectory from first order behaviour [78, 84], in which the trajectories are assumed to have small angles and distances from the flight axis, as well as a negligible spread in energy in comparison with the potentials of the ion optics. An aperture at the pupil plane (velocity mapping plane) should remove high transverse velocity trajectories, reducing aberrations at the cost of lowering signal. Simulations [86] of the system including

a contrast aperture of 1 mm at the pupil plane showed an improved spatial resolution from $26\text{ }\mu\text{m}$ to $20\text{ }\mu\text{m}$, an improvement of 23%. The mass resolution was also improved from $m/\Delta m = 93$ to $m/\Delta m = 101$. The use of an even smaller aperture should increase these improvements.

Another method of improving the spatial resolution is the implementation of post-extraction differential acceleration (PEDA), based on the methods of Aoki *et al.* [87]. Simulations using the method so far [85] indicate that with an addition of a simple three aperture electrode Einzel lens, an $m/\Delta m$ in excess of 2500 may be attained.

Chapter 3

Experimental

The experiments presented in this thesis were performed using an apparatus modified from conventional velocity-mapped [47] ion imaging [46]. Sec. 3.1 will describe the apparatus used in the experiments, in both velocity- and spatial-mapped ion imaging. The modifications carried out to facilitate spatial imaging are also described. Characterisation of typical data obtained from the detectors is described in Sec. 3.2. Finally, the analytical techniques to extract data from velocity-mapped images, for which the experiment was initially built, are presented in Sec. 3.3, together with an example analysis.

3.1 Apparatus

To illustrate the principles involved, a schematic of the experiment is shown in Fig. 3.1. The ions to be studied are created within the set of ion optics, using an ionising laser pulse. In the case of conventional velocity-mapped ion imaging, these are generally formed from a molecular beam. The molecule species of interest is seeded in an inert gas and introduced through a supersonic nozzle and skimmer to achieve rotational cooling. The molecule of interest is subsequently dissociated and/or ionised using appropriate laser pulses. In the case of surface imaging, part of the ion optics are adapted into a mount for the samples and a laser pulse incident on the surface triggers the desired desorption and ionisation. In either case, these

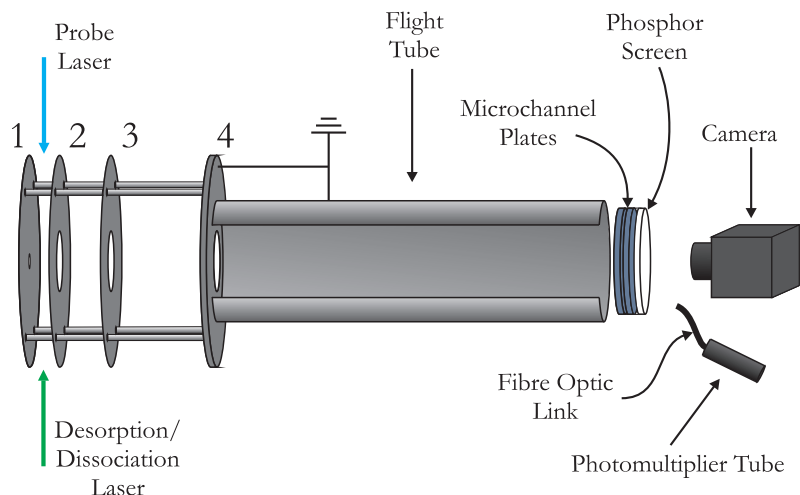


Figure 3.1: Schematic of the experiment.

nascent ions are accelerated by the potentials of the ion optics into the field-free flight tube. The velocities along the time-of-flight axis are determined by the mass/charge ratio of the ions, allowing the separation of different masses by time-of-flight. Impact on to a set of microchannel plates at the end of the flight tube triggers a cascade of electrons with a gain of approximately 10^6 electrons per incident ion. The ejected electrons produce a flash on the phosphor at the position of the arrival of the ions in the plane of the detector. These flashes are recorded by a camera and the data sent to a computer for analysis and recording. As described in Chapter 2, the behaviour of the ion optics can be controlled by manipulating the potentials on the electrodes, in order to determine the nature of the mapping. The ratio of the ion optics are set to either velocity- or spatial-map the ions. The arrival positions of the ions are then dependent solely on either the velocity normal to the time-of-flight axis or origin in space, respectively.

3.1.1 Reaction Chamber

A schematic of the apparatus used to perform the experiment is shown in Fig. 3.2. The apparatus is separated into two main chambers. The two are connected when performing velocity-mapped gas-phase experiments by a skimmer with a 2 mm hole,

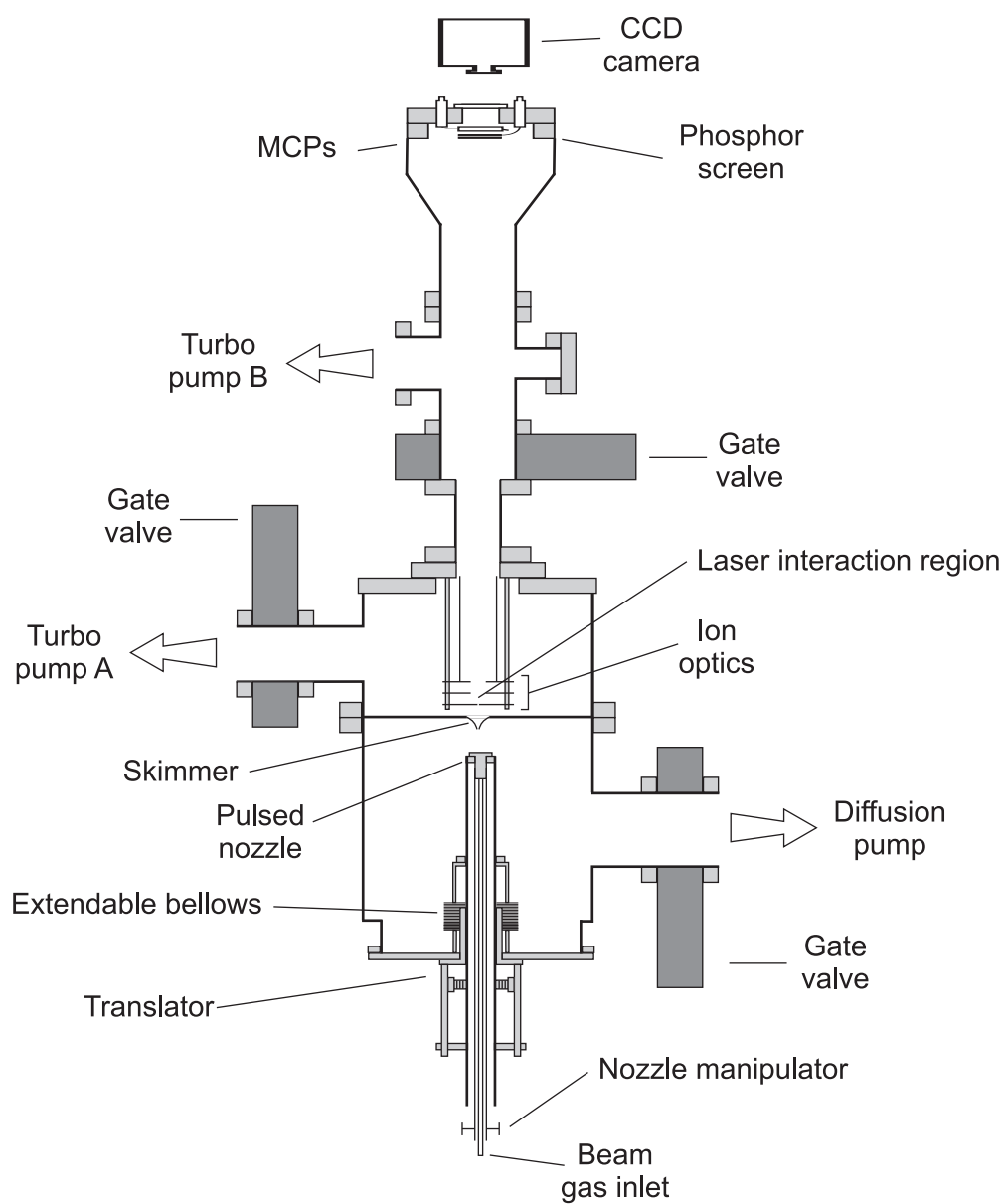


Figure 3.2: Schematic of the apparatus reaction chamber. The two chambers are separated by a blank flange when performing spatial imaging.

and are differentially pumped. The chamber containing the molecular nozzle (the “source” chamber) is evacuated by a 3700 ls^{-1} diffusion pump (Varian VHS-250) backed by a rotary pump (Leybold Trivac D40V). The diffusion pump is fitted with a water baffle to reduce the flow of oil into the source chamber. The source chamber typically reaches stagnation pressures of $\sim 5 \times 10^{-7}$ Torr, which may rise to $\sim 1 \times 10^{-6}$ Torr when the nozzle is in use. The source used to introduce the gas to the chamber is a pulsed solenoid nozzle (General Valve series 9), with $500\text{ }\mu\text{m}$ diameter orifice. The nozzle is mounted on an $x/y/z$ translating stage, allowing the manipulation of the position of the nozzle in relation to the skimmer while the chamber is under vacuum. The gas mixture is stored in a stainless steel bomb behind the nozzle at a pressure of 2 bar. Previous experiments have indicated the rotational temperature of the resultant beam to be $\sim 25\text{ K}$ [88].

The reaction chamber is pumped by two turbomolecular pumps. A larger 510 ls^{-1} Pfeiffer Vacuum TMH-521P is located under the ion optics and laser interaction region (“Turbo Pump A”). The smaller 60 ls^{-1} Pfeiffer Vacuum TMU-071P serves to evacuate the flight tube (“Turbo Pump B”). Both pumps are backed by another Leybold Trivac D40V rotary. Stagnation pressures in the reaction chamber are typically around 7×10^{-8} Torr, rising to 1×10^{-7} Torr when the nozzle was operating. When surface samples were used, the experiment was run at $\sim 1 \times 10^{-7}$ Torr. The chamber containing the ion optics, flight tube and detector (the “reaction chamber”) can be separated from the source chamber with a blank flange when performing surface imaging experiments in which the molecular nozzle and skimmer are not necessary. This greatly reduces the volume to be evacuated, improving the amount of time required to reach operating pressure in the chamber after the changing of the sample and hence experimental turnover rate. Gate valves separating the large turbomolecular pump and the flight tube from the reaction region further reduced the volume that had to be let up to atmosphere, allowing the detector to remain in vacuum whilst the surface samples were replaced.

The laser beams were introduced through 5 mm thick fused silica plates in the

sides of the chamber, providing $\sim 90\%$ transmittance at the UV wavelengths used in this thesis. When performing gas-phase experiments, the beam is sent through the chamber normal to these windows, such that it exits the other window and is stopped by a beam block. When performing surface imaging, the laser is introduced at a 15° angle from the normal so that the beam is incident on the mount and irradiates an area of the sample.

3.1.2 Sample Types and Ionisation Processes

Broadly speaking, there were four types of ionising methods used for the experiments in this thesis. For the velocity-mapped ion imaging experiments, ionisation of the photofragments was achieved with resonantly-enhanced multi-photon ionisation (REMPI). For spatial-mapped imaging of surfaces, Desorption/Ionisation On porous Silicon (DIOS), Matrix-Assisted Laser Desorption/Ionisation (MALDI), and direct laser ablation/ionisation were employed. These three methods required different adaptations of the sample mount due to their different requirements. Each of these modifications, as well as their sample preparation methods, will be discussed in their relevant chapters.

3.1.3 Ion Optics

The ion optics of the experiment consist of four stainless steel plates of 92 mm diameter, mounted on stainless steel rods. The lengths of each of the spaces between plates 1–4 (see Fig. 3.1) are 15 mm, 21 mm, and 29 mm respectively. The plates are kept insulated from each other and the mounting rods by means of MACOR ceramic spacers. The rods are in turn fixed to a flange that connects the chamber to the flight tube. The repeller plate (labelled 1 in Fig. 3.1) is varied for different types of experiment. For velocity mapping experiments, a plain stainless steel plate is used with a 2 mm hole to allow the molecular beam to pass through. Simulations of the ion trajectories indicate that the aperture is small enough that it does not adversely affect the velocity-mapping trajectories of the ions. For surface imaging

experiments, the repeller was adapted to take a variety of samples. Each of these sample mounts could be easily interchanged without damaging the mapping ability of the apparatus. The next three plates (labelled 2 to 4 in Fig. 3.1) are of stainless steel and have apertures of 20 mm. The potentials of plates 1-3 may be individually controlled to up to a maximum of 10 kV. Plate 4 is held at ground and serves to preserve a field-free flight path to the detector. For velocity-mapping experiments, the potentials of the repeller to extractor plate was set to 1 : 0.738, with the absolute potentials varied to control the magnification of the image on the detector. For spatial mapping experiments, the potentials on all three plates were varied, and the potentials used described in Sec. 2.2.2.

3.1.4 Laser Systems

Depending on the nature of the experiment, the laser systems used were very different. In velocity-mapped imaging, a conventional pump-probe experimental setup was used, with counter-propagating pump and probe laser beams perpendicular to the molecular beam and flight path. However, for surface imaging experiments, only one laser was used at a shallow incidence angle to the sample, with the ionisation mechanism dependent on the nature of the sample preparation. To facilitate these different requirements, three different lasers were used.

An excimer laser (Lambda Physik EMG 103 MSC) was used as a pump laser in the velocity-map imaging experiments. An ArF gas fill was used to provide radiation at 193.3 nm. The laser was triggered at 10 Hz, with a pulse duration of 25 ns. Approximately 160 mJ was produced per pulse. The beam was narrowed by an iris upon exit from the laser, and then collimated by a 2 m focal length lens. It was then linearly polarised by a Rochon polariser, with the direction of the polarisation controlled by the orientation of the polariser. The ordinary beam was used, with the extraordinary beam discarded with a beam dump. The polarised light was focussed into the chamber through a 30 cm focal length lens.

A dye laser (Sirah Cobra Stretch) was used to generate the probe wavelengths

of light when conducting velocity-mapped imaging experiments. This was pumped with the third harmonic (355 nm) of an Nd:YAG laser (Continuum Powerlite Precision II 8010). Rhodamine 101 was used as a dye in methanol to give, after frequency doubling, a wavelength of around 310 nm. The frequency-doubled output of the dye laser was separated from the fundamental with a set of four Pellin-Broca prisms and the fundamental discarded into a beam dump. The dye laser was capable of producing, at this wavelength, up to 12 mJ of energy per pulse. At a repetition rate of 10 Hz, this corresponded to a power of 120 mW. A photoelastic modulator (PEM) was used to impart either horizontal or vertical polarisation. For velocity mapping experiments, the beam was focussed into the chamber with a 30 cm focal length lens so as to overlap with the molecular beam and the focus point of the pump laser. For surface imaging experiments, the laser was defocussed with a 30 cm cylindrical lens in the vertical plane. The laser was introduced into the chamber such that it had an incidence angle of about 15° from the sample plate. This shallow incidence angle, together with the vertical defocussing, ensured that the whole sample was irradiated by the laser.

When a greater laser energy was required for the surface imaging, an Nd:YAG laser (Continuum Powerlite Precision II 8000) was used directly. The potential energy of the laser pulse was far greater than desired, which would lead to a large amount of signal saturating the detector, and high damage to sample within a few experimental cycles. Hence, the tripled 355 nm output was internally reduced, then sent through two 50% beamsplitters to further reduce the power. As the light emitted from the Nd:YAG is strongly polarised, the Rochon polariser used for the excimer in the velocity-mapping experiments was also used as a fine control for selecting a desired proportion of the laser beam by setting the angle of the polariser to allow only part of the light to pass through undeflected. After the steering optics and the defocussing lenses, the energy of the laser entering the chamber was about 25 mJ per pulse, but could be finely controlled between 10 mJ–45 mJ. The pulse width of the light was between 5–7 ns.

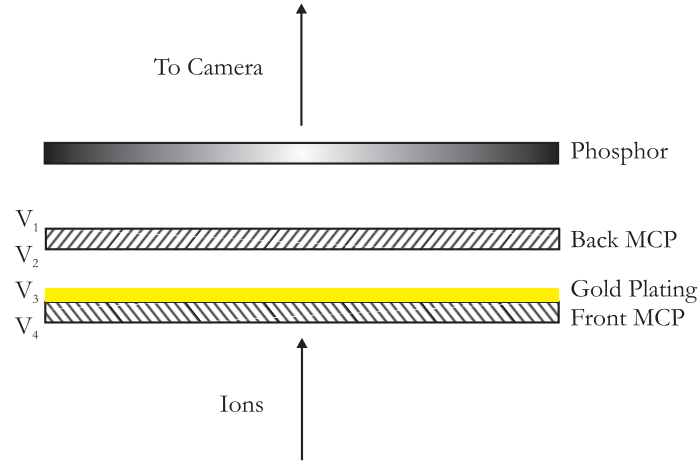


Figure 3.3: Diagram of the MCP and phosphor potentials. A potential gradient between V_2 and V_3 theoretically prevents the electrons exiting the input MCP triggering the second cascade in the second MCP by returning them to V_3 . An alternative method of reducing signal is to raise the potential the input face in order to reduce the initial gain.

3.1.5 Detection Assembly

The detection system used in the experiments consisted of a set of microchannel plates (MCP) coupled with a phosphor screen, followed by a camera. The MCPs (Burle Technologies, now Photonis) were a pair of 78 mm diameter plates arranged in chevron configuration. Each plate consists of millions of channels going through the plate at an angle of 12° to the surface. The channels have a centre-to-centre pitch of $32\mu\text{m}$ and pore diameter of $25\mu\text{m}$. The electronic potential of each face of the MCPs (V_1 , V_2 , V_3 and V_4 in Fig. 3.3) could be controlled separately, with a maximum bias of 1.2 kV across each individual MCP.

In the cases when there was a large amount of signal other than the mass peaks of interest, the extra signal may saturate the detector. In these cases, the large signal could be removed by pulsing the detector. The back face of the input MCP was gold-plated in order to reduce the capacitance of the MCP, thereby allowing faster changing of the potential on plate. By having the potential of the output face of the input MCP (V_3) more positive than the input of the output face (V_2), electrons ejected from the first MCP will be repulsed by the second and not trigger

the second cascade of electrons. However, it was found that this method was not effective enough at pulsing out unwanted signal as the bias between the plates was not large enough to repulse all electrons. An alternative method was used, in which the potential of the input face of the MCP was raised by up to 500 V, thus greatly lowering the gain of the first MCP.

Electrons ejected from the back of the MCPs were accelerated onto a P47 phosphor screen, which converted the electron impacts to flashes of light. The P47 phosphor screen had a 100%-10% decay of time of 60 ns. The maximum bias between the output of the MCPs and the phosphor screen was 5 kV. However, the maximum potential was never used as a good amount of signal could be attained, with further increases in screen potential resulting in saturation of the detector.

The flashes of the phosphor were detected by three different camera systems, a conventional intensified charge-coupled device (CCD) camera, a fast-framing CCD camera, and a new time-stamping detector. For each of these, the data was read out to a PC *via* a framegrabber for analysis.

3.1.6 Camera Systems

Conventional CCD Camera

The conventional intensified CCD camera (Photonic Science) had a pixel resolution of 576×768 . The camera integrated signal on-chip over an amount of time determined by the trigger. All signal that is detected within the trigger gate is therefore integrated into a single image. The gate could be set from a minimum length of 50 ns to an arbitrarily long period of time. When the acquisition period is over, this integrated signal has to be read out to a PC, allowing the camera to collect the next image. This camera is fitted with a controllable MCP intensifier, and thus is the most sensitive of the cameras used in this work, with the highest pixel resolution. It was used to acquire preliminary images, in the alignment of the sample and lasers, and optimisation of the signal intensity. It was also used in determining the resolution of the experiment without being limited by the detector pixel resolution.

Fast-Framing CCD

The fast-framing CCD camera (Dalsa [89]) is based on using *in-situ* storage on the CCD in order to collect a number of integrated images in sequence before reading out to the PC. Full details of the camera are described in ref. [90], but a description of its operation will be provided here. In a conventional CCD camera, the framerate is limited to the experimental cycle, as only one image may be stored before readout is necessary. Additionally, reading out the data from the camera is also a relatively long process, which cannot be done within the timescale of the separation between mass peaks in a time-of-flight spectrum. In the fast-framing camera, each pixel is assigned 2 sets of 8 memory registers dedicated to the storage of the acquired images. After the camera begins acquiring signal, it will, at pre-programmed times, transfer the acquired signal from the collection area alternately into one of the two blocks of memory registers. Thus, the detector effectively collects 16 sequential images of pre-determined length and stores them on-chip in each experimental cycle. The reading out of the data is performed in the time in the experimental cycle after the masses of interest have finished arriving at the detector and before the next cycle begins.

The times of integration have to be programmed into the camera before the acquisition begins, thus requiring some previous knowledge of the masses that may be observed. In the experiments of this work, the time-of-flight spectrum signal acquired with the photomultiplier tube was used to program the lengths of the frames. The lengths of the frames could be programmed in steps of 5 ns. The number of steps could be set in the range from 2 to 255, meaning frame lengths between 10 ns and 1275 ns. At 10 ns frame lengths, this represents a burst rate of 100 Mfps (megaframes per second), making this the highest time-resolution camera available for this work. In order to attain the highest signal-to-noise ratio, frames were timed such that they encompassed only the mass peaks of interest. Since the masses were generally separated by some time in which there is no signal of interest, and the frames had to be acquired sequentially, sacrificial frames were used

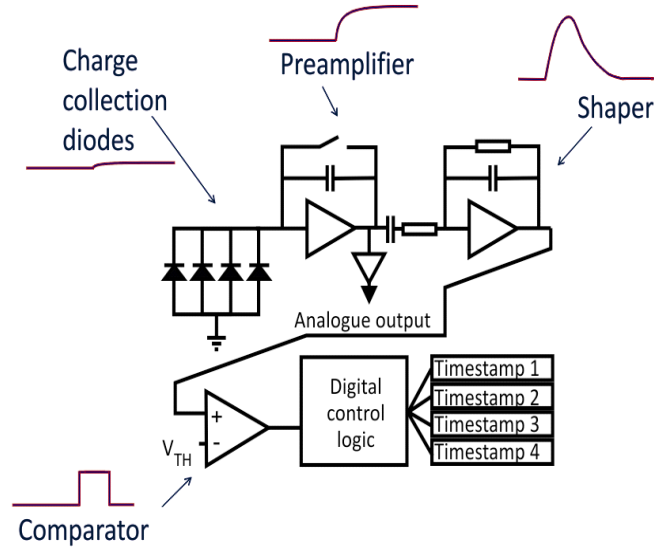


Figure 3.4: Pixel diagram for the PImMS detector. The signal collected in the charge collection diodes is amplified in the preamplifier. The amplified signal is passed to a shaper to convert the rise in charge to a pulse, which is then compared to a threshold. If the signal is large enough, the digital control logic stores its timecode in the first unfilled timestamp. Analogue readout is also possible from just after the preamplifier. Adapted from Ref. [91].

to acquire during periods with only noise between each mass peak in order so that these would not be integrated into the signal frames. Effectively this means that it was possible to use only half of the frames to acquire signal in, and collect images of eight mass peaks.

Because of the large amount of space taken up by storage, readout and other elements, the fill factor of each pixel is extremely small (1%), and microlenses have been implemented in order to improve the sensitivity of the detector. The fast framing camera has a pixel resolution of 64×64 , and thus has the lowest pixel resolution of the cameras available for this work. However, this low pixel resolution was not limited by the architecture of the detector, but a choice by the manufacturers: it is one of the advantages of *in-situ* storage detectors that the frame rate is not greatly affected by the pixel count of the detector.

PImMS Detector

Over the course of this thesis, a new detector was developed in collaboration with the Rutherford Appleton Laboratories and Oxford Physics. The concept, named PImMS (Pixel Imaging Mass Spectrometry), is a timestamping detector based on CMOS and INMAPS technology [92]. A schematic of its processes is shown in Fig. 3.4. A clock is started at the beginning of the acquisition cycle. The time steps of the clock may be set down to 25 ns. Each pixel has four charge collection diodes, which receive the signal in the form of photons from the phosphor screen. The signal is passed to a preamplifier, which increases the signal. At this point, an analogue readout allows the total signal, together with its intensity information, to be viewed. After the signal has been amplified, the shaper turns the rising curve into a pulse. This pulse is then analysed by the comparator, which determines if the signal is above a pre-programmed threshold. This threshold may be individually programmed for each pixel in order to compensate for slight differences in pixel sensitivity [93] so that the response level for each pixel is the same for the same amount of signal. If the signal is higher than the threshold, the digital control logic saves the value of the clock at that time into the first unused register. Each pixel has the capability to store up to four timestamps, labelled timestamp 1 to timestamp 4 in Fig. 3.4, before running out of memory.

At the end of each experimental cycle, the time data may be read out from the memory buffers of each pixel into a PC. Each datum then provides information on which pixel (x/y information) signal was detected, as well as the time of the event (t). The three-dimensional information can then be analysed and reconstructed. For example, by counting the number of events at each time step and plotting the frequency against time, a one-dimensional mass spectrum may be produced from the data. By plotting the positional information of the pixels within the desired time steps, images within time windows of interest may be generated. The images may also be centroided and event-counted in time in addition to in space to improve mass and time resolution [86, 94].

An improvement of PImMS over previous time-stamping detectors is the ability to store multiple timestamps. With the Medipix family of detectors and Timepix in particular [95], only one value for the time may be obtained per experimental cycle. In “arrival time” mode, the clock is started when signal above the threshold is detected and stopped at a synchronised time at the end of the experimental cycle. The value of the clock at the time therefore gives information on the arrival time of the ion. However, this means that once started, the clock must continue running until the end of the cycle: it is not available to keep track of the arrival of any further ions. This is the cause of the one-hit limit. This limit presents a serious problem in ion imaging, particularly in the context of spatial imaging mass spectrometry. In this situation, a large number of masses is expected to desorb from each region on the sample, and these will be mapped to the same position on the detector. Multiple hits are therefore expected for each pixel, and valuable information on the later peaks would be consistently lost. A possible method of circumventing this is to ensure a low count rate in order to reduce the chances of double hits on a single pixel. This method was used by Jungmann *et al.*, for velocity-mapped slice imaging [41]: the authors used a repetition rate of 5 Hz and an average of 2.6 events per frame. However, this strategy defeats the major advantage of fast detectors of imaging multiple masses with a higher throughput.

With the four registers of PImMS, multiple ions striking the same pixel may be recorded. The chances of each individual pixel being struck by multiple events decreases exponentially with the number of events [96]. The register in which the data is stored is also read from the detector, and the occupancy of each register is investigated in the results chapters of this work. This can be used to determine the degree of saturation of the memory buffers, and if four is a sufficient number of registers for use in biological imaging mass spectrometry. Finally, future versions of PImMS will have in excess of 300×300 pixels, which will further reduce the chances of the same pixel being hit by two different events.



Figure 3.5: Ion image taken of signal from a single laser shot. Each flash on the screen is shown over an area of pixels approximately 2 mm in diameter.

3.1.7 Acquisition

The data from each of the detector systems was read out onto a PC. For the conventional CCD camera, this was transferred *via* a framegrabber (Euresys Picolo Pro 2). Flashes on the detector would be recorded by an area of pixels on the camera, as the cloud of electrons from the output face of the MCPs are spread out over a number of pores and are ejected in a cone from each pore. An image taken of ions released from a single laser shot is shown in Fig. 3.5. The images could be centroided [97] by assuming a Gaussian intensity distribution of the flashes on the phosphor, caused by the electron spread from the pores of the MCP. Thus, a pixel which was brighter than all its nearest neighbours would indicate the centre of the electron cloud and hence the position of impact of the ion on the input face of the MCP. This centroiding was performed on-the-fly with the acquisition software and the images summed together. The output data of the acquisition program consisted of a single intensity map, the sum over the number of laser shots taken.

The fast-framing CCD camera transferred its data through a breakout box and framegrabber (Vivid Engineering). The data recording and processing was performed with custom LabVIEW programs. It had been found that due to the lower

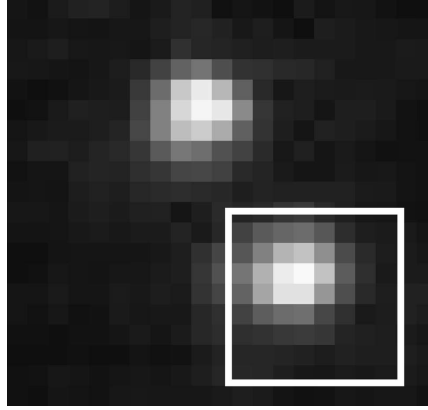


Figure 3.6: Close-up image of two events detected with the conventional 576×768 CCD camera. Each event covers multiple pixels of the higher pixel resolution camera. The white square shows the equivalent area that would be covered by one pixel of the fast-framing CCD. The spot is significantly smaller than the pixel size of the detector. Adapted from Ref. [77]

pixel number of the fast-framing camera, each ion hit on the detector illuminated only one pixel [77]. The difference in pixel sizes is shown in Fig. 3.6. Additionally, with the low pixel resolution, the probability of two individual hits on adjacent pixels is much higher, and centroiding may even be detrimental to the data by mis-identifying the two adjacent events as one hit. Hence, centroiding algorithms were used during the acquisition of data with the fast framing camera, improving the speed of the acquisition program. The output was a series of 64×64 intensity maps, summed over the number of experimental cycles. The effects of centroiding are investigated further in Sec. 3.2.

The PImMS camera data was transferred to a PC via a USB port and was collected by custom-built software from aSpect Systems [98]. The software allowed further analysis of the data using our own custom-built LabVIEW modules.

3.2 Characterisation of Typical Signal

When MCPs are struck by an ion, the cascade of electrons through the pores give a gain of approximately 10^3 electrons per MCP. The electrons are ejected from the

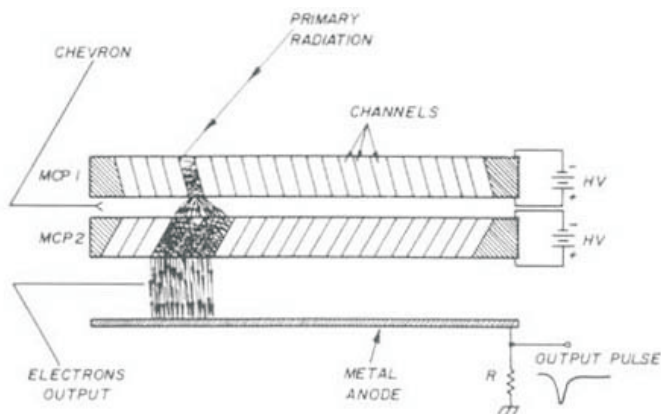


Figure 3.7: Illustration of operation of two MCPs in chevron operation. The electrons from a single channel spread and cause a flash over a finite area on the phosphor. Illustration adapted from Ref. [39].

output face of the plate, in a cone with a given solid angle. This will trigger a group of pores on the second MCP, leading to an even larger electron footprint upon exit from the second MCP (Fig. 3.7). This spread of electrons produces a spot with a two-dimensional Gaussian intensity on the phosphor. Characterisation of these spots is required to determine the size of the clusters and the necessity of centroiding for the different cameras. Additionally, this data was used to determine the required sensitivity and threshold for the PImMS detector during its development. This section presents the methodology and results of such an analysis, conducted to characterise the clusters produced by the MCP and phosphor array used in this work.

3.2.1 Experimental Procedure

The experiment was used in velocity-mapped imaging mode. A mix of $\sim 5\%$ CS_2 in He was prepared at 2 bar. This was stored in a stainless steel bomb. This was introduced to the source chamber through the pulsed nozzle along the time-of-flight axis. The beam was intersected with 193 nm light from the pump laser. This served to both dissociate the molecules and ionise the fragments. The MCP potentials were set to have a 1 kV bias across each plate, with $V_1 = 2 \text{ kV}$, $V_2 = V_3 = 1 \text{ kV}$,

and $V_4 = 0\text{ V}$. The screen was set to 4 kV . These potentials were chosen as near the upper limit of the operating voltages in order to maximise the effect of clusters during normal operation.

The detector used for these experiments was the “Vanilla” sensor [99], provided by the Rutherford Appleton Laboratories. This sensor was designed as a testing sensor, with high fill factor and sensitivity, as well as minimisation of noise. It is an active pixel sensor (APS) also based on CMOS technology. It consists of 520×520 pixels of $25\text{ }\mu\text{m}$ pitch. This detector was chosen as it has been carefully characterised in the Rutherford Appleton Laboratories, with its quantum efficiency and digital number calibration well-known. The quantum efficiency is the fraction of photons striking the photo-sensitive area that create an electron-hole pair in the detector, thus releasing electrons into the device. In the case of Vanilla, this value is 0.74. The calibration of the digital number is then used to set the dynamic range of the detector to the electrons released. The Vanilla detector has an 8-bit dynamic range of 0-255 digital numbers (DN), and each digital number corresponds to a signal of 26.3 electrons. In short, quantum efficiency $\times$ digital number calibration (camera gain) gives the number of photons striking the detector for each increase in DN. This then corresponds to 35.4 photons per digital number for the Vanilla detector.

The detector was allowed to acquire at 30 Hz with the experiment running at 10 Hz, in order to collect a variety of windows of the time-of-flight. A set of thirty such “data” images were collected. Another series of thirty “dark” images, which were taken with exactly the same conditions, but without the ionising radiation, was also taken. Each of these frames could then be analysed individually, using code written in FORTRAN 77.

3.2.2 Random Noise Characterisation

To begin with, a characterisation of the random noise produced by the detection system provides valuable insight into the required thresholds that one might use to separate the signal from the noise. This analysis was performed by using the

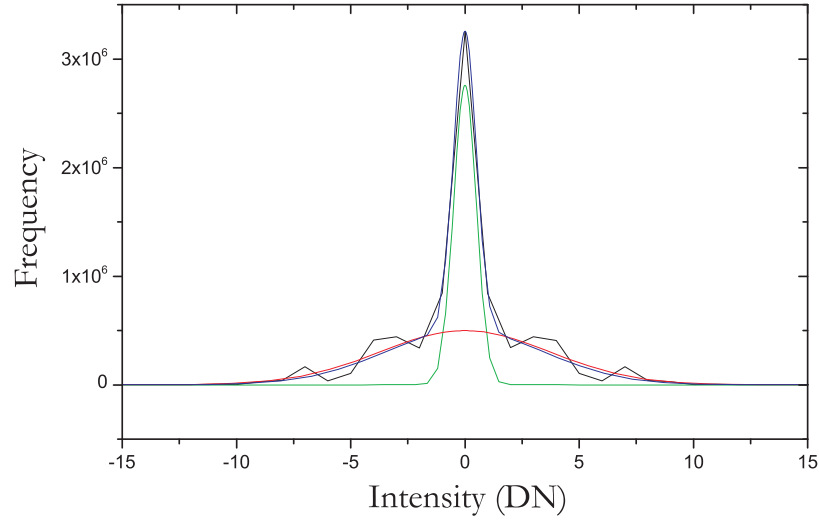


Figure 3.8: Frequency plot of intensity distribution of the background signal noise collected by Vanilla. Each frame was subtracted from the following frame, resulting in both positive and negative values centred around 0. Fit with the sum of two Gaussian functions (green, red) gives a σ of 0.496 DN and 3.732 DN respectively. The sum of the fits is shown in blue.

“dark” data. With the thirty frames, each frame was subtracted from the next, *i.e.*, $2 - 1, 3 - 2, \dots, 30 - 29, 1 - 30$. This subtraction served to remove the systematic background signal of the detector itself, leaving only the fluctuations from random noise.

A plot of the intensity of the pixels is shown in the black line of Fig. 3.8. The frequency of each intensity pixel from each of the resultant difference images has been plotted against their intensity. As the images were subtracted from each other in a cyclical manner, the expected symmetrical distribution of noise centred around 0 intensity can be observed. In order to characterise the distribution of the noise, it is necessary to fit the peak. However, the shape of the plot indicates that it may not be well described by a single Gaussian function, suggesting that there may be multiple sources of noise with different distributions. A summed fit of two curves is shown in blue in Fig. 3.8, which fits the distribution of the noise well. The individual Gaussian functions are also shown in red and green. Their contributions to the total noise may be determined by their areas under the curve.

Hence, 42.4% (green curve) of the noise has a σ of 0.496 DN, while the remaining 57.6% (red curve) has $\sigma = 3.732$ DN. Since the original distribution came from the addition of two sets of random noise, it is also necessary to divide the functions by $\sqrt{2}$ to determine the σ of the original noise, giving 0.3508 DN and 2.659 DN. In order to have a unambiguous identification of signal, a signal-to-noise ratio of greater than 5σ is desirable. Since signal should be ideally be higher than all the noise, the larger value of 2.659 DN is used to calculate a 5σ of 13.295 DN. All signal should therefore be ensured to be above this level, and, for the removal of noise, this is the minimum value a threshold should be set at. To convert this information back into the number of photons impacting the Vanilla detector, the noise has a σ of $2.659 \text{ DN} \times 35.4 = 94.1$ photons, and the minimum threshold should be set to 470.6 photons.

3.2.3 Cluster Intensity and Size

With the degree of noise and the minimum threshold values determined, these data may be used in the characterisation of the signal and clusters that are produced. In order to extract the signal from the data, the images taken with signal were subtracted from the corresponding frames of the dark images. The effect of this is shown in Fig. 3.9. (a) shows a raw data image, taken by the camera of the detector. The image is relatively noisy and the flashes of the phosphor unclear. Panel (b) shows the corresponding frame in the dark series, similarly noisy but with no signal. The image resulting from the subtraction of (b) from (a) is shown in (c). The image is much clearer and the signal is clearly visible. The flashes on the image can be seen to have a Gaussian intensity spread with a single maximum in each flash. This is in agreement with the observations of Chang *et al.* [97].

Chang *et al.* describe a method for identifying the centre of the spot using local maxima in the intensity of the image [97]. This was done by comparing the intensity of a pixel with the intensities of its surrounding pixels. If it is higher in intensity than all of its neighbours, then it is regarded as the centre of an event. During normal

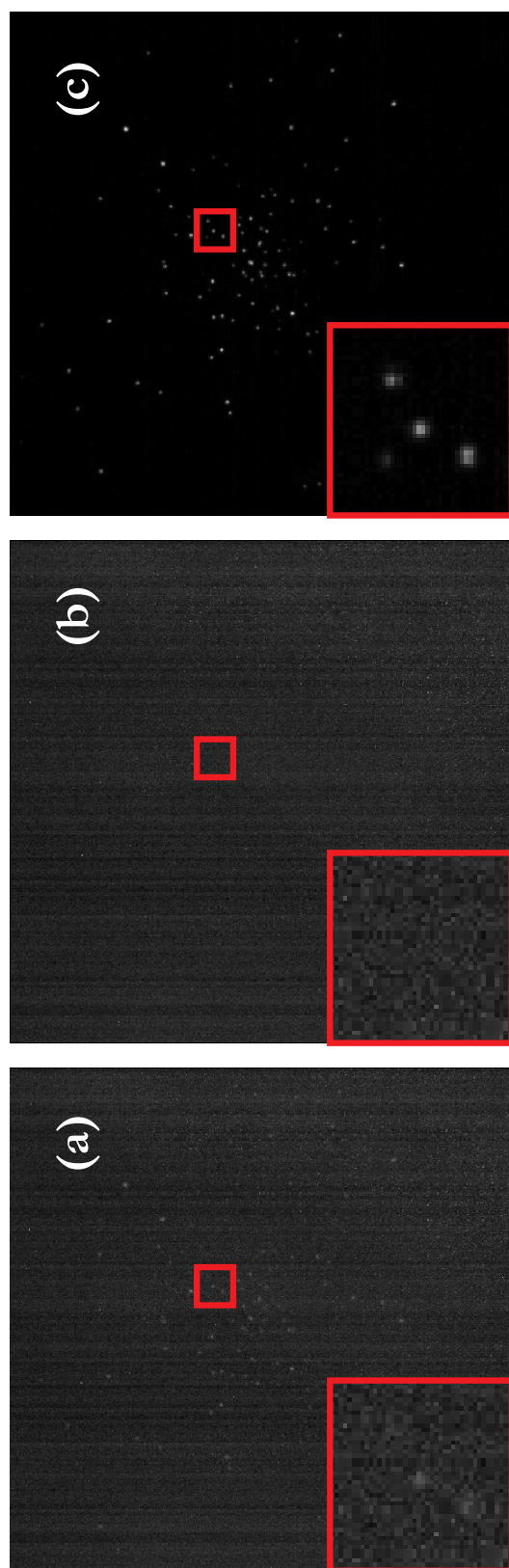


Figure 3.9: Representative single shot ion images collected with “Vanilla”. (a) Shows a raw data image, (b) a background subtracted image, and (c) a background subtracted image. The spots on the subtracted image show a clean Gaussian intensity distribution with the intensity in the centre. Insets in each panel show magnified views of areas within the small red squares. The magnified region is the same in each panel.

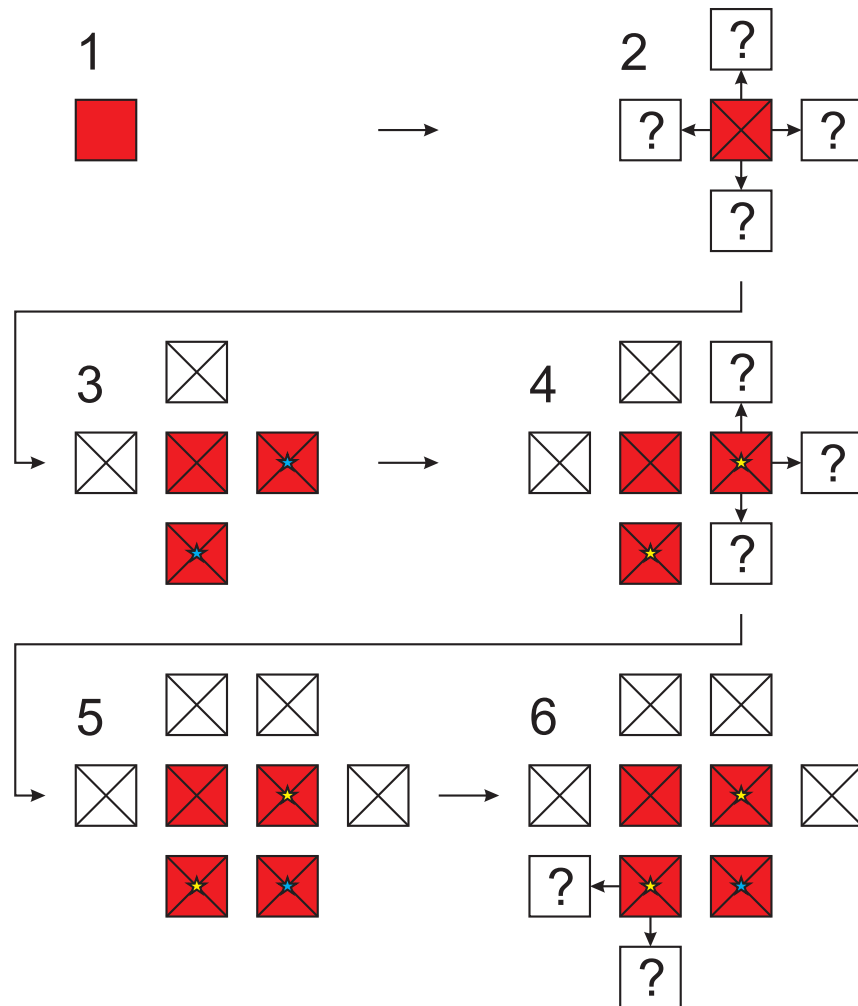


Figure 3.10: Illustration of process of cluster analysis program. **1.** A pixel above threshold is identified. **2.** The surrounding pixels are checked to see if they are also above threshold. **3.** The pixels above threshold are identified and tagged for checking (starred). **4.** Pixels around newly identified members of the cluster are also checked. Pixels that have been checked before (crossed) are not considered. **5-6.** The cycle is continued until no more pixels with intensity higher than threshold are found.

data acquisition for conventional velocity-mapped experiments on this apparatus, the centre of the ion impact has been found by using this method. However, this does not give information on the cluster itself. For a more quantitative analysis of the subtracted images, a new program was written in order to analyse the size of the clusters. The approach of this program is illustrated in Fig. 3.10. The program moved through the pixel array, searching for pixels above the desired threshold (1). This was marked as a part of the cluster. All pixels immediately adjacent to that were then identified (2), and those which were above the threshold also marked as part of the same cluster (3). The pixels adjacent to these new members of the cluster were then also marked for testing (4). The process continued (5, 6) until an “island” of pixels above threshold surrounded by pixels of low signal had been identified. The most intense pixel in the “island” was identified as the centre of the cluster and the total intensity of the cluster calculated. The alternative method of calculating the centre-of-mass (intensity) to identify the centre of the cluster was not used as this would have required a much more complex analysis, and the main parameter of interest was the size and intensity of the clusters. The highest intensity method of locating the centre of the mass peak should be valid under a Gaussian spread of intensities. Once the analysis of the cluster was completed, the program then continued to find the next cluster in the image. Finally, the total number of clusters was calculated.

In addition to providing information on the size and shape of clusters, a major advantage of this algorithm is that the intensity spread of the pixels through the cluster and the shape of the clusters itself can be examined. There are, however, two disadvantages of using this method of cluster analysis. The first is that more processing power was required to run the algorithm, and hence it was not feasible to implement this analysis on-the-fly during image collection. The other possible disadvantage of using this algorithm is that two clusters which hit the detector very close to each other may be considered as the same event. If the pixels above the threshold in the two clusters are spread out enough such that they would be in the

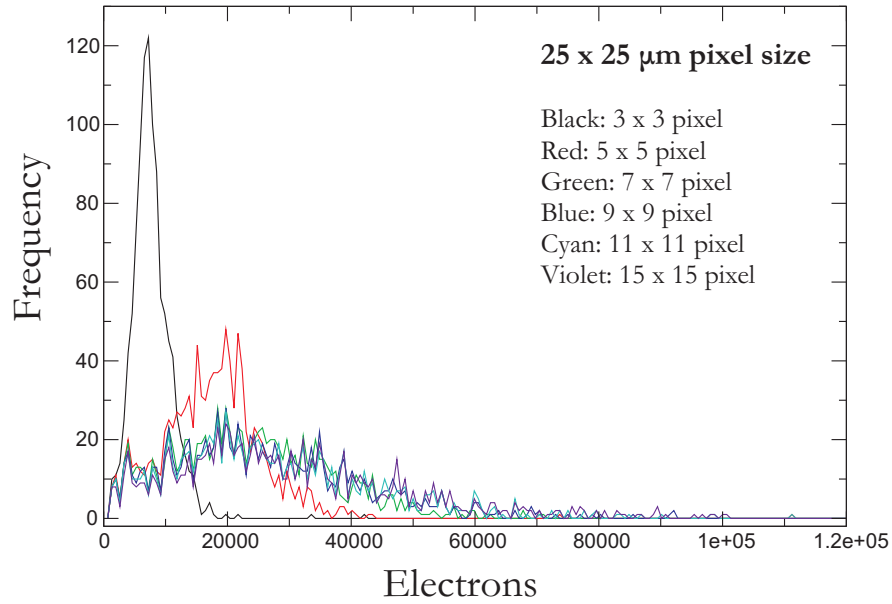


Figure 3.11: Plot of total intensities of each cluster. As the size of the shape in which intensity is summed is increased, the average intensity of the cluster increases: more of the pixels in the cluster are summed. From summing a square of 9×9 pixels upwards, the intensities can be seen to converge.

contact with each other they would be seen as the same cluster. This would not be the case if the local maxima were used to identify clusters. To see if this would have a significant effect, both cluster identification methods were run on the same data, and the number of clusters returned compared. Analysis on the frames with the highest amount of signal showed that the method devised by Chang *et al.* did not find significantly more clusters than this new method, indicating that the vast majority of events were spread sufficiently far apart that they did not overlap.

The cluster analysis program was used to investigate the intensities and shapes of the clusters in the data. In order to obtain this information, the intensity over the cluster was found. This was found by summing the intensities of differently-sized squares with the most intense pixel in the cluster as the centre. Single pixels, 3×3 , 5×5 and so forth until 15×15 squares were summed. The intensity distribution is shown in Fig. 3.11. For this analysis, a threshold of 25 DN was used. This was comfortably above the required minimum value of 13.295 DN determined in Sec. 3.2.2, and chosen in order to ensure the removal of all noise.

As one expands the size of the integration area beginning from the most intense pixel to the squares around it, the mean total intensity can be expected to increase as the high intensity pixels around the centre of mass of cluster are included. This can be seen clearly on the plot in curves corresponding to a 1×1 (single pixel) to 7×7 , in which the mean of the peaks is shifting to the right. The integral of the curves are constant, as the total number of clusters are the same. At a certain point, the box within which the integration of the signal will become large enough to include all the pixels in the cluster. It can be seen that this is the case for squares of 9×9 and greater, which have similar curves. Finally, there will be a point at which the square will begin to include other clusters and the mean intensities will begin to rise again. This point has not been reached on the plot.

In summary, it can be concluded from these curves that with the operating conditions used in this study, the cluster size is approximately nine pixels in diameter on the Vanilla detector, corresponding to $225 \mu\text{m} \times 225 \mu\text{m}$. Also, the ion impact positions appear to be far enough apart that, even in the most densely populated frames, the intense pixels of the clusters are unlikely to overlap.

3.2.4 Simulation of PImMS Pixel Size

In order to relate the results to the development of the PImMS detector, an additional layer of processing was used. The pixels of Vanilla are $25 \mu\text{m} \times 25 \mu\text{m}$ in size, while the design of the PImMS detector was for pixels of $75 \mu\text{m} \times 75 \mu\text{m}$ in order to allow space for the required transistors. Using the same magnification of the image onto the detector, that would mean that each pixel on the PImMS detector would cover nine times the area of the vanilla detector. This would have an effect on the number of photons striking each pixel and hence implications for the efficiency and sensitivity of the detector. This could be taken into account by the coarsening of the acquired data into 3×3 blocks, and summing the intensities within the blocks into one virtual pixel (Fig. 3.12). This effectively simulates the image that would be taken with a detector of the same overall size but with the coarser pixels, turning

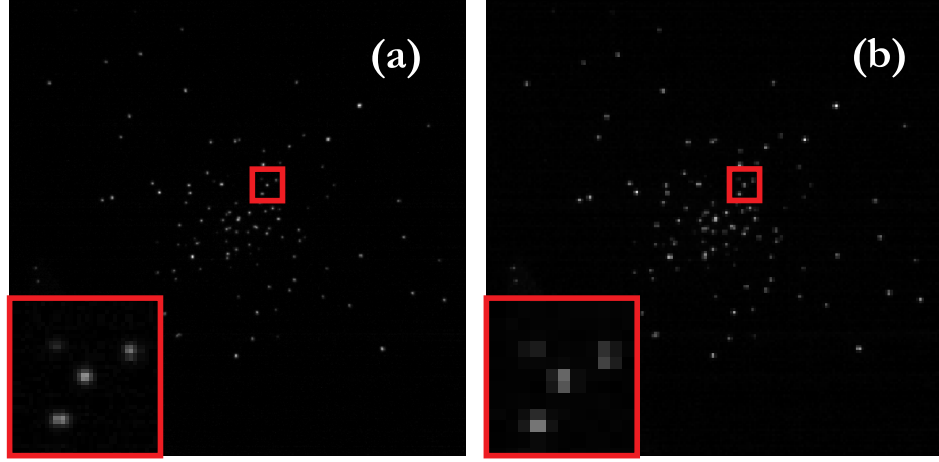


Figure 3.12: Effect of coarsening of the image by the Vanilla detector. The intensities in the nine $25\ \mu\text{m} \times 25\ \mu\text{m}$ are combined into a single $75\ \mu\text{m} \times 75\ \mu\text{m}$ pixel. Insets in each panel show magnified views of areas within the small red squares. The magnified region is the same in each panel.

an image of 520×520 pixels into one of 174×174 pixels.

The images produced by the grouping of signal into larger pixel sizes has been analysed in a similar fashion to the original data. The same clustering program was also used to analyse the coarsened images, and the results shown in Fig. 3.13. The threshold used for this analysis was 75 DN: as the integrated area has been increased by 9, the data intensity would be increased by the same amount, as it is the sum of the intensities within those nine squares. The noise would then be expected to increase by a factor of $\sqrt{9}$, and so the threshold was increased by three times that used for the $25\ \mu\text{m} \times 25\ \mu\text{m}$ pixel images. The data for the coarsened image have been plotted together with selected data from the analysis of the original images for comparison. The dashed lines are the results corresponding to the $75\ \mu\text{m} \times 75\ \mu\text{m}$ coarsened image, while the solid lines are from the original data. The 3×3 and 9×9 clusters of the original data have been plotted in comparison with the single pixel and 3×3 pixel of the coarsened image, as they integrate the signal over the same area.

Slight differences in the plots can be observed in Fig. 3.13. For the integrated area of $75\ \mu\text{m} \times 75\ \mu\text{m}$, there is a larger spread of intensities with a lower minimum

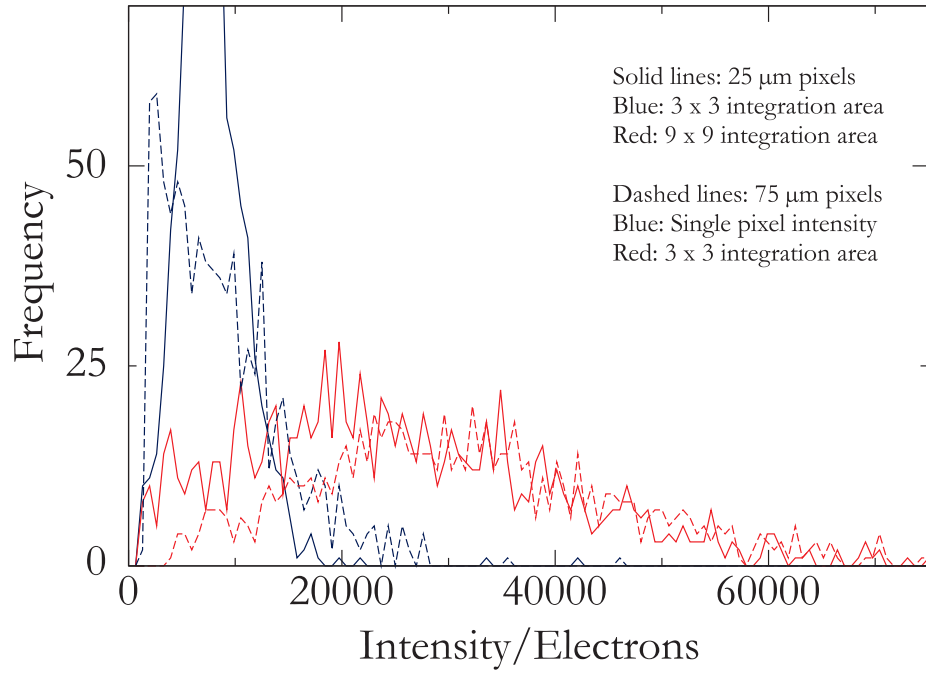


Figure 3.13: Plots of the cluster intensities for both original and coarsened data. The solid lines are from the $25\ \mu\text{m}$ pixel data while the dashed lines are from the simulated $75\ \mu\text{m}$ pixels. The red lines correspond to intensities integrated in a $75\ \mu\text{m} \times 75\ \mu\text{m}$ area on the detector, while the blue line shows integration over $225\ \mu\text{m} \times 225\ \mu\text{m}$. See text for full details.

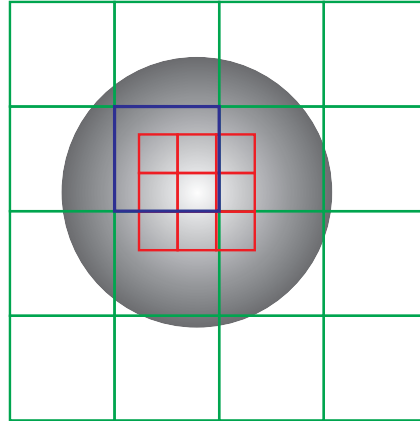


Figure 3.14: Illustration of the difference in intensity integrated into a single $75\ \mu\text{m} \times 75\ \mu\text{m}$ pixel and a 3×3 square of $25\ \mu\text{m} \times 25\ \mu\text{m}$ pixels. The cluster (grey spot) falls within the $75\ \mu\text{m} \times 75\ \mu\text{m}$ coarsening grid (green). The centre of the cluster is identified within this grid and is recorded as the integrated intensity within the pixel (blue square). In contrast, the 3×3 square of the $25\ \mu\text{m} \times 25\ \mu\text{m}$ pixels (red) is centred on the cluster, and therefore integrates the highest intensity part of the cluster.

in the coarsened single pixel (red dashed line) than for the 3×3 $25\mu\text{m}$ pixels (red solid line), while the maximum value remains the same. The reason for this is illustrated in Fig. 3.14. In analysing the original data with $25\mu\text{m} \times 25\mu\text{m}$ pixels, the program identifies the centre of the spot, and the 3×3 integration area will be centred around this maximum. This is represented by the small red grid. This grid integrates the most intense part of the spot. During the coarsening process, however, the image is split into a grid of $75\mu\text{m} \times 75\mu\text{m}$ without accounting for the position of the clusters. The centre of the spot is identified as the pixel with the highest intensity. However, as the grid may not be exactly centred on the spot, the intensity is no longer necessarily taken from the most brightest area of the flash, and so even though the integrated area is the same, the total intensity of the pixels is less.

Another point to note is the lower number of low-intensity clusters. This is clear in the comparison between the 9×9 $25\mu\text{m} \times 25\mu\text{m}$ pixel integration intensities (blue solid line) and the 3×3 $75\mu\text{m} \times 75\mu\text{m}$ pixel intensities (blue dashed line) below 20,000 electrons. This is due to the higher threshold used in the analysis program: the lower intensity clusters are now being removed from the analysis. The same effect can be seen in the red plots, but with the cluster population dropping at around 7500. This is a result of different sizes of clusters being affected by the thresholding.

In summary, these results suggest that the clusters under these experimental conditions fall within an area of $255\mu\text{m} \times 255\mu\text{m}$. This gives an idea of the point in which centroiding would be required on the new detector; if the entire cluster is likely to fall within one pixel, then no centroiding would be required. Since the coarsened image shows that the cluster would still fall within a 3×3 square, but is likely to be larger than a single pixel, a centroiding algorithm would be required if the ion image would be projected onto the detector with the same magnification. Additionally, the threshold values would have to be carefully investigated to optimise the amount of useful signal received.

Finally, there are a few issues that need to be taken into consideration when drawing conclusions from these simulations. Generally, the light optics that are coupled to the detector are adjusted such that the entire phosphor of the MCP assembly is projected onto the detector (as discussed in Sec. 1.3.2). The size of the clusters in relation to the pixels is then closely related to the pixel resolution. The image can be thought of as the phosphor screen being split into a number of squares equal to the number of pixels in the detector. In the case of the fast-framing CCD camera, with 64×64 pixels, it has been found that grid was coarse enough that clusters would fall entirely within the pixel area [77], and that not using a centroiding algorithm did not adversely affect the quality of the results. In the case of the prototype PImMS detector, the pixel resolution of 72×72 is very close to that of the fast-framing CCD, and so no centroiding was employed when using the PImMS detector in the experiments in this work. However, future versions of the PImMS are planned to have in excess of 300×300 pixels, and would be in a similar range as these simulations. Centroiding in these images would then be effective in improving the quality of these images.

3.2.5 Threshold and Efficiency

Finally, the value of the threshold is particularly important in a time-stamping detector, as a threshold that is too low would fill up the memory registers with noise and cause loss of signal, while too high a threshold would lose low-intensity signal. In order to determine an appropriate level at which to set the threshold, the efficiency dependence of the signal was examined. The cluster analysis algorithm was modified to determine the dependence of the number of clusters detected on the threshold. The results are shown in Fig. 3.15. (a) shows the results for the original data with the $25 \mu\text{m} \times 25 \mu\text{m}$ pixels, while (b) shows results for the $75 \mu\text{m} \times 75 \mu\text{m}$ pixels, the image simulated to be acquired by PImMS. The solid lines show the percentage of the clusters that are observed against the value of the threshold. Since the on-chip thresholding will be most directly sensitive to the number of electrons produced

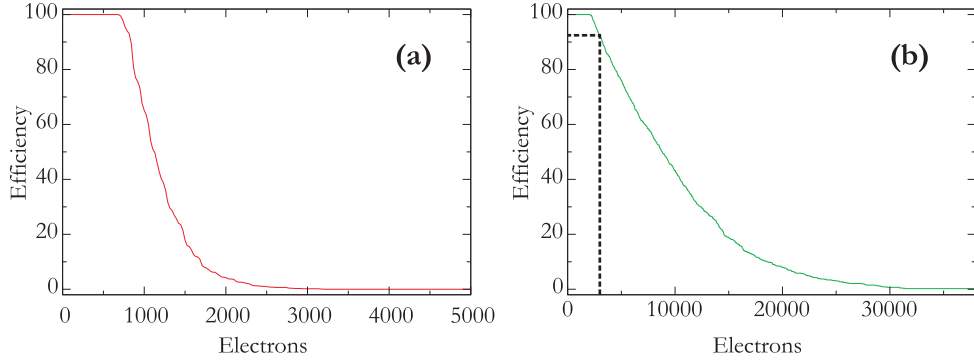


Figure 3.15: Plots of efficiency against threshold. The plot for $25\ \mu\text{m} \times 25\ \mu\text{m}$ pixels is shown in (a) while the plot for the simulated PImMS image with $75\ \mu\text{m} \times 75\ \mu\text{m}$ pixels is shown in (b). The line plots the percentage of clusters that would still be recorded with that particular threshold. The black dashed line on (b) shows the value of the planned threshold of the PImMS detector during development, which would result in detecting 92% of the clusters.

when the signal hits the detector, the results are plotted as a function of electrons. The number of electrons that were produced was calculated from digital number using the camera gain of the Vanilla detector (26.3 electrons per DN).

Examination of the curves show the smooth expected dropping-off of detector efficiency as the threshold is increased, as more clusters fall below the threshold value and are removed. It is important to note that the quantum efficiency of the Vanilla detector and the expected quantum efficiency of PImMS is significantly different; for Vanilla it is known to be 0.74, while the expected Q.E. of PImMS is in the region of 0.06, slightly less than 10% of Vanilla. This means that a number of photons which produces 1000 electrons on Vanilla can be expected to produce only about 100 electrons on PImMS. The value of 300 electrons was set by the developers of the PImMS detector in RAL, which would correspond to signal that would produce 3000 electrons on Vanilla. The dashed line Fig. 3.15(b) shows the percentage of clusters that would be detected with this threshold to be 92%, an acceptable amount.

3.2.6 Summary

In this section, the typical signal produced by the MCP and phosphor screens has been characterised, and considered in relation to the requirements for, and specifications of, the development of PImMS. With the conditions used, the random noise has been identified to have σ in the region of 94 photons, and thus a minimum required signal of 470 photons. The clusters have been identified to hit an area of smaller than $225\ \mu\text{m} \times 225\ \mu\text{m}$ on the detector, giving an indication of when clustering algorithms may be required in the acquisition of data. The images have been used to simulate those which may be seen by the larger pixel size of PImMS, and the effect of these larger pixel sizes on the required threshold and efficiency levels. These results have been used in the development of the PImMS detector, which was produced during the course of this work. Results from the detector will be presented in the later experimental chapters.

3.3 Ion Image Analysis: CS₂ Dissociation

In this section, a typical velocity-mapped experiment conducted on the apparatus before its modification for spatial imaging is presented. The technique has been applied to the investigation of the photodissociation dynamics of CS₂ after dissociation at 193 nm. The S(³P_{*J*}) products have been studied in particular. These results provide an example of the type of images and data for which the apparatus was constructed to collect. The analysis demonstrates the extraction of angular momentum polarisation information from this data. The principles behind the experiment are first described, together with the theoretical techniques used. Following this, the results are presented and analysed, and their implications discussed.

3.3.1 Theory

The main aim of velocity-mapped imaging experiments is the characterisation of the velocity distribution of the polarised photofragments. These have been described

quantum mechanically within the axial recoil approximation neglecting Coriolis-type interactions by the formalism of Siebbeles *et al.* [100] and generalised to two open-shell fragments by Bracker *et al.* [101] and Balint-Kurti *et al.* [102]. The $\mathbf{J}$ distribution of the photofragments are described by ρ_{KQ} . These state multipoles of rank K and component Q in the laboratory frame are given by:

$$\begin{aligned} \rho_{KQ} &= \frac{3}{4\pi} \left(\frac{2K+1}{2j_A+1} \right)^{\frac{1}{2}} \sum_{q,q',Q'} \sum_{k_d,q_d} (-1)^{K+q'} (2k_d+1)^{\frac{1}{2}} \\ &\times E_{k_d q_d}(\mathbf{e}) \begin{pmatrix} 1 & 1 & k_d \\ q' & -q & -Q' \end{pmatrix} \frac{f_K(q, q')}{f_0(0, 0) + 2f_0(1, 1)} \\ &\times D_{QQ'}^{K*}(\theta, \phi, 0) D_{q_d Q'}^{k_d}(\theta, \phi, 0), \end{aligned} \quad (3.1)$$

where j_A is the angular momentum of the probed atom, q, q' are the vector spherical harmonic components of the molecular electric dipole moments with respect to the recoil axis [103] and $Q' = q' - q$. The large brackets indicate a 3- j symbol and $D_{q_d Q'}^{k_d}(\theta, \phi, 0)$ are the Wigner rotation matrix elements [104]. $E_{k_d q_d}(\mathbf{e})$ is the matrix element of the polarisation tensor of the photodissociation light of geometry j and rank k_d [104]:

$$\begin{aligned} E_{k_d q_d}(\mathbf{e}_j) &= (-1)^s (2k_d+1)^{\frac{1}{2}} \begin{pmatrix} 1 & 1 & k_d \\ s & -s & 0 \end{pmatrix} \\ &\times C_{k_d q_d}(\Theta_d^j, \Phi_d^j), \end{aligned} \quad (3.2)$$

with $s = 1, -1$ and 0 for left circularly, right circularly and linearly polarised light respectively. $f_K(q, q')$ are the dynamical functions [100], which contain all the dynamical (*i.e.* non-geometric) information on the process. Explicitly, these are

given by [102]:

$$\begin{aligned}
f_K(q, q') &= \sum_{n, \Omega, \Omega_A, n', \Omega'_A} \begin{pmatrix} j_A & j_A & K \\ -\Omega_A & \Omega'_A & q - q' \end{pmatrix} \\
&\times T_{j_A \Omega_A j_B \Omega_B}^{n \Omega} (T_{j_A \Omega'_A j_B \Omega_B}^{n' \Omega'})^* (-1)^{K+j_A+\Omega'_A} \\
&\times \langle \Psi_{n, \Omega}^-(R, \mathbf{r}, E) | \hat{\mathbf{d}}_q | \Psi_{\Omega_i} \rangle^* \\
&\times \langle \Psi_{n', \Omega'}^-(R, \mathbf{r}, E) | \hat{\mathbf{d}}'_q | \Psi_{\Omega_i} \rangle,
\end{aligned} \tag{3.3}$$

where $\Psi_{n, \Omega}^-(R, \mathbf{r}, E)$ is the scattering wave function for the channel (n, Ω) . Ω is the total angular momentum component about the z axis, with Ω_i its initial component: $\Omega = \Omega_i + q$. $\langle \Psi_{n, \Omega}^-(R, \mathbf{r}, E) | \hat{\mathbf{d}}_q | \Psi_{\Omega_i} \rangle$ are the photofragment $\mathbf{T}$ matrix elements and the terms $T_{j_A \Omega_A j_B \Omega_B}^{n \Omega}$ are the expansion coefficients of the adiabatic molecular electronic states in the fragment basis at the asymptotic limit.

The recoil velocity dependence of the renormalised dynamical functions may be described as a linear combination of gaussians [105]:

$$\begin{aligned}
f'_K(q, q'; v) &= \frac{f_K(q, q'; v)}{\langle f_0(0, 0; v) \rangle + 2\langle f_0(0, 0; v) \rangle} \\
&= N \sum_i a_K(q, q'; i) e^{-\sigma(v_i - v)^2}
\end{aligned} \tag{3.4}$$

where N is a normalisation constant, $a_K(q, q'; i)$ are the Gaussian specific polarisation parameters, σ the width parameter, and v_i the centre of i^{th} gaussian. The angled brackets here indicate integration over all recoil velocities. The dynamical functions will in this work be recast for convenience in the form of polarisation parameters derived by Vasyutinskii and coworkers ($s_K, \alpha_K, \gamma_K, \gamma'_K, \eta_K$) [101, 106, 107] for their direct relation to orientation and alignment of the angular momentum, as well as the fact that they can be determined directly from the experimental images.

3.3.2 Methods

The experimental setup used was typical of velocity-mapped [47] time-of-flight mass-resolved ion imaging, as described in Sec. 3.1. The sample was seeded to $\sim 5\%$ partial pressure in He at 2 bar, and then passed into the chamber through a pulsed molecular nozzle and skimmer. The molecular beam was dissociated at 193.3 nm by the ArF excimer, then ionised *via* (2+1) REMPI using the Nd:YAG-pumped dye laser. The probe wavelengths used were 310.0 nm, 308.1 nm, and 310.8 nm for selective ionisation of the $S(^3P_0)$, $S(^3P_1)$, and $S(^3P_2)$ fragments respectively. The resultant ions were then accelerated with ion optics set to velocity-mapping potentials along a field-free flight tube to the MCP/phosphor detector. The images were captured with the CCD camera (Photonic Science) and sent to a PC for thresholding, centroiding and event counting. The images presented here were summed over 32,000 shots.

The method of data analysis employed is the Fourier moment analysis of Bass *et al.* [108]. The experimental image can be expressed as a Fourier series:

$$\mathcal{I}^j(r, \Phi) = \mathcal{C}_0^j + 2 \sum_{n>0} \mathcal{C}_n^j(r) \cos n\Phi + \mathcal{C}_n^j(r)' \sin n\Phi, \quad (3.5)$$

where $\mathcal{C}_n^j(r)$ and $\mathcal{C}_n^j(r)'$ are the Fourier moments of the image, determined by performing the appropriate Fourier integral:

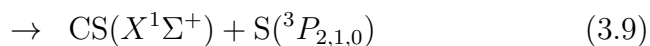
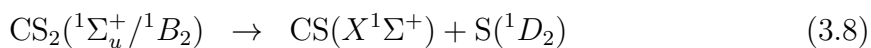
$$\mathcal{C}_n^j(r) = \int_0^{2\pi} \mathcal{I}^j(r, \Phi) \cos n\Phi r d\Phi \quad (3.6)$$

$$\mathcal{C}_n^j(r)' = \int_0^{2\pi} \mathcal{I}^j(r, \Phi) \sin n\Phi r d\Phi. \quad (3.7)$$

These experimental Fourier moments can then be fitted to those of an image simulated from polarisation parameters selected using a genetic algorithm, and errors calculated using a Monte-Carlo based method.

3.3.3 Previous Work

The ultraviolet absorption spectrum of CS₂ has been the subject of much interest and controversy for many years. Upon irradiation at 193 nm, the linear $X^1\Sigma_g^+$ ground state is excited *via* the fully allowed transition to the quasi-linear $^1\Sigma_u^+$ state above the barrier to linearity, labelled as 1B_2 in C_{2v} symmetry [109]. The absorption spectrum itself is complex and highly structured, due to near degenerate vibrational stretching and bending modes in the excited state with $\nu_1 \approx \nu_2 \approx 400\text{ cm}^{-1}$ [110–112], with absorption of 193 nm leading to the population of combined symmetric bending and stretching modes [110,113]. From the excited state, there are two possible dissociation pathways giving sulfur atoms of either singlet or triplet multiplicity:



The generally accepted branching ratio of products between these two pathways is $^3P/^1D = 2.8 \pm 0.25$, given by Waller and Hepburn [114], with previous measurements ranging from 0.25 to 6 [115–120]. However, two more recent studies have reported differing branching ratios of 1.5 ± 0.4 and 1.6 ± 0.3 [121, 122]. Multiphoton effects have been suggested as possible causes for the discrepancies in these values [118, 123], as CS($v > 5$) may absorb at 193 nm to produce S(3P_J) [117]. The electronically excited states sampled on the pathway to the asymptotic products are not well known; few *ab initio* potential energy surfaces are available for the CS₂ system, with only the lowest lying excited states calculated [124, 125]. Mank *et al.* have suggested a possible dissociation mechanism for the production of the S 1D products, using preliminary *ab initio* calculations [126]. After initial excitation to the $^1\Sigma_u^+/^1B_2$ state, the system undergoes an avoided crossing with the higher energy (repulsive) $^1\Pi$ surface which splits into $^1A_2 + ^1B_2$ components in the bent C_{2v} geometry, facilitating predissociation to the singlet products. For the formation of the triplet products two repulsive states, $^3\Pi_g$ and $^3\Sigma_g$ are expected to correlate to the CS+S(3P_J) products,

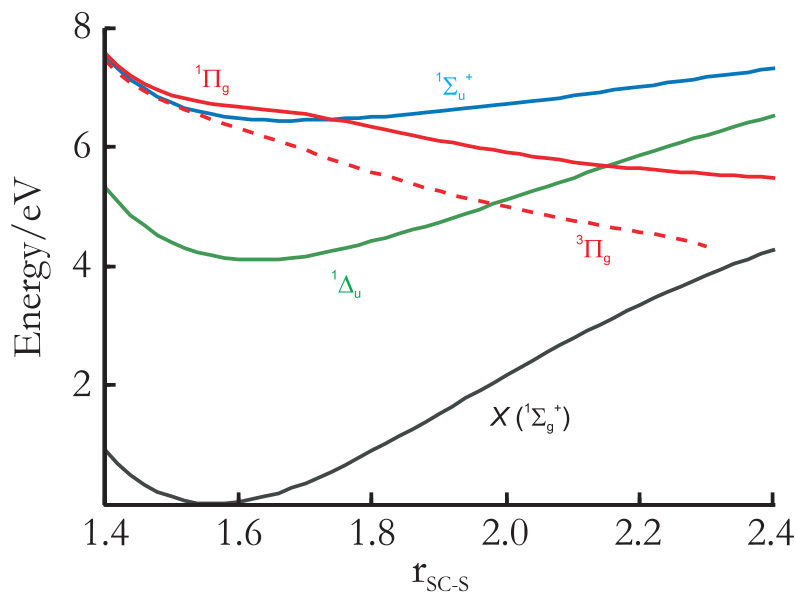


Figure 3.16: Potential energy curves taken from a slice through the surface at linearity of the potentials calculated by Bisgaard *et al.*. The dissociation is shown as a function of the asymmetric stretching coordinate. Adapted from Ref. [127]

with the mechanism involving coupling to these states. Recent *ab initio* potential energy surfaces by Bisgaard *et al.* [127] are in agreement with this, indicating a repulsive $^3\Pi_g$ surface that leads to the triplet product. A cut through the potential surface at linearity is shown in Fig. 3.16.

3.3.4 Results and Discussion

Ion images were collected for various geometries to allow sensitivity to all polarisation parameters and to ensure a unique fit, producing the first complete spin-orbit state resolved polarisation characterisation of the $S(^3P_J)$ products of CS_2 dissociation. In the case of images collected with linearly polarised light, the geometries were defined by the relation between the polarisation of the electric dipole vector of the light and the time of flight axis, with “V” being parallel to TOF axis and “H” perpendicular. Four linear pump/probe geometry combinations are thus available: “HH”, “HV”, “VH”, “VV”, where the first letter indicates the pump geometry and the second the probe. Additionally, light with polarisation at 45° to the TOF axis

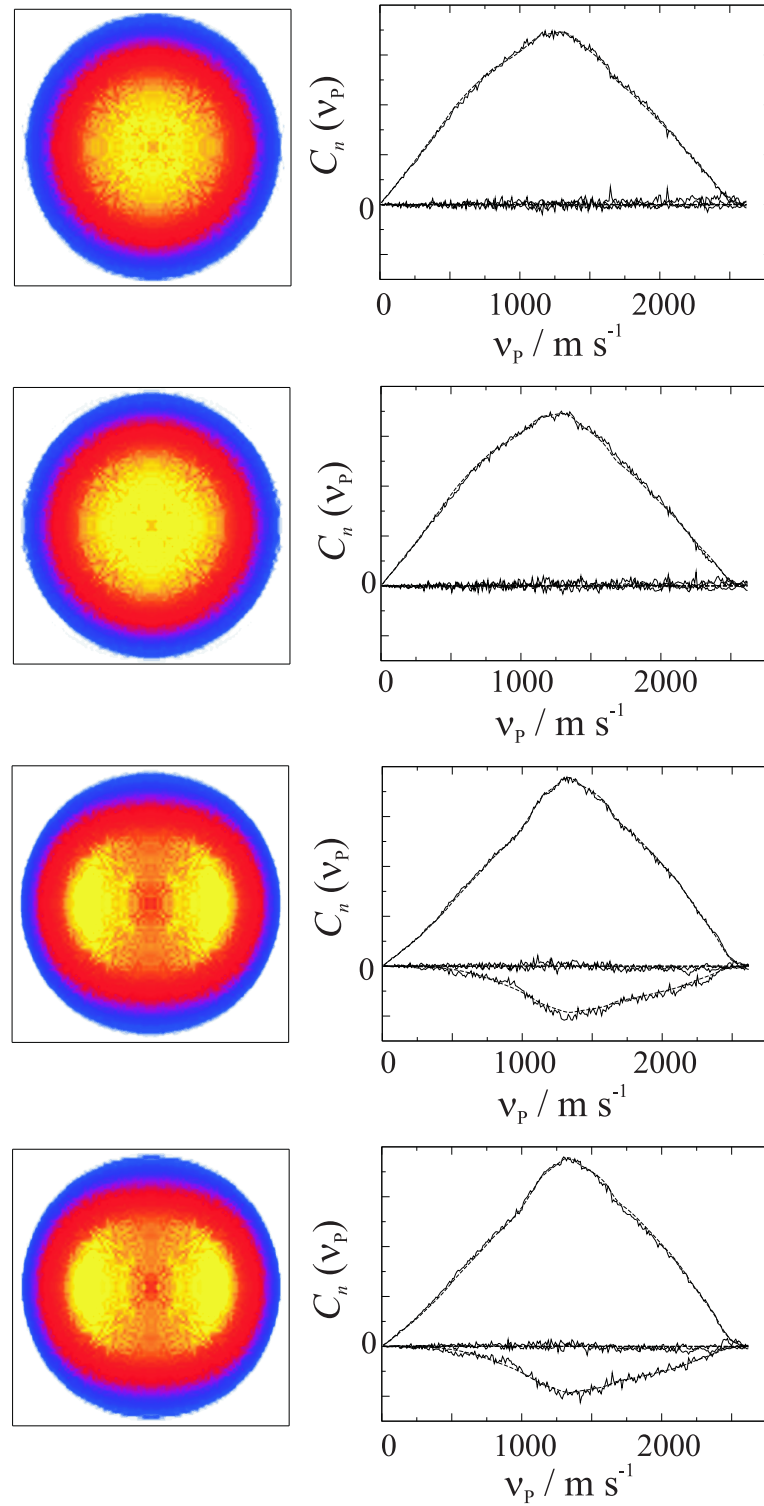


Figure 3.17: Ion images (false colour), fourier moments (—) and fits (- - -) of the $S(3P_0)$. Pump probe geometries from top to bottom: VV, VH, HH, HV.

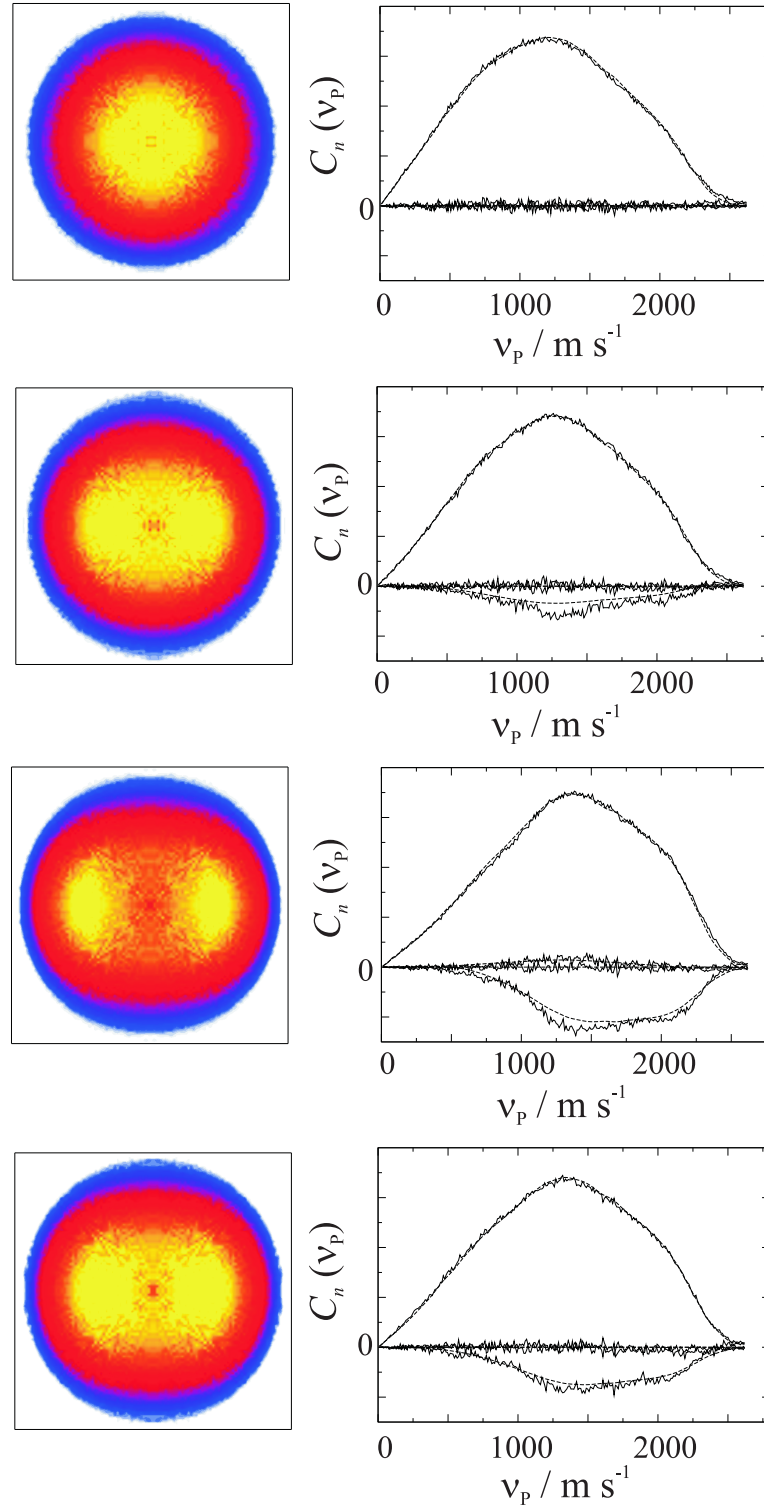


Figure 3.18: Ion images (false colour), fourier moments (—) and fits (---) of the $S(^3P_1)$. Pump probe geometries from top to bottom: VV, VH, HH, HV.

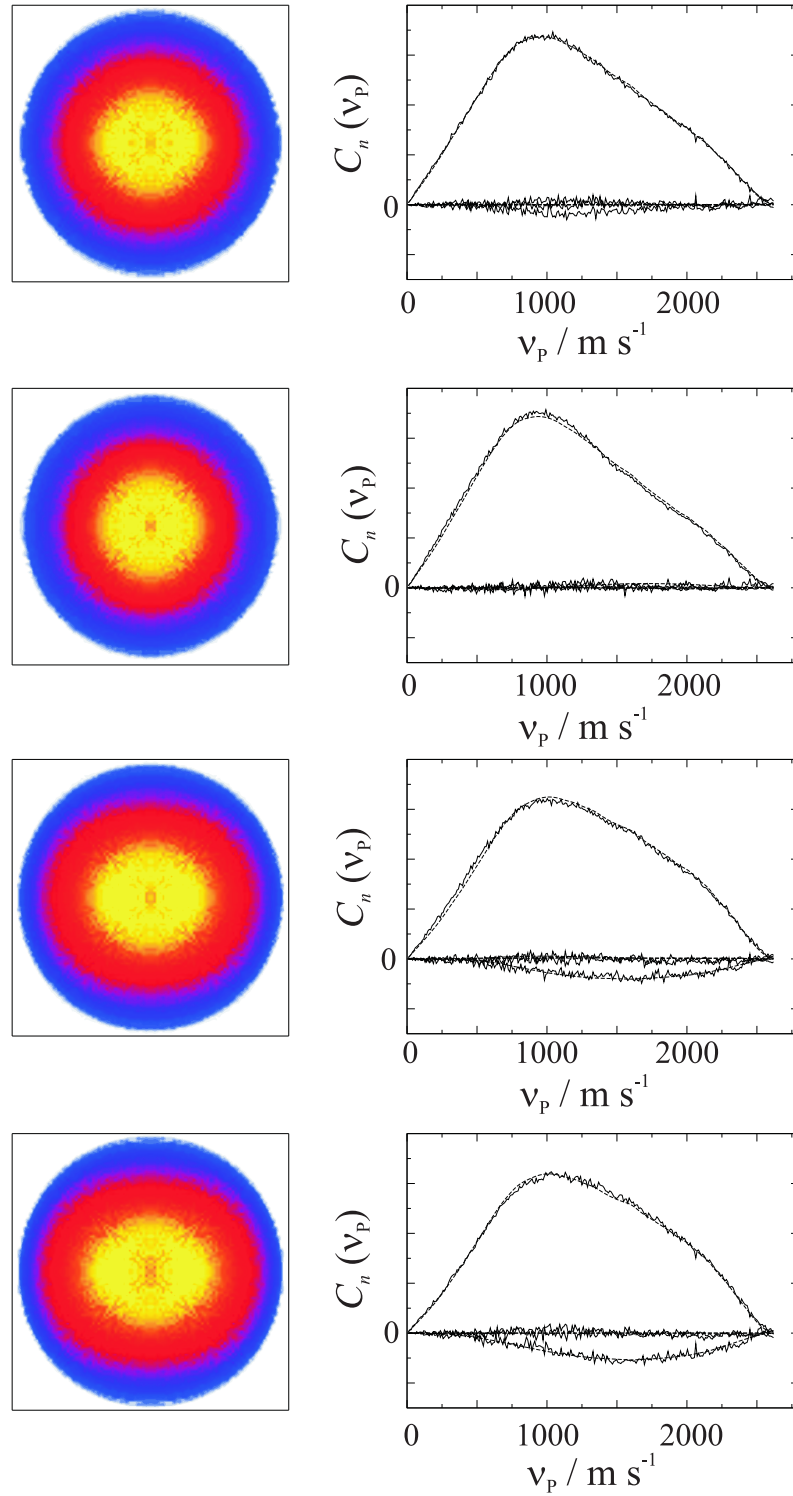


Figure 3.19: Ion images (false colour), fourier moments (—) and fits (---) of the $S(^3P_2)$. Pump probe geometries from top to bottom: VV, VH, HH, HV.

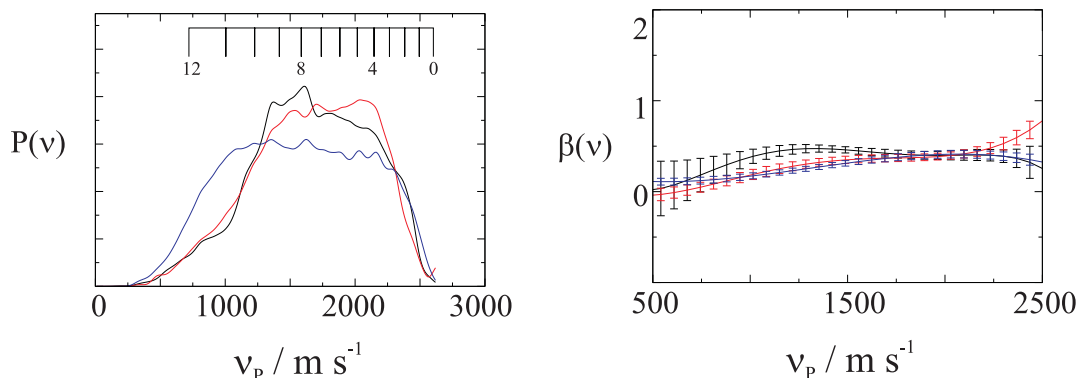


Figure 3.20: Speed distribution (left) and spatial anisotropy (right) of $S(^3P_0)$ (black), $S(^3P_1)$ (red), $S(^3P_2)$ (blue).

was used together with right- (“R”) or left- (“L”) circularly polarised light to give another four geometries for each REMPI transition. For the $S(^3P_2)$ product, the two transitions $S(^3P_1) \leftarrow S(^3P_2)$ and $S(^3P_0) \leftarrow S(^3P_2)$, were used, while $S(^3P_2) \leftarrow S(^3P_1)$ and $S(^3P_2) \leftarrow S(^3P_0)$ were used for $S(^3P_1)$ and $S(^3P_0)$ respectively. Only four geometries were collected for this final state, as $J = 0$ states have no polarisation and so only enough images to assign the speed distribution and anisotropy were required. Selected images and Fourier moments for the $S(^3P_0)$, $S(^3P_1)$, and $S(^3P_2)$ products are shown in Fig. 3.17, Fig. 3.18, and Fig. 3.19 respectively. Together with these are the simultaneous global fits, carried out for each separate spin-orbit level.

The speed distributions and anisotropy parameter (β) for each spin orbit level returned from the fits are shown in Fig. 3.20. From this figure, it can be seen that the speed distributions of $S(^3P_1)$ and $S(^3P_0)$ are similar to each other, whereas that of $S(^3P_2)$ is broader, with a larger fraction of slow products. This broad distribution is in agreement with a study by Kitsopoulos *et al.* [128], as well as one using 202.3 nm photolysis by Jones *et al.* [129]. The difference in speed distribution between spin-orbit levels, also very similar to that observed by Kitsopoulos *et al.* [128], indicates some measure of adiabaticity in the dissociation process, as one would expect identical speed distributions for a completely diabatic process. Further evidence for adiabatic processes lies in the distribution of populations between the spin-orbit states observed in a collected REMPI scan, shown in Fig. 3.21. As the linestrengths

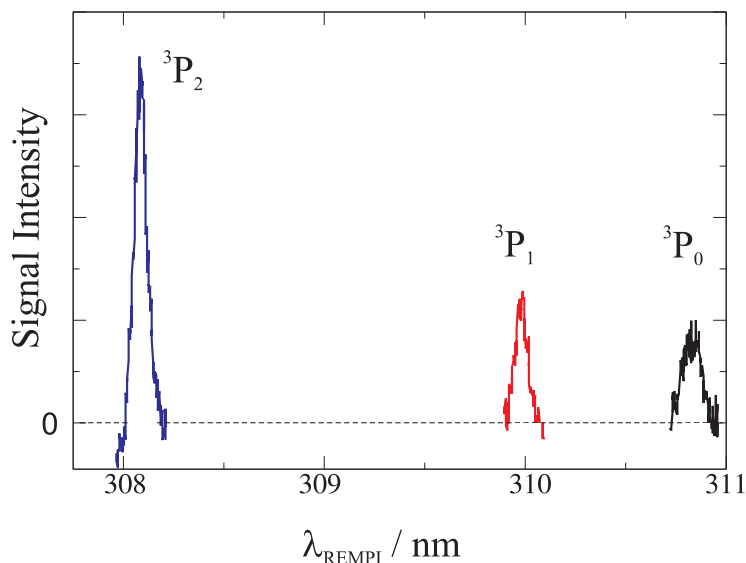


Figure 3.21: REMPI spectrum of $S(^3P_J)$ products. Linestrength-weighted integrals under the peaks give spin-orbit populations of $S(^3P_2):S(^3P_1):S(^3P_0) = 2.7:1:1.1$. The peaks have been expanded by $\times 10$ around their centres to demonstrate differences in area.

for these transitions have been estimated to be approximately equal [88, 130], the integrals under the peaks give spin-orbit populations of $S(^3P_2):S(^3P_1):S(^3P_0)$ of $2.7:1:1.1$. The ratios deviate greatly from the statistically expected distribution of $1.67:1:0.33$. Finally, this difference in population by spin-orbit level is similar to that observed following excitation to the Σ band [131].

Inspection of the spatial anisotropy parameter in Fig. 3.20 and table 3.1 shows β to be quite unstructured, small and positive for all speeds, with similar values for all spin-orbit levels. A slight increase in β can be seen with increasing velocity. The positive value of the anisotropy parameter is as expected from a parallel ($\Delta\Omega = 0$) transition in the axial recoil approximation, with the reduction from the limiting value of 2 perhaps stemming from deviation from linearity at dissociation, rotation of the parent molecule, or non-axial recoil arising, for example, from rotation of the cofragment. The trend of increasing β with recoil speed has been observed by Kitsopoulos *et al.* [128] and Frey and Felder [132], and again reflected in dissociation after excitation into the Σ bands [131]. The values of β obtained here are larger

Parameter	S(3P_2)	S(3P_1)	S(3P_0)
β	0.301(22)	0.378(17)	0.409(14)
s_2	0.007(3)	0.019(4)	—
α_2	-0.001(5)	0.033(7)	—
γ_2	-0.009(6)	0.079(17)	—
η_2	-0.003(4)	0.046(12)	—
s_4	0.000(1)	—	—
α_4	0.000(3)	—	—
γ_4	0.001(2)	—	—
η_4	0.001(2)	—	—
α_1	0.001(2)	-0.005(9)	—
γ_1	0.001(2)	0.005(7)	—
γ'_1	0.017(11)	0.018(26)	—
α_3	0.002(15)	—	—
γ_3	0.000(17)	—	—
γ'_3	0.000(4)	—	—

Table 3.1: Speed averaged polarisation parameters extracted from fitting of experimental data for S(3P_J). Numbers in brackets represent 1σ confidence limit and refer to the final digits of the values.

than those of Kitsopoulos *et al.* (~ 0.2) but smaller than those of a number of other studies, such as Waller and Hepburn (0.85 ± 0.15) [114], Frey and Felder (~ 0.7) [132] and McGivern *et al.* ($0.6\text{-}0.7 \pm 0.1$) [121]. Rotational temperatures of the parent molecule beam have been suggested as the cause of the discrepancies between reported values [121, 132], with Frey and Felder finding a decrease in β of ~ 0.3 with an increase of beam temperature from 10 K to 20 K. Given that the temperature of the molecular beam used here is 25 K, the values for β follow this trend of temperature dependence.

It can be seen from the polarisation parameters in Table 3.1 that the only triplet spin-orbit state with appreciable polarisation is that of $J = 1$. The relative magnitudes and signs of the parameters give some insight into the nature of the dissociative process. Firstly, s_2 describes the contributions to alignment components that vanish after averaging over all recoil angles [101], and is the only parameter not reduced by rotation of the parent molecule. From the fact that its value is the smallest of the $K = 2$ parameters, it can be deduced that little rotation is involved in the genera-

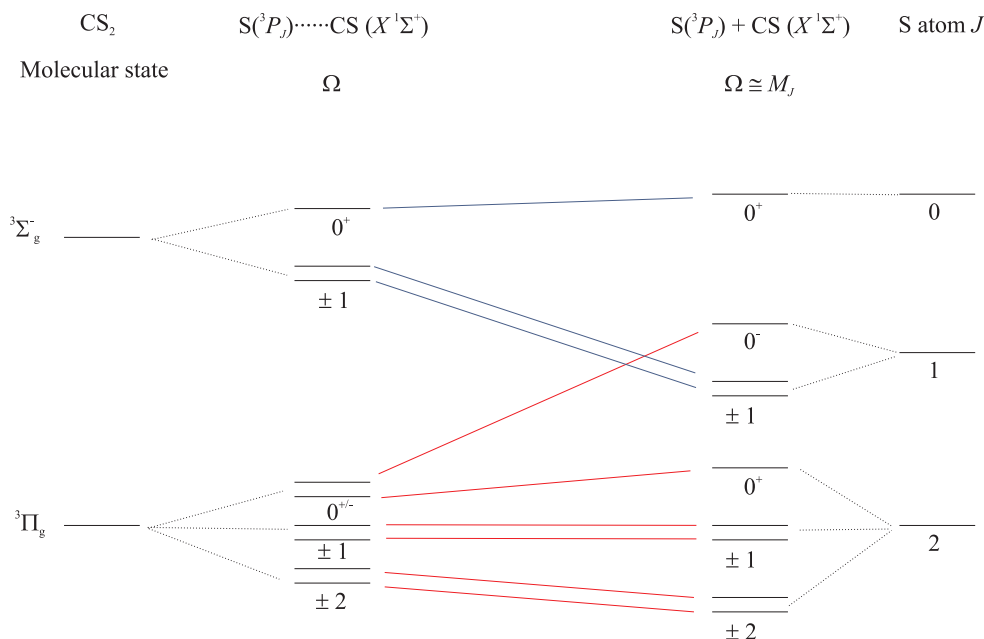


Figure 3.22: Ω -conserving adiabatic correlation diagram at linearity, connecting molecular and atomic states.

tion of this product. Also, in the spin-unpolarised fast dissociation model [133], the ratios of the polarisation parameters between spin-orbit states may be predicted. In the case of the parameters for $\text{S}(^3\text{P}_J)$, the parameters for $\text{S}(^3\text{P}_1)$ and $\text{S}(^3\text{P}_2)$ should be approximately equal in magnitude and opposite in sign. This is clearly not the case, indicating once more the presence of adiabatic effects in the dissociation.

The non-statistical distributions of the spin-orbit populations, differences in speed distribution and polarisation parameters all indicate that some measure of adiabaticity is involved in the generation of the $\text{S}(^3\text{P}_J)$ products from the dissociation of CS_2 . An adiabatic interpretation may perhaps then shed some light on the processes involved. An adiabatic correlation diagram constructed with the conservation of angular momentum along the quantisation axis is shown in Fig. 3.22. In light of this diagram, adiabatic dissociation along the energetically accessible $^3\Pi_g$ with statistical distribution amongst its Ω levels would lead to the unpolarised $\text{S}(^3\text{P}_2)$ products observed experimentally. Population of $\text{S}(^3\text{P}_0)$ and $\text{S}(^3\text{P}_1)$ should arise from distribution on the $^3\Sigma_g^-$ state, and a difference in population between the $^3\Pi_g$

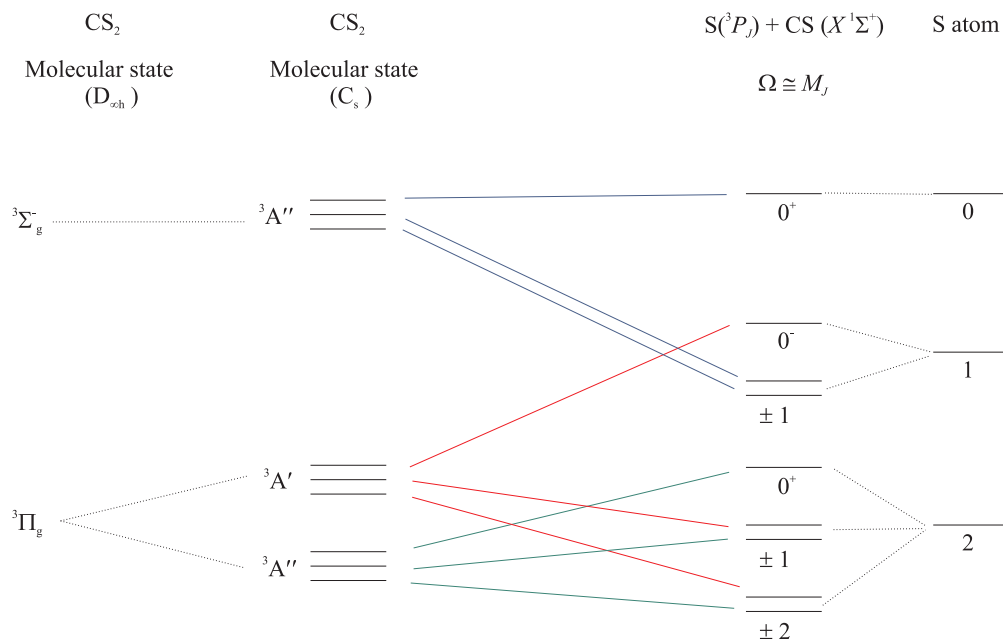


Figure 3.23: Ω -conserving adiabatic correlation diagram for bent molecule, connecting molecular and atomic states.

and $^3\Sigma_g^-$ would then produce the polarisation only seen in S(3P_1). The same conclusions may be obtained using a correlation diagram constructed for the bent state of CS₂ as opposed to at linearity (Fig. 3.23). The mechanism cannot, of course, be completely explained in such a simplistic manner. M_J populations for S(3P_1) products backed out from the polarisation parameters indicate a preference for the ± 1 levels, which would be possible if the population of the $^3\Sigma_g^-$ state were more than half that of $^3\Pi_g$. The REMPI scan intensities, however, give the populations S(3P_2) : S(3P_1) as 3 : 1, indicating a 1 : 6 ratio between populations of $^3\Sigma_g^-$ and $^3\Pi_g$ states in the adiabatic limit. In the same way the process is not completely diabatic, so a totally Ω -conserving adiabatic model cannot be entirely accurate: the true picture clearly would lie somewhere between these two limiting cases. Additionally, the point of crossing onto the $^3\Sigma_g^-$ surface is unclear; in the Franck-Condon region the surface can be taken to be inaccessible, and has not been observed in any *ab initio* calculations for low-energy surfaces. This coupling point may well play a role in the population of the S(3P_1) state. However, the similarities of the polarisation and

anisotropy parameters noted here between the $S(^3P_J)$ products from dissociation at 193 nm, and into the Σ_0^h or Σ_0^f bands, seem to indicate that similar effects are responsible for their resultant polarisation.

3.4 Summary

The experimental apparatus and techniques used within this work have been detailed in this chapter. Techniques for the acquisition and analysis of the data have been described. Typical data recorded on the apparatus have been presented and characterised, and the relevance to the newly-developed fast detector discussed. An example analysis of velocity-mapped imaging data, for which the apparatus was originally constructed, has been performed.

Chapter 4

Desorption and Ionisation on Silicon

Initial experiments carried out on spatial imaging are presented in this chapter. The aim was to determine if spatial imaging could indeed be performed on the apparatus built for velocity-mapped imaging, with simple three-electrode aperture ion optics. The apparatus was thus relatively unmodified, with as much of the original experiment retained as possible. A sample desorption and ionisation method was chosen for simplicity of preparation: desorption/ionisation on silicon (DIOS) plates. The samples themselves were chosen to provide simple and easily identified mass spectra, and the detection system identical to that used in the velocity-mapped imaging experiments. This chapter presents the proof-of-concept experiments carried out using the DIOS plates as a method of producing ions from a solid sample.

4.1 Introduction

Laser-induced desorption and ionisation from a surface was first described in conjunction with mass spectrometry in the 1960s [134]. Vastola *et al.* successfully ionised alkali salts of 1-hexyl sulphonate with low fragmentation using ruby laser irradiation at 694.3 nm. The masses were analysed using a time-of-flight mass spectrometer detecting positive ions. The authors were able to identify the organic masses as either an entire salt or dimer together with an additional alkali ion. This technique was improved upon in the years following this, with extension to polar

organic molecules such as oligosaccharides and glycosides [17], and refinement of the apparatus such as with laser microprobe mass analysers (LAMMA) [135]. However, the technique did not become widespread as there was still significant fragmentation of the analytes, as well as a limited mass range of about 5–10 kDa.

Investigation was made into other techniques of ionising non-volatile samples for mass spectrometry, such as field desorption [136], ^{252}Cf plasma desorption [137], secondary ion mass spectrometry (SIMS) [1], and fast atom bombardment (FAB) [138]. In 1985, Karas *et al.* [139] noted that different amino acids had greatly different irradiance thresholds for desorption at different wavelengths of laser light. The irradiation of a mixture of tryptophan and alanine at the threshold of tryptophan showed a strong signal of the alanine, even though the tryptophan threshold was only about one-tenth of the threshold of alanine. This was attributed to high absorbance of tryptophan at the irradiation wavelength helping to ionise the alanine, and was termed “*Matrix-Assisted*” *Laser Desorption*. Karas and Hillenkamp and co-workers extended the combination of laser desorption with matrix assisted desorption to ionise other non-volatile analytes using matrices of tryptophan, nicotinic acid, sucrose, 3-nitrobenzyl alcohol and 2-nitrophenyl octyl ether [140], as well as to the analysis of proteins of above 10 kDa [141]. The techniques described in these works have become standard for matrix assisted laser desorption/ionisation (MALDI) experiments. Further review of these techniques and their mechanisms are described in Sec. 5.1.

A significant drawback to the techniques used in MALDI has been in the analysis of low molecular weight compounds (< 500 Da). Many pharmacologically active constituents or metabolites are of a similar or lower mass than commonly used matrices, leading to their obfuscation by the large amount of signal from the matrix parent ions or fragments which are present in great excess [142]. Additionally, the quality of results from MALDI MS are highly variable and dependent on sample preparation methods [143]. For these reasons, matrix-free soft ionisation/desorption techniques have been sought, such as material enhanced laser desorption and ioni-

sation (MELDI) or surface-assisted laser desorption and ionisation (SALDI). There have been investigations into direct desorption of various samples from different substrates [144–147]. The high absorptivity of silicon in the ultraviolet was noted by Wei *et al.* and was examined as a potential surface medium for desorption and ionisation [19]. The authors created a highly hydrophobic porous surface with high surface area by galvanostatic etching. The surface was found to exhibit bright photoluminescence on irradiation with ultraviolet light and could be functionalised to serve as an ionising medium for a variety of chemicals. There was little degradation of the surface after use and the analytes could be easily washed off once the analysis was complete. Desorption and ionisation on silicon (DIOS) was proposed as a re-usable matrix-free alternative to MALDI, suitable for a large range of analytes from 150–18,000 Da [148]. The silicon plates were produced commercially in the same shapes as existing stainless steel MALDI plates such that they could be directly replaced in the mass spectrometers. The plates used for the work in this thesis were obtained from Waters [149].

A number of mechanisms have been proposed for the matrix-free desorption and ionisation from a surface. Wei *et al.* [19] initially suggested that the porous silicon could cause the desorption and ionisation by energy transfer from the silicon to the analyte by vibrational pathways, or by rapid heating causing the release of H₂. It was also noted by the authors that the porous silicon easily picked up hydrocarbon and solvent contaminants, which were evident in the spectra at high laser intensities. It was suggested that these may perhaps also play a role in the desorption/ionisation.

A detailed investigation into the mechanism was carried out by Alimpiev *et al.* [150], studying the effects of surface porosity, wavelength, solvent, basicity, laser repetition rate and extraction field. It was found that although a large surface area and roughness was an essential for the desorption and ionisation, porosity was not. However, the porosity would improve the signal, perhaps by re-supplying the surface with analyte as it was depleted by the laser. The laser fluence required to desorb the analyte was found to vary greatly with wavelength, but shared the trend that a

surface temperature of ~ 800 K was needed for desorption. When the sample was applied to a carefully cleaned and dry surface in the vacuum, little or no analyte ions were observed with infra-red radiation, but a much stronger signal was obtained when deposited in the solvated liquid phase and then dried. This, together with the dependence of the signal on liquid-phase (as opposed to gas-phase) basicity, indicated that some charge separation occurred around the sharp edges of the silicon during crystallisation, as observed with other forms of surface-assisted LDI [151]. The dependence of signal on solvent physisorption for UV radiation was not described in the work. It was suggested by the authors that the laser's electric field, enhanced by surface-enhanced Raman spectroscopy (SERS) [152], would be sufficient to fully separate the ions and the analyte would be desorbed from the surface by thermal excitation.

Nagoshi *et al.* [153] investigated the contributions of surface plasmon (SP) excitation, one of the effects of which included SERS. When nano-scale metals are irradiated resonantly, a large number of the excited electrons become localised on the metal surface. These not only have the effect of strengthening the local electric field as in SERS, but also potentially interact with the adsorbed molecules. By maximising the SP interactions using gold nanoparticles, Shibamoto *et al.* [20] were able to greatly improve the sensitivity and efficiency of SALDI, indicating that these effects could play a large role in the ionisation mechanism.

Despite its strengths, DIOS has slowly fallen out of use commercially over the last few years due to three main disadvantages [154]. The first is its limited lifetime: the hydrogen-terminated porous silicon surface slowly oxidises to form a dielectric layer, even with careful storage. Also, the fabrication processes of wet etching produced inconsistent performance between each manufactured surface. Finally, the ease of physisorption of solvents and hydrocarbons lead to easy contamination from a variety of sources such as human handling, ambient air, and even residual gases in the chamber. Many alternative matrix-free SALDI methods have been investigated, which have been extensively reviewed recently by Law and Larkin [154]. However,

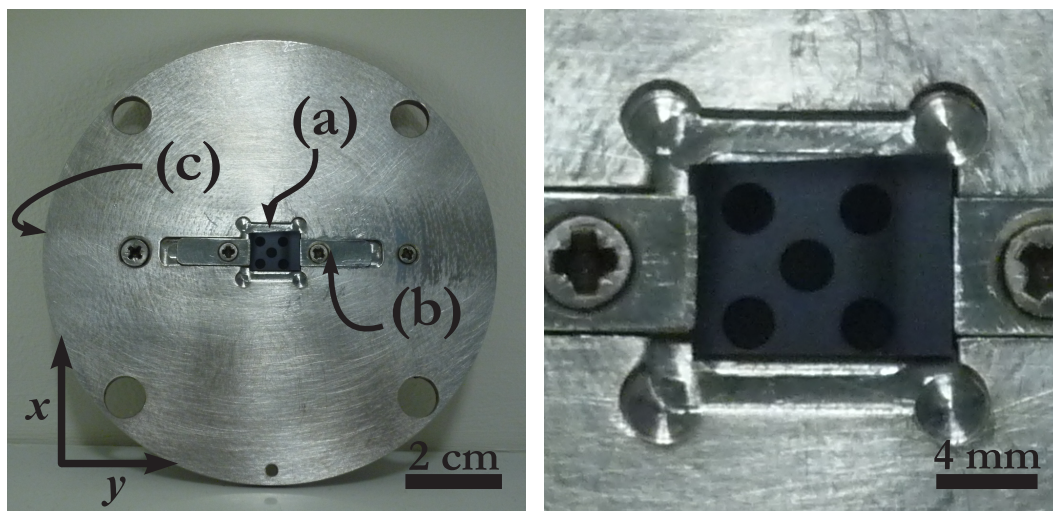


Figure 4.1: Left: Photograph of repeller plate used with DIOS sample chip. The chip (a) is held in place by a spring-loaded jaw (b). The x position can be controlled by a screw (c, behind the plate) adjusting the position of the non-sprung part of the jaw. **Right:** Zoomed in image of chip mount area.

the large majority of these techniques were not commercially available at the time of the experiments in this work, and would have required specific expertise and equipment to prepare. DIOS was chosen as the ionising medium for the initial proof-of-concept experiments as it was commercially available and its simplicity of preparation.

4.2 Sample Preparation

Plates of silicon prepared for DIOS were obtained from Waters. These consisted of plates of smooth silicon wafers which had been treated to produce porous wells. The surface was patterned with groups of five of these wells in a similar manner to standard MALDI sample plates, as they were initially designed as an alternative to MALDI. Each of these groups was of the ideal size for our field of view, $\sim 6 \text{ mm} \times 6 \text{ mm}$. The plate was cut with a diamond scribe into square chips around each group of five wells. The chips were stored in Nalprene containers of isopropyl alcohol in order to minimise contamination in between use.

The repeller plate of the ion optics was adapted as a sample mount. A stainless steel disc was manufactured with an outer diameter of 93 mm, similar to the repeller it replaced. The plate had a thickness of 2 mm, with a recessed well in which the DIOS plate (a) could sit such that the top of the sample plate was flush with the face of the repeller plate. This was done to keep the electric field in the interaction region as similar to those simulated as possible. The chip was held in place by a spring-loaded jaw (b). The position of the chip could be centred in the x direction by a screw which controlled the position of the non-sprung part of the jaw (c). There was sufficient leeway in the holding mechanism in the y direction to allow the manual centering of the wells on the repeller plate.

Two main types of samples were used on the DIOS plates, ionic salts and matrices that were used in MALDI. These were chosen for their ease of handling and simple signal in this stage of the experiments; they could be easily prepared using the dried-droplet method. The ionic salts consisted of RbI, CsI, and NaI. Each of these salts should give a single light positive ion upon ionisation and desorption. This could be easily detected as it is within the mass range that the apparatus was initially developed to detect. 0.1 M solutions of each salt were prepared; 2 μ l drops were applied to each well of the DIOS plate using a micro-pipette and allowed to air-dry. This resulted in approximately 200 pmol of the salt being deposited on the plate. The sample was not optically visible on the plate once it was dried. In addition to the ionic salts used, matrices commonly used with MALDI techniques were examined on the DIOS plates. These included sinapinic acid (SA), α -cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB). These matrices and their uses are described in more detail in section 5.1, and their preparation in section 5.2. The solutions of the matrices were also deposited on the wells with a micropipette in a similar fashion to the salts: 2 μ l of solution was deposited on the wells, leaving approximately 5 pmol of matrix on the surface. This, too, was left to air dry before being introduced to the chamber.

The samples were irradiated using the defocussed dye laser as described in sec-

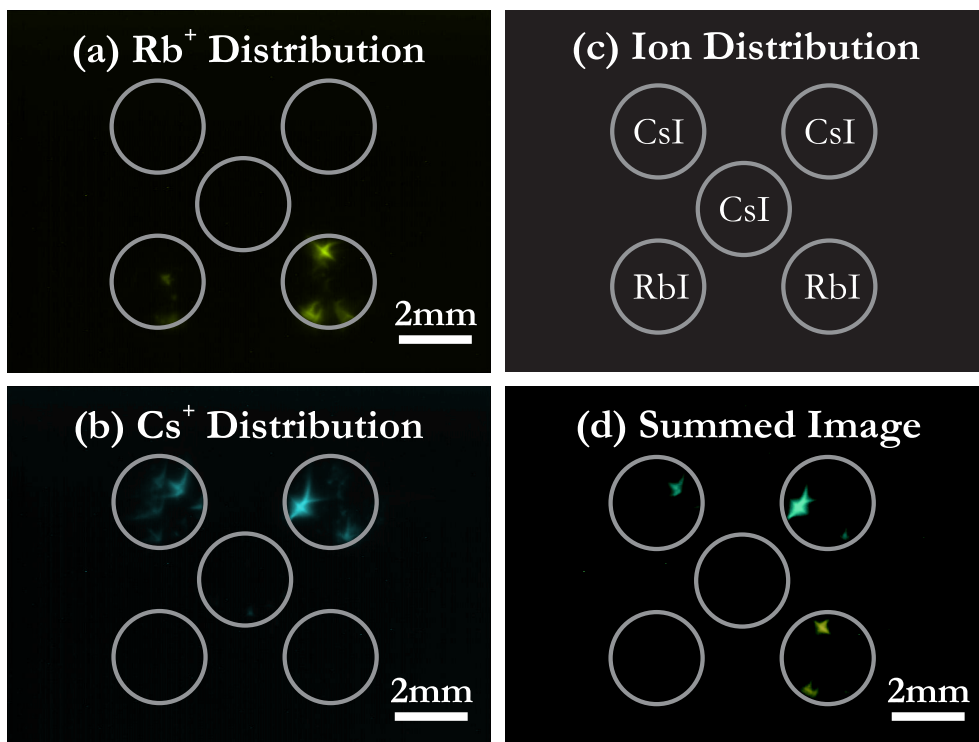


Figure 4.2: Ion images of DIOS plate sample with RbI and CsI salts. The image was obtained with a conventional intensified CCD camera. Images were summed over 1000 laser shots. Clear differences in spatial distribution can be seen at different times of flight corresponding to the mass of **(a)** Rb^+ ions and **(b)** Cs^+ ions. The salts placed into each of the wells is indicated in **(c)**. The grey circles denote the positions of the porous silicon wells. The summed image is shown in **(d)**.

tion 3.1.4. A wavelength of 310 nm, from doubling the 620 nm radiation of Rhodamine 101, was used to perform the desorption and ionisation. The ions were imaged using the three-electrode potentials described in Sec. 2.2.2. The images were acquired using the conventional intensified CCD camera over 1000 shots.

4.3 Results

Results for the ionic salts and MALDI matrices on the DIOS plates are presented here. Fig. 4.2 shows a typical set of results obtained from using the salts on the DIOS plates. It was found that the Na^+ salts did not give a strong signal in the

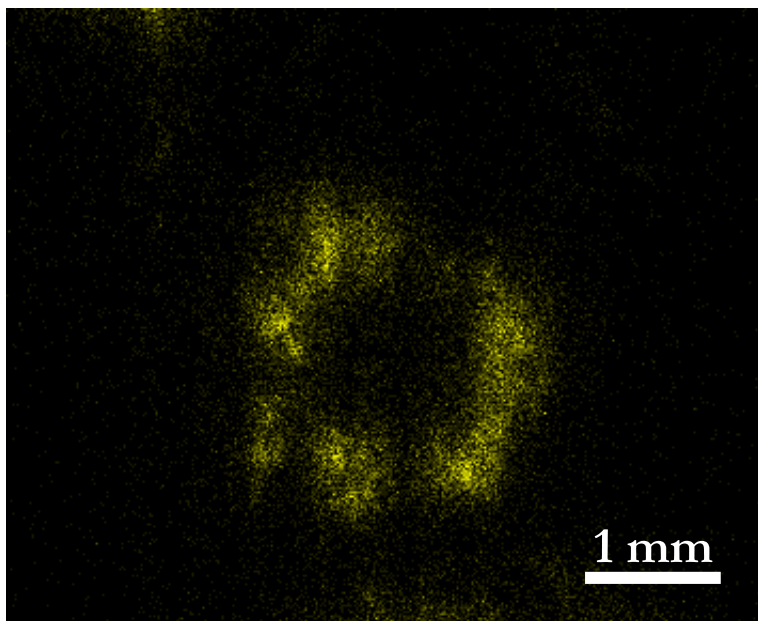


Figure 4.3: Ion image of a single DIOS plate well collected at a mass of 189 Da. The image was acquired over 1000 laser shots. The distribution observed is consistent with that seen for dried droplet application of matrices, such as used by Klerk *et al.* in Ref. [155].

ion images, either due to the low desorption efficiency of the ion from silicon, or difficulties with sample preparation. Hence, a sample which did not involve the use of Na^+ is presented here. The image was acquired over 1000 laser shots. An optical image shows the positions of the wells on the DIOS plate. RbI was spotted onto the bottom two wells, while CsI was put onto the top three wells. The acquired distributions of the Rb^+ ions are shown in (a) and the Cs^+ ions in (b). (c) is the summed distributions of the two images. The grey circles on the image indicate the positions of the wells on the ion images.

Fig. 4.3 shows an ion image of a sample of CHCA at mass 186 Da. The mass peak was determined by comparison of the time-of-flight with a mass spectrum acquired using a commercial mass spectrometer (see Sec. 5.3.1 for more details). The sample was prepared using the dried-droplet technique described in section 5.2, using 10 mg/ml of CHCA in 50:50 water and acetonitrile. The image was also acquired over 1000 laser shots, using ion-counting and centroiding. The image shown is

centred around a single well in the centre of the DIOS plate in order to show the distribution of the matrix within the well.

4.4 Discussion

The differences in spatial distribution for the Cs^+ and Rb^+ ions can be clearly seen in the distributions collected. The differing distributions, together with their order in the time-of-flight, clearly identify the ions on the plate. The summed image in panel (c) shows that the distributions of the ions are clearly located in the wells shown in the optical image (d). The effect of large crystals of RbI and CsI formed during the drying of the droplets can also be seen in the clusters of high intensity within the well. It can be seen from the summed image that the ions are well localised within the areas indicated by the grey circles and that they correspond correctly to their origin positions on the sample chip.

The ion distribution of the MALDI matrix over the porous spot of the silicon can be seen clearly with the ion image in Fig. 4.3. The matrix can be seen to be concentrated around the outside of the well, which is characteristic of samples prepared using the dried-droplet method. For comparison, the image is shown together with images obtained in work by Klerk *et al.* in Fig. 4.4. The distribution of the acquired signal from the DIOS plate is shown in (a), while results by Klerk *et al.* for an array of 3×3 such dried droplets is shown in (b). The distribution in (a) is in good agreement with any one of the spots in the array in (b). This shows that the apparatus is operating under correct spatial mapping conditions.

Finally, it is possible to obtain an approximate measure of the magnification from these preliminary results. Comparison of the centre-to-centre separation between wells on the DIOS plate and the spots on the detector gives a ratio of $\sim 4 \text{ mm} : 56 \text{ mm}$, indicating a magnification of about $\times 14$.

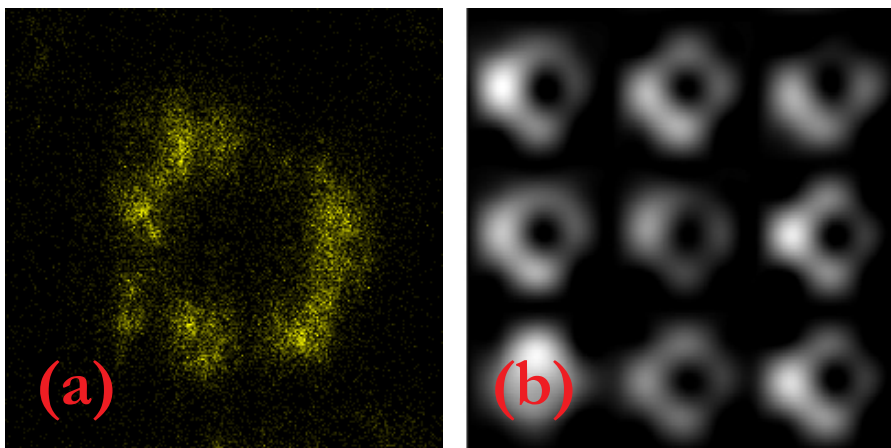


Figure 4.4: Comparison of matrix distribution deposited using dried-droplet technique (a) on DIOS in this work and (b) in work by Klerk *et al.* image adapted from Fig. 7 of Ref. [155]

4.5 Summary

This chapter has presented some initial experiments intended to investigate the possibility of spatial imaging on apparatus designed for velocity-mapped imaging. The results obtained using DIOS plates as a method for producing ions from the solid phase have proved promising: the ion images taken correspond closely with the optical images and expected distributions, as well as showing a mass dependence. These experiments confirm the suitability of the apparatus used for spatially-resolved imaging mass spectrometry. However, the lack of a sharp or repeating pattern on the sample make the determination of spatial resolution difficult. Further experiments were required to determine the spatial and resolutions that may be attained with these apparatus. These will be presented in the following chapters, in which more flexible sample preparations allow for more quantitative investigation into these parameters and their causes.

Chapter 5

MALDI Imaging Mass Spectrometry

5.1 Introduction

In this Chapter, experiments that were carried out using matrix-assisted laser desorption/ionisation (MALDI) techniques as a method of desorption/ionisation are presented. The MALDI matrices were used both as a method of desorption/ionisation for the analytes, and as analytes themselves. The aim of the experiments in this chapter is to determine the different factors such as magnification and resolution, and the effect of experimental characteristics such as extraction voltages. These experimental results allow for comparison with the predictions made by simulations in Chapter 2, and thus aid in determining the origin of the limits to resolution of the apparatus.

An overview of the history and mechanism of MALDI will first be presented in Sec. 5.1.1. This is followed by details on the experimental methods particular to these samples in Sec. 5.2. Results are then presented and discussed in Sec. 5.3, and the conclusions and future directions summarised in Sec. 5.4.

5.1.1 Origins and State of the Art

Matrix-assisted laser desorption and ionisation (MALDI) has become one of the two most widely-used “soft” ionisation techniques in mass spectrometry, suited to

detection of molecules of mass greater than 10 kDa [141]. With high tolerance to salts and buffers, and small sample amount requirements [142], it is the ionisation method of choice for proteins, peptides, and a variety of other biologically significant molecules, making matrix-assisted laser desorption mass spectrometry (MALDI MS) an extremely powerful tool in fields such as proteomics and metabolomics [68, 156, 157].

Following on from the work of Vastola *et al.* on laser ionisation for mass spectrometry [134], much investigation was carried out in attempting to desorb thermally labile molecules without fragmentation [17, 158–160]. These early works found strong mass peaks corresponding to cation attachment such as with Na^+ . Also, generally, thermal decomposition was reduced by short laser pulses of less than 100 ns. Thus, the pulsed nature of the desorption made the ionisation techniques particularly suitable for time-of-flight [161, 162] and Fourier transform ion cyclotron resonance (FTICR) mass spectrometers [163].

Karas and Hillenkamp and coworkers [139, 164, 165] noted that soft desorption from the substrate only occurred within a small window of laser fluence, slightly higher than the desorption threshold of the sample analysed and that greater irradiance led to extensive photofragmentation. Furthermore, attempts at desorption of non-resonant molecules resulted merely in destruction of the sample on the substrate. As in matrix techniques used for fast atom bombardment (FAB) [166] and secondary ion mass spectrometry (SIMS) [167], a solution to these difficulties was believed to lie in compounds which were highly absorbing in the wavelength range of the laser, from within which the analyte could be co-desorbed [168]. Many different organic molecules with absorption in the near-UV to work with N_2 (337 nm) and tripled Nd:YAG (355 nm) were investigated.

The first successful desorption of large (> 100 kDa) intact biomolecules was reported by Tanaka *et al.* using cobalt powder of 300 Å diameter in glycerol [169]. For the most part only a few organic molecules such as nicotinic acid derivatives are commonly used nowadays, selected empirically from the investigated matrices. Of

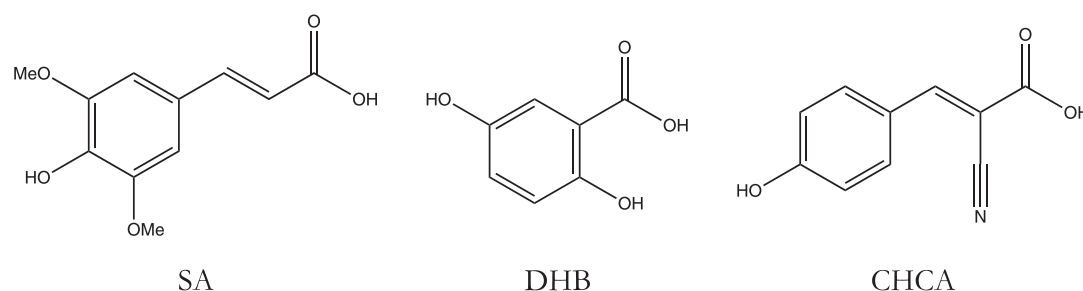
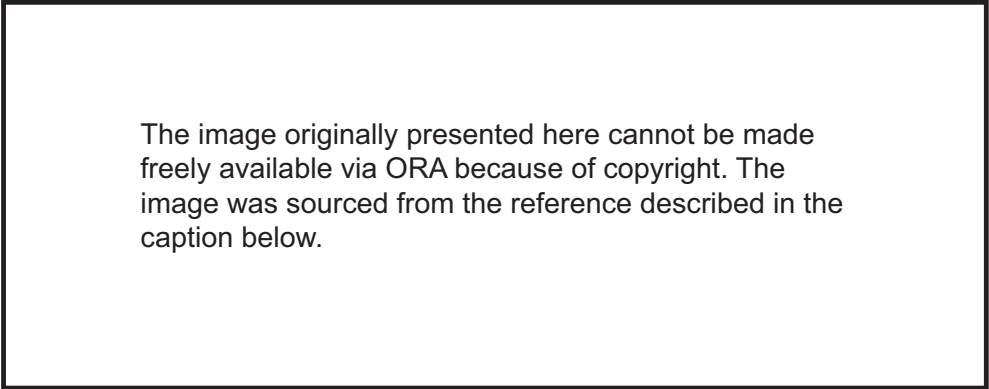


Figure 5.1: Structures of matrices commonly used in MALDI-MS Sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB) and α -cyano-4-hydroxybenzoic acid (CHCA).

particular note are the use of sinapinic acid (SA) derived from cinnamic acid [170], 2,5-dihydroxybenzoic acid (DHB) [171], and α -cyano-hydroxycinnamic acid (CHCA) [172], the most commonly used matrices in the present day. Their structures are shown in Fig. 5.1. More recently, however, molecules have begun to be designed and synthesised specifically for use in MALDI, such as 4-chloro- α -cyanocinnamic acid (Cl-CCA) [173]. This was developed with the goal of enhancing sensitivity, improving the range of peptides which could be desorbed. Additionally, signal-to-noise ratios were improved.

In 1995, Vestal *et al.* [174] greatly improved mass resolution by quantifying the use of delayed extraction of ablated ions, using the principles of “time-lag energy focussing” of Wiley and McLaren [32] for apparatus of various dimensions. Knochenmuss *et al.* noted that the large signal resulting from matrix desorption could be suppressed by carefully controlling the matrix : analyte ratio and using a relatively high concentration of analyte [175]. However, the technique was found to be not applicable to large molecules where the excluded volumes at the high concentrations hindered desorption, and so remains relatively unused. Some novel applications of MALDI include its coupling with thin layer chromatography [176] and gel electrophoresis [177], in a similar manner to the coupling of liquid chromatography and electrospray mass spectrometry [178]. The ability to prepare entire surfaces for analysis have made it particularly applicable to the rapid advances in spatial imaging mass spectrometry in recent years, as described in Sec. 1.1.



The image originally presented here cannot be made freely available via ORA because of copyright. The image was sourced from the reference described in the caption below.

Figure 5.2: Illustration of the principal processes of the lucky survivor model. The analytes, matrix or counterions may have some degree of ionisation in the bulk. Ablation produces clusters which may have some net overall charge, and the charge is transferred within the clusters. Full vaporisation leaves protonated analyte molecules. Adapted from Ref. [179]

5.1.2 Ionisation Mechanism

Many investigations have been undertaken into the mechanism of ionisation and desorption in MALDI, with a variety of different mechanisms proposed. However, the mechanisms generally agree on a two-phase process [179]: a fast irradiation step is followed by a desorption step vaporising the substrate into the gas phase. The main contention lies in the exact processes involved in these two steps. There are two main theories for the ion generation, known as the “Lucky Survivor” and “Gas Phase Protonation” models.

Lucky Survivor Model

The Lucky Survivor model was proposed, and later refined, by Karas and co-workers [180, 181]. An illustration of its basic principles is shown in Fig. 5.2. It is based on the possibility that the analytes that are incorporated into the matrix crystals retain the charges that they may have in solution in a similar fashion to the pre-formed ions recorded in secondary ion mass spectrometry [182–184], laser desorption/ionisation [162, 185] and fast atom bombardment [186–189]. Krüger *et al.* have used pH dye indicators as a method to measure degree of protonation in the solid phase, co-

crystalising the matrix with a variety of indicators [190]. The authors reported that the colours of the dyes remained the same in both solution and crystal phase, indicating that charge states and degree of protonation remained the same in both cases. The matrix/analyte mix thus consists of a degree of charge separation in the form of protonated analyte and matrix molecules, as well the corresponding counterions, distributed throughout the crystals.

Upon irradiation and desorption, clusters of matrix and analyte are ablated. These clusters are not necessarily charge-neutral, with potentially an excess of protonated matrix $[m + H^+]$, protonated analyte $[A + H^+]$ (net positive charge), or deprotonated matrix $[m - H^+]$ and analyte counterion $[R^-]$ (net negative charge). The charge may be redistributed in the cluster before complete vaporisation in the plume; it is accepted that there is sufficient time for thermal equilibration in the expansion plume of the matrix [191]. The desorption process will be described in more detail in section 5.1.3. After complete vaporisation, the remaining charged ions that have not been neutralised by photoelectrons or recombination (the “lucky survivors”) may be detected by the apparatus.

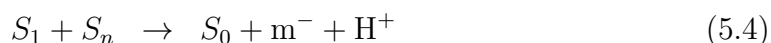
Gas Phase Protonation Model

Also known as the photoexcitation/pooling mechanism, this postulates that although the direct multiphoton processes that would be required to photoionise the matrices are highly unlikely, it is still possible for the matrix molecules to acquire sufficient energy to ionise by pooling the energy absorbed by multiple matrix molecules. The possibility of pooling as a method of energy concentration was initially conceived by Ehring *et al.* [192] and developed by Knochenmuss and coworkers [179].

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Figure 5.3: Processes potentially involved in the photoexcitation/pooling model. In the single center processes, the molecule is excited or relaxes to a different energy level. Two S_1 molecules may then pool their energy to produce a ground state (S_0) and a higher excited state (S_N). The phonons may move through the plume by the transfer of excitation from one molecule to another (hopping). Finally, sufficiently excited molecules may pool their energy to form a ground state molecule together with an ion. Adapted from Ref. [179]

The scheme is shown in Fig. 5.3 and the following series of equations.



Upon irradiation of the solid matrix by near-UV wavelength lasers, some matrix molecules may become excited to a state below dissociation of the acidic H^+ (Eqn. 5.1). Irradiation at these wavelengths is insufficient for single- or double-photon ionisation [193]. This exciton of the S_1 state may then move within the expanding plume by “hopping”, transferring the energy to a neighbouring molecule (Eqn. 5.2). The excitons move around the matrix until they encounter each other, at which point they may form a higher excited molecule S_n , which still has insufficient energy for ionisation, together with a ground-state molecule S_0 (Eqn. 5.3). Finally, when a

molecule in the S_n state encounters another excited molecule, either S_1 or S_n , there will be enough energy to ionise the matrix (Eqn. 5.4). This produces the ionised matrix fragment, m^- , and a proton, H^+ . The proton may then attach to the analyte as the molecules equilibrate in the expanding plume.

Energy transfer between molecules has been known in solid-state aromatics for a long time [194, 195], and self-quenching of the excited state has been demonstrated in DHB by Ehring and Sundqvist in 1995 [196, 197]. It was later shown by Setz and Knochenmuss [198] that the excitons within solid DHB were mobile and could undergo pooling annihilation reactions, and the dynamics required for the mechanism have been extensively studied by Knochenmuss *et al.* [179]. However, the degree of contribution of this effect to the ionisation, as well as the extent of these effects in other matrices, were not fully characterised.

Unified MALDI Analyte Protonation Mechanism

Very recently, Jaskolla *et al.* [173] have gone some way to reconciling the differences in the mechanisms using deuterated analytes and matrices, proposing the “Unified MALDI Analyte Protonation Mechanism”. It was found that *both* the Lucky Survivor model and the Gas Phase Protonation models could contribute to some extent, which was dependent on the experimental parameters. The authors synthesised matrices that only became sufficiently acidic after irradiation-induced rearrangements. By deuterating all acidic sites on the matrix, it was possible to determine the extent of the ionisation that occurred in the gas-phase expansion and how much stemmed from pre-charged analytes.

The proportion of analytes formed by the gas phase protonation model increased with the basicity of the analytes: neutral analytes underwent exclusive deuteration by the gas phase model as they were unprotonated in the solution (and hence crystalline) phase. Greater protonation was already in place in the more basic analytes and so products of the lucky survivor model were observed. Similarly, the lower the proton affinity of the matrix, the more effective the gas-phase deuteration: the

deuterium ions are more easily desorbed. Finally, raising the laser fluence promoted the gas-phase deuteration process over the lucky survivor model, the cause of which may be the high-order dependence on excitons of the mechanism. A reduced laser fluence hence suppressed the process and protonation occurred almost exclusively.

5.1.3 Desorption Process

There is much more agreement on the process of desorption into the gas phase in MALDI, with much study having been conducted on the different possible mechanisms. The experimental work and theoretical modelling has been extensively reviewed by Dreisewerd [191] and Zhigilei *et al.* [199] respectively.

As mentioned in Sec. 5.1.1, MALDI matrices are generally irradiated with near-UV wavelengths, such as from an N₂ laser (337 nm) or tripled Nd:YAG (355 nm). The matrices are chosen such that they absorb strongly at this wavelength, while the majority of analyte compounds will be non-absorbing at this wavelength. This prevents the direct excitation of the analyte, reducing effects such as photofragmentation as are seen when using lasers of shorter wavelengths [191]. The best results have been obtained when the absorption maximum of the matrix and the wavelength of the irradiation were similar [200, 201]. In addition to the near-UV radiation used, which is absorbed by the matrices *via* electronic excitation, IR-MALDI has been an alternative method, with the infra-red radiation being absorbed into vibrational modes of the matrices such as the C—O stretches or C—H bends at around 10 μ m using CO₂ lasers [202, 203]. However, the mechanism for their processes lies outside the scope of this thesis, as all desorption in this work has been achieved with UV radiation.

Widely noted in the desorption processes of MALDI is the threshold effect, in which little or no signal is observed up to a certain laser fluence, after which there is a sudden increase in signal [204, 205]. This is generally considered to correspond to the ablation threshold [205, 206]. Ingendoh *et al.* and Dreisewerd *et al.* [204, 207] found the irradiated area to have a strong influence on the threshold fluence, with a

larger area requiring a smaller fluence to obtain signal. The reasons for this remain unclear, but Zhigilei *et al.* [208] speculate that they may lie in relative amounts of molecular desorption to clusters, with clusters generated more easily at small spot sizes, as well as changes in the plume characteristics.

The requirement for a short laser pulse gives a little insight into the mechanism of the desorption. Generally, laser pulses of between 0.5 ns and 10 ns are used, with the same power over longer pulses leading to thermal excitation of the analyte and disintegration of the sample. Vertes *et al.* [209] describe a “bottleneck” for energy transfer: after the matrix molecules have absorbed the energy from irradiation, the large difference in frequency of the vibrational phonons of the matrix and the modes of the analyte means a slow thermal equilibration of the two in the solid phase. If the power is supplied in a sufficiently short time, the matrix and analytes will desorb before equilibration and the analytes will remain intact.

In the cases in which the laser pulses are much shorter than the mechanical relaxation times (“stress confinement”), a high pressure caused by the rapid energy deposition creates a void under the surface which quickly spreads and ejects a large amount of the solid [199]. This large amount of solid equilibrates in the plume and largely vaporises, but still produces clusters. The effects of the desorption mechanism have also been shown to successfully model the dominance of positive ions in the desorbed analyte/matrix [210].

As discussed in section 2.2.1, the velocity distributions of the MALDI and analyte plume are important considerations in designing spatial-mapping ion optics. A range of very different axial velocities have been reported, from 200 ms^{-1} [211] up to in excess of 1000 ms^{-1} [212]. A study by Glückmann *et al.* [211] investigated the dependence of the desorption velocities on a variety of factors such as preparation, choice of matrix, and analytes or additives used.

The authors described a range for the recoil velocities from 273 ms^{-1} to 543 ms^{-1} for a variety of different matrices, with 291 ms^{-1} for α -CHCA. However, within each matrix, the peptides and proteins used showed similar initial velocities. Also,

once above the irradiation threshold, the laser power did not have a large effect on the initial ion velocities. The smaller carbohydrates used in the study showed a significantly lower initial velocity ($\sim 200 \text{ ms}^{-1}$). However, it is important to note that these were detected from different regions of the sample, with different matrix crystal sizes. There was no clear correlation between the laser wavelength and matrix used, mainly due to the different matrices required for the different wavelengths employed.

Finally, the authors noted that factors inhibiting direct comparison between studies by different groups is the lack of standard spot size and well-defined focussing conditions [207]. However, the majority of the data agrees that the ions (both matrix and analyte) produced from the MALDI method are in the range of a several hundred to one thousand meters per second [191].

As mentioned before in Sec. 2.2.3, the desorption from the surface is believed to take the form of a plume of mainly neutral matrix molecules in which the analyte is entrained [82]. This shape arises as a result of the vaporisation from a finite area, acting as a source for the jet [191]. The plume expansion was observed by Zhang and Chait [83] to have a bias normal to the surface according to

$$h(\theta) = (n + 1) \cos^n \theta, \quad (5.5)$$

where $(n + 1)$ is a normalisation factor. The values of n have been observed all the way up to 28. The values of n vary greatly depending on the analytes and matrices, but the authors speculated that the trend appeared to be that the higher the mass of the ion, the higher the value of n .

5.2 Sample Preparation

For the experiments in this thesis, MALDI matrices were used both as samples and as an ionisation method. These included sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB) and α -cyano-4-hydroxycinnamic acid (CHCA). The structures of these are

shown in Fig. 5.1 of Sec. 5.1.1. Each of these matrices were prepared with different solvents and concentrations. The matrices were obtained from Aldrich and used directly as supplied. Solvents were obtained from Fisher Scientific and Rathburn Chemicals.

SA was prepared in a concentration of 10 mg/ml. The solvent was a 4:6 mixture of acetonitrile (ACN): 0.1% aqueous trifluoroacetic acid (TFA).

DHB was prepared at two different concentrations: 10 mg/ml in 70:30 water:ACN for use with the DIOS plates (Sec. 4.2), and a saturated solution in 20:80 water:ACN for use on the stainless steel plates.

CHCA was prepared with a variety of concentrations depending on the application methods. A concentration of 10 mg/ml was used in 1:1 ACN:0.1% TFA was prepared for use with the DIOS plates and direct application to the polished plate. 15 mg/ml in 20:80 0.1% TFA: methanol for application with the air-brush. For the electrospray application, 1:1 methanol/water was used as a solvent. A concentration of 8.9-10 mg/ml CHCA was made up in this.

Crystal violet was also used as an analyte in conjunction with the matrix, as it produced a distinctive mass distribution around 372 Da, as well as being easily ionised and desorbed by CHCA.

The ion optics were also adapted in order for them to act as a sample plate for the matrices. There were two types of sample mounts used in these experiments, plates for direct application and plates for mounting indium tin oxide (ITO) coated glass slides. For direct application, stainless steel discs of similar dimensions to the velocity-mapping repeller plates, 98 mm diameter and 2 mm thickness, were cut. The surface was polished to provide a smooth surface upon which to place the sample. The polished surface also prevented the movement along the scratches on the initial brushed metal surface by capillary action of the sample when applied in solution. Two concentric circular grooves of 1 mm depth, 2 mm width were cut into the surface at the center of the plate. They had 20 mm and 24 mm inner

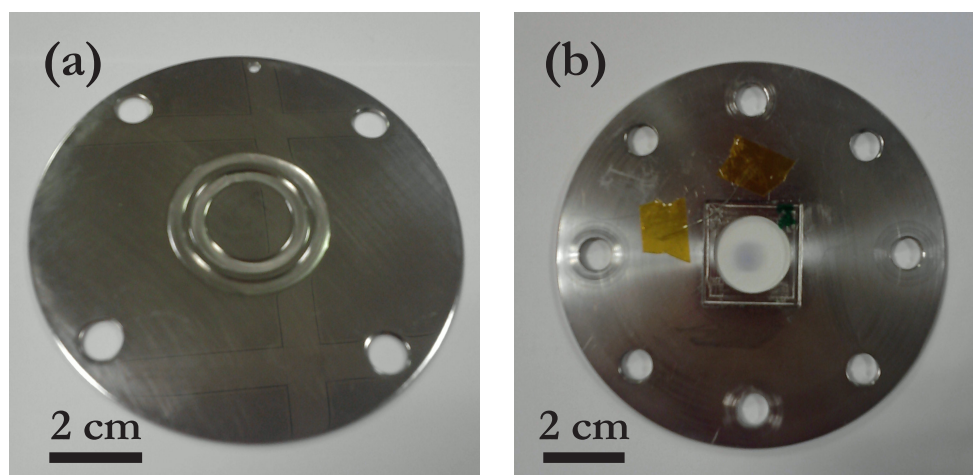


Figure 5.4: Photographs of stainless steel repeller plates used for MALDI samples. (a) is used for samples to be placed directly on the repeller. (b) Repeller with recession for ITO-coated glass slide, with mounted slide. The face of the slide and the repeller are kept in electrical contact with wire attached with high-vacuum tape.

diameters. These were used when it was desirable to affix an electroformed grid over the sample region. For use with ITO-coated glass slides, plates of the same outer diameter were manufactured, but with a recession the dimensions of the glass slide. After the sample has been applied to the ITO-coated slide, the slide was glued into the recession, and the ITO-coated surface kept in electrical contact with the repeller by a thin wire. The wire was attached to the plate by high-vacuum tape. Photographs of the mounts are shown in Fig. 5.4.

A number of methods were used in order to apply the matrix to the sample plate. The matrix could be directly applied to the plate with a capillary tube and spread over the surface with a glass rod and air-dried. Where additional spatial information was desired, an electroformed nickel mesh was used in conjunction with the matrix. A variety of meshes were used; their pitches, transmission and dimensions are listed in Table 5.1. Each grid was made up of square holes. When used with direct application on the mount in Fig. 5.4(a), this could be placed over the applied sample to act as a mask. The grid could also be dipped in the matrix solution and attached to a clean plate. When attaching the grid to the plate, the grid was held down with an o-ring

Supplier	Grid	Pitch/ μm	Hole Size/ μm	Transmission
Goodfellows	NI008720/9	500	430×430	75%
Goodfellows	NI008711/11	381	340×340	80%
Industrial Netting	BM0100-02-N	254	234.2×234.2	85%

Table 5.1: Dimensions of electroformed nickel mesh used in this work.

in the outer groove of the plate, and bonded with cyanoacrylate glue on the ridge between the grooves. The inner groove served to prevent the running of the glue, the solvent of which would disturb and contaminate the sample. The grids were also used to create a spatial structure to the applied matrix. An airbrush (Iwata CM-C Plus) was used to apply the matrix through the grid, which had been placed over the plate. The plate was placed on a heating plate to improve the drying speed. Once the matrix was applied, the grid could be removed, leaving a negative grid pattern.

As the variability of matrix crystals were observed to have a negative impact on the images obtained (see Sec. 5.3), it was desirable to produce a fine and even layer of matrix on the sample. To achieve a more homogeneous layer of matrix, a home-built electrosprayer was used (Fig. 5.5). This used a syringe pump (KDS Legato 100) together with a syringe/tubing/needle assembly (Hamilton). The needle was floated to 3 kV while the sample was kept at ground, resulting in a fine spray of matrix and solvent. A flow rate of $12 \mu\text{l/hr}$ was used, with a total volume of $4 \mu\text{l}$. This was compatible with the grid stencilling technique, as well as useful for applying the matrix to biological samples (Chapter 7). The ITO-coated glass slides and the mount in Fig. 5.4(b) were used in conjunction with this method of matrix deposition.

There are a number of advantages of using an electrospray deposition method. It was applied to the deposition of matrix in 1997 by Hensel *et al.* and Axelsson *et al.* [213, 214]. The authors of both works reported improved reproducibility of the results and lower shot-to-shot and spot-to-spot variation of the signal. The crystals of matrix produced are extremely small ($< 1 \mu\text{m}$) and evenly dispersed. This means

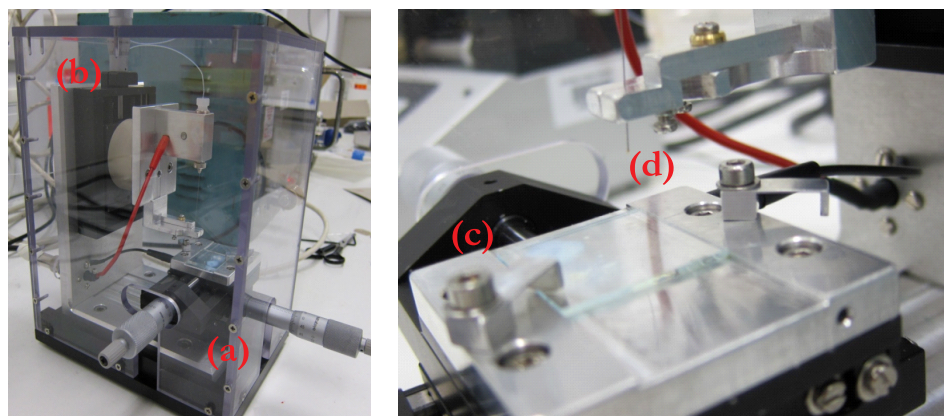


Figure 5.5: **Left:** Picture of custom-built electrospraying apparatus for application of matrix. The x/y translational stage **(a)** provides fine adjustment of location of the spot, while the distance of the needle from the sample may be controlled by the z adjuster **(b)**. **Right:** Close-up of the spraying region. The electrical contacts **(c)** keep the top of the sample plate at ground, while the spray is produced from the needle **(d)**.

that the matrix layer is very fine and homogeneous, thus having minimal effect on the sample topology [215]. Also, the size of the solution droplets is extremely small, meaning that the analyte transport by the solvent is minimised. The matrix deposition process may also be independent of the operator and automated: once the electrosprayer is set up, and the conditions optimised, the results from the application are much more reproducible than with manual application. However, a disadvantage is that, due to the small size of the matrix solution droplets, only small amounts of analyte are incorporated into the crystals and detection sensitivity has been reported to be low [216].

5.3 Results and Discussion

A number of variables have been investigated in the experiments with the MALDI samples in this chapter. These include the effects of matrix application techniques and the imaging of different spatial distributions. On a more technical side, the effects of applying centroiding to the image quality as well as the applied extraction voltages are tested. The ion images presented here have been collected using

defocussed tripled Nd:YAG irradiation (355 nm), with typical power of 20 mJ. The images were acquired over 1000 laser shots with the conventional intensified CCD camera described in Sec. 3.1.6.

Initial experiments were conducted in which the matrix ions were placed directly onto the stainless steel mount and imaged. Attempts to acquire images with sinapinic acid and di-hydroxybenzoic acid did not produce sufficient amounts of signal for clear images with the wavelengths and fluences used, with only CHCA producing acceptable signal. DHB was found to form large but sparse crystals inconsistently across the sample, while sinapinic acid did not reliably produce significant degrees of signal. Additionally, SA is particularly suitable to the ionisation of proteins lying in the high kilo-Dalton range as opposed to the smaller peptides produced by CHCA [217]. As the MCP detectors have a decreasing sensitivity at higher masses [218, 219], CHCA was chosen as the matrix to use in this work.

5.3.1 Time-of-Flight and Mass Spectra

In order to determine the masses seen in the time-of-flight spectrum, samples that were used in the imaging experiments were also analysed in a commercial electrospray ionisation mass spectrometer (Waters). Commercial MALDI-TOF MS instruments available were not suitable for this purpose: low-massed ions of less than ~ 300 Da were automatically blanked out. These mass spectra could then be compared with the time-of-flight spectrum acquired from the phosphor screen with the photomultiplier tube in order to determine the masses of the peaks observed. A mass spectrum of CHCA is shown in Fig. 5.6. The mass spectrum was acquired in negative ion mode, as signal was not observed in positive ion mode for electrospray ionisation. One may speculate that the proton affinity of the cinnamic acid was too high to produce significant levels of positive ions in electrospray ionisation. The dominant peak can be seen to be that corresponding to $[m - H]^-$, the parent ion. In the ion imaging experiments of this work, the ions observed are positive. It is therefore expected that any of the parent ion detected would be the un-deprotonated

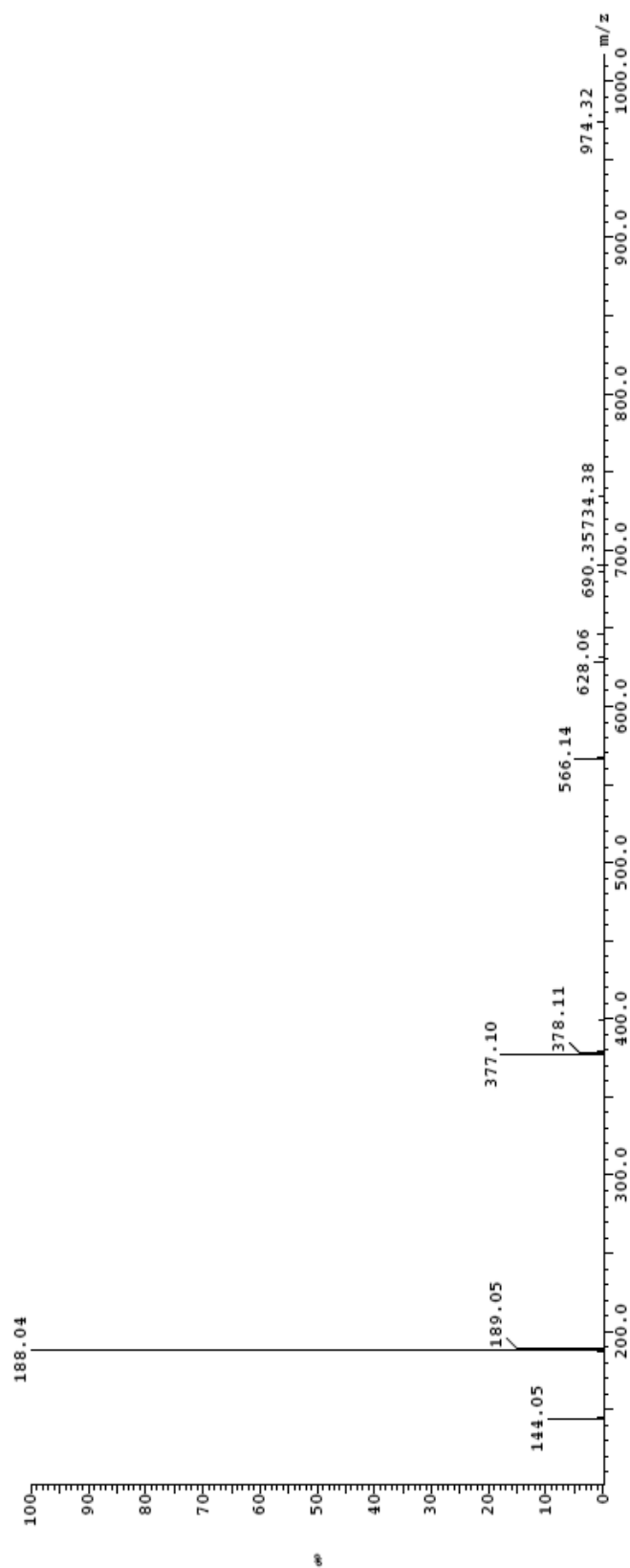


Figure 5.6: Mass spectrum of CHCA acquired on a commercial ESI mass spectrometer in negative ion mode. The strongest peak at 188.04 Da corresponds to $[M - H]^-$, with a smaller fragment peak at 144.05 Da. The heavier, lower intensity peaks are attributed to matrix clusters.

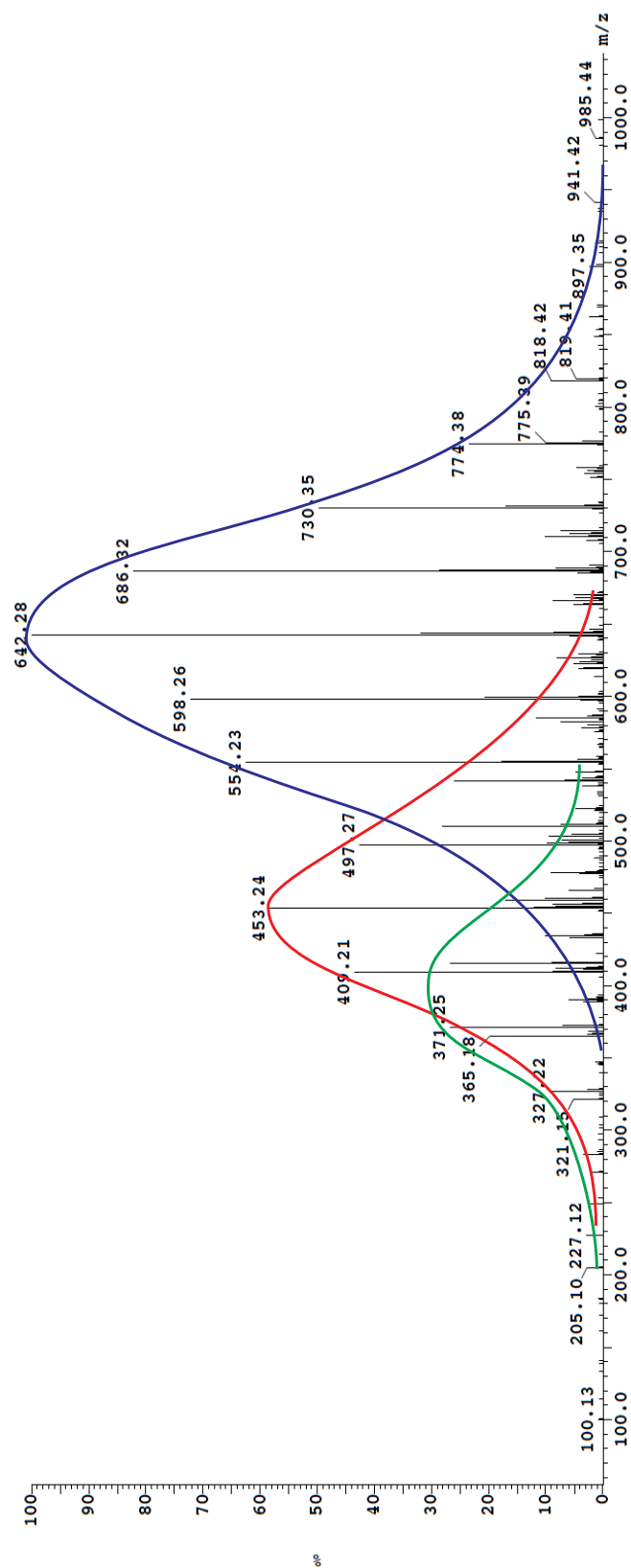


Figure 5.7: Mass spectrum of polyethylene glycol (PEG) acquired on a commercial ESI mass spectrometer on positive ion mode. The PEG can be seen to have well-defined spacing between the peaks of 44.03 Da. The red, green and blue curves highlight different combs of such separation. See text for full details.

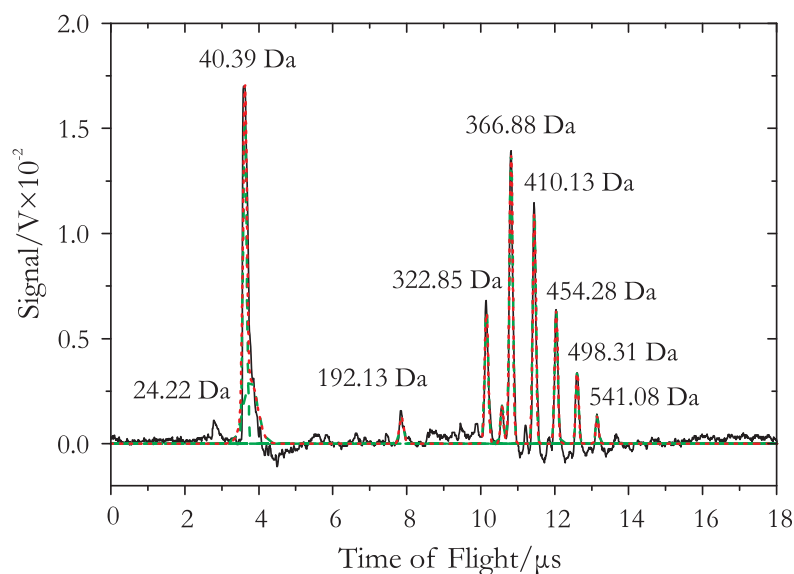


Figure 5.8: Mass spectra of CHCA matrix and PEG obtained using the photomultiplier tube in conjunction with the time-of-flight apparatus. The features of the CHCA peaks together with the comb structure of the PEG can be clearly seen. The masses have been assigned by comparison with the mass spectra acquired in Figs. 5.6 and 5.7. The green dashed line shows the fitted peaks, while the red dotted line shows the sum of the fits.

acid, as observed by Greenwood *et al.* [220]. This parent ion would correspond to m^+ and a mass of 189.17 Da. Unfortunately, with the mass resolution of the apparatus, it would currently not be possible to distinguish between the protonated or deprotonated ions by their masses.

As a measure of obtainable mass resolution with the current apparatus, polyethylene glycol (PEG), a standard calibrant used in mass spectrometry, was used in conjunction with the matrix. Upon ionisation, PEG dissociates to form masses with a well-defined separation of 44.03 Da [221]. Fig. 5.7 shows a mass spectrum acquired with the commercial electrospray ionisation mass spectrometer. The comb structure of peaks separated by 44.03 Da can be clearly observed. Closer inspection reveals multiple series of even separation, which are slightly staggered. These are highlighted within the green, red, and blue curves. A possible origin of this is differing ends of the chain of the polymer fragments, or differing fragmentation positions.

Fig. 5.8 shows the mass spectrum of a sample acquired using the photomultiplier

tube on the spatial imaging apparatus. PEG was mixed into a solution of CHCA prepared as described in Sec. 5.2. The solution was directly applied to a stainless steel plate. An accelerating potential of 10 kV was applied. The main features of the mass spectrum acquired with the commercial mass spectrometer can be identified within the PMT spectra. A distinctive comb corresponding to the regularly spaced peaks of PEG fragments may be seen from 10 μ s–14 μ s. Using the known mass separation of 44.03 Da, together with the mass/flight time dependence (Eqn. 1.4), these have been assigned approximate masses between 322.85 Da–541.08 Da. It appears that a smaller range of peaks in the comb of the PEG fragments can be seen in the TOF spectrum. A possible cause is a difference in types of fragmentation in the different ionisation processes. As the different combs identified in Fig. 5.7 correspond to different fragments, it is feasible that certain fragmentation processes which produce them are suppressed in MALDI, and only fragments corresponding the present processes are observed in the TOF spectrum.

The peaks corresponding to the PEG fragments in the TOF spectrum was fitted with a sum of Gaussian functions, with a function for each mass peak. The individual functions are shown by the green dashed line in Fig. 5.8, and their sum by the red dotted line. A full width half maximum of ~ 100 ns was determined, leading to a mass resolution of $m/\Delta m \equiv t/2\Delta t \approx 60$ at this mass. Comparison of this value with the $m/\Delta m$ of ~ 90 obtained in simulations presented in Sec. 2.2.5 suggests that there are additional factors limiting the time resolution of the apparatus. The probable cause of this is the decay time of the P47 phosphor screen, which has a 120 ns decay time from 100 % to 1 % intensity. Inspection of the time-of-flight spectrum reveals the effect of the exponential decay on the time of flight spectrum: the peaks are asymmetrical, with a sharper rise time and longer decay, producing a longer tail and peak broadening.

Approximate values for the early peaks at around 2.8 μ s and 3.6 μ s have also been calculated using the mass/TOF dependence determined from the PEG calibration as around 24.22 Da and 40.39 Da. These assignments are more tentative than those

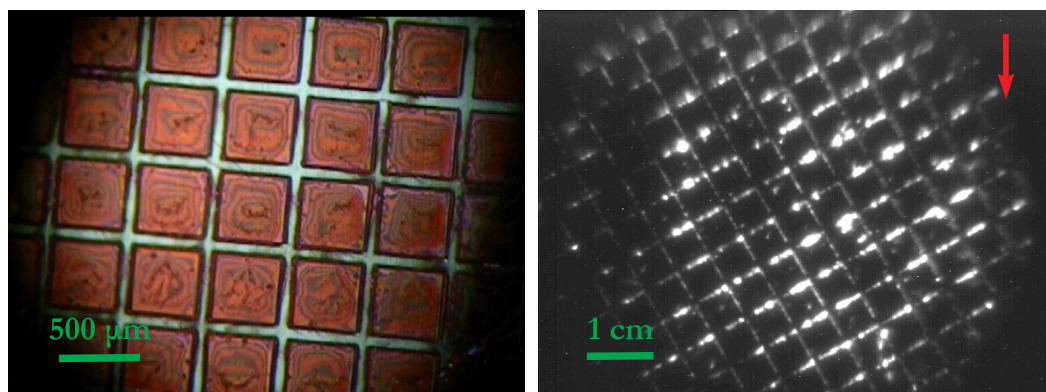


Figure 5.9: (a) Optical image of a sample prepared with a 500 μm pitch grid. Red dye has been added to the CHCA to make the sample visible. The sample has been directly applied through the grid to form the squares seen. (b) Ion image of the same sample. The ions were taken at mass 188 Da, corresponding to $[m - H]^+$. The bar in (b) indicates the dimensions on the detector. A repeating pattern is clear but is of lines as opposed to squares. The larger matrix crystals may be seen as brighter spots on the image. The red arrow indicates the direction of the incident laser.

for the series of peaks just calculated: the mass range for which the calibration is accurate is that which falls within the masses from the PEG fragments. However, it can be seen the fragments are much smaller than would correspond to CHCA parent ions. These may correspond to fragments of the matrix, or ionic salt contamination (Na^+ , Mg^+ , K^+ , or Ca^+) of the mount or sample during preparation. The signal at 7.83 μs corresponds to a mass of ~ 192 Da, and may be identified as the parent ion. The difference in abundance of the parent ion peak between ESI and MALDI is perhaps unsurprising, despite them both being soft ionisation techniques. While ESI ionises all the sample producing intact ions, in MALDI, the matrix absorbs the vast majority of the laser energy. Fragmentation of the matrix may serve to “protect” the analyte, allowing its soft ionisation.

5.3.2 Pitch and Magnification

Figs. 5.9 and 5.10 show optical and ion images of CHCA. The matrix solution has been applied through grids of two different sizes in order to produce the square

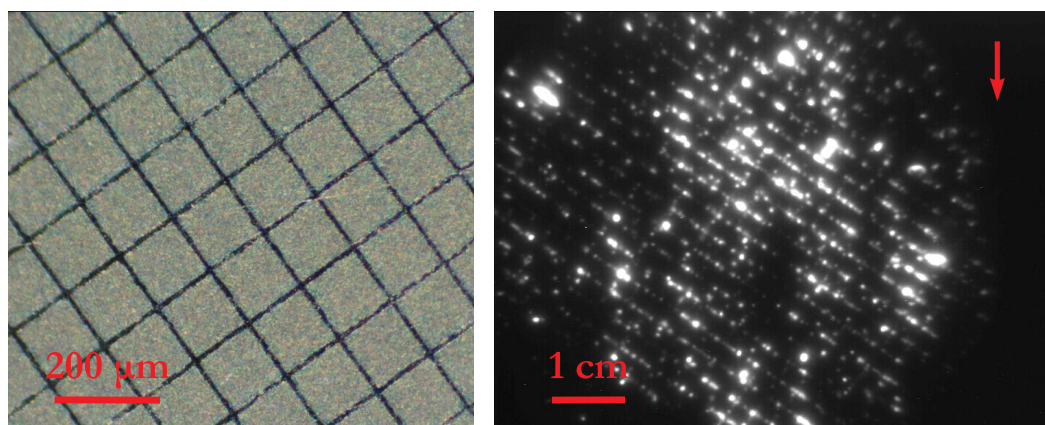


Figure 5.10: (a) Optical image of a sample prepared with a $254\text{ }\mu\text{m}$ pitch grid. CHCA was applied with the airbrush through the grid to form squares of matrix. (b) Ion image of the same sample, taken at 188 Da , corresponding to $[m - H]^+$ for CHCA. The bar in (b) indicates the dimensions on the detector. A smaller repeating line pattern is again clear, as well as the larger matrix crystals. The magnification of the ion image is the same as in Fig. 5.9. The red arrow indicates the direction of the incident laser.

pattern seen in the optical images. The grids used to produce the patterns in Figs. 5.9 and 5.10 were of $500\text{ }\mu\text{m}$ and $254\text{ }\mu\text{m}$ respectively. The images were acquired with 10 kV accelerating potentials.

The regular repeating pattern is clearly distinguishable in the ion images. The edges of the circular detector can be seen in the ion images, where the pattern ends on the ion image. The difference in pitch is clear as the magnification of the system remains constant. By comparing the point-to-point distance on the optical and ion images, it can be determined that the field of view of the apparatus under these operating conditions is approximately 6.5 mm on the sample. As the detector has a diameter of 70 mm , this gives a magnification of $70\text{ mm}/6.5\text{ mm} = \times 10.8$ on the detector. This is in good agreement with the value of $\times 9.7$ predicted by the simulations in Sec. 2.2.4. The effect of uneven crystallisation may be seen on the image where larger matrix crystals have formed. These produce a larger amount of signal and so show up as bright spots on the image. Additionally, the effect of the uneven laser profile may also be observed, with larger regions of varying brightness.

It can be seen that although the sample is present on the surface in squares, a

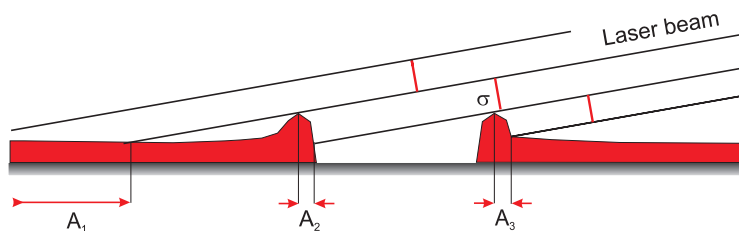
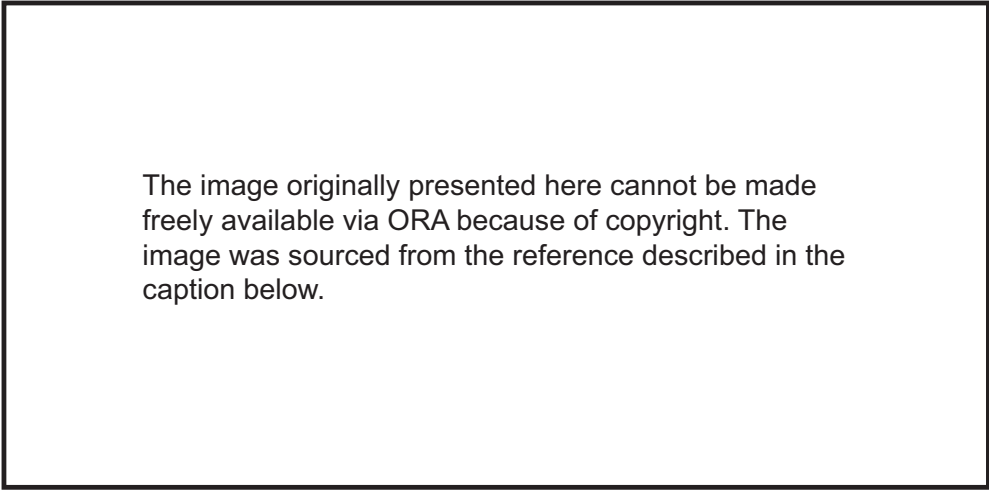


Figure 5.11: Diagram illustrating the reason the edges of sample are seen more clearly in ion images. Due to the incidence angle and the protrusion of the sample from the surface, the same power (σ) is spread across a smaller area at the edges (A_2 , A_3), leading to a greater laser flux at the edges than at the flat areas (A_1). This leads to greater desorption and hence signal in these areas.

grid pattern of lines is observed in the ion images. A potential cause of this may be the shallow angle of incidence of the laser. This results in a greater laser fluence at the edge of the squares where the matrix is raised from the surface. An illustration of this effect is shown in Fig. 5.11. The sample laid down on the surface will have a finite thickness and be raised from the surface. At the edges of the sample, there will be an edge which presents a face of sample more normal to the incident radiation. Thus, the area at the edge (A_2) receives a greater concentration of energy, producing larger amounts of signal than in the flat regions (A_1). The edges may also have a raised section (A_3) due to the meniscus during application, or by larger crystal formation of the edges (as observed for the dried droplet preparation: Sec. 4.4). This would also present a more perpendicular region for irradiation, which is the cause of the double lines observed later in Fig. 5.15.

Another possible cause of observing lines as opposed to squares is an effect similar to that observed by McCombie and Knochenmuss [222] upon studying the effects of sample thickness. They observed a strong dependence of signal on sample thickness, with thin layers of matrix and analyte on the metal substrate providing much greater signal than thick layers. The authors attributed this to the proximity of the analyte to a conducting surface allowing the electronic states of the matrix molecules to interact with the conduction band. In the cases in which the conduction band is higher in energy than the LUMO of the isolated matrix, the LUMO becomes occupied and ionisation from this level is reduced to second order with respect to



The image originally presented here cannot be made freely available via ORA because of copyright. The image was sourced from the reference described in the caption below.

Figure 5.12: Profile of intensity against position across the spots, from Ref. [179]. The numbers indicate the amount of time for which the matrix was applied: longer times therefore correspond to thicker layers. Thin layers of matrix result in higher amounts of signal. Where the spot is thick, the edges at which there may be lower thickness or the matrix is closer to the substrate, there is higher signal. The characteristics of the line profile are strikingly similar to that in Fig. 5.16.

photons. A profile of signal intensity against position adapted from Ref. [222] in Ref. [179] is shown in Fig. 5.12. The improved signal effect was also attained by Altelaar *et al.* [223] from the sputter-coating of a sample with gold. In relation to the results observed here, the edges of the sample may be closer to the metal surface, and thus ionise more efficiently. This would then produce the intensities observed in these experiments.

In order to explore the causes of the square/line discrepancy, the electrospray applicator was used to deposit thin layers of matrix. In order to reduce the effect of insufficient laser fluence, crystal violet, which readily ionised and produced signal, was used together with the matrix as an analyte. However, the signal from the crystal violet was short-lived, lasting in the region of 10,000 laser shots. The electrospray applicator was set to flow rate of 5 μl /hour, for 10 minutes. The optical (b) and ion (b) images for crystal violet are shown in Fig. 5.13. The images collected at masses corresponding to crystal violet and CHCA showed similar patterns. It can be seen that the electrospray deposition produces a much more homogeneous layer

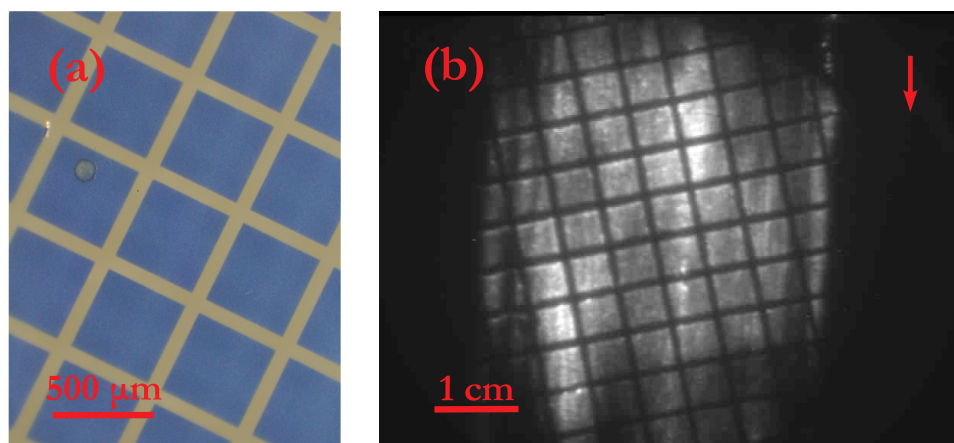


Figure 5.13: (a) Optical image of thin layer of CHCA with crystal violet. The sample was deposited by electrospray through the 500 μm grid. (b) Ion image of the same sample collected at mass corresponding to crystal violet fragments. The bar in (b) indicates the dimensions on the detector. The red arrow indicates the direction of the incident laser. The pattern of squares is clear. Note the additional intensity at the top edge of each square, corresponding to the impinging radiation. The uneven laser profile can again be identified.

with finer crystals.

The ion image can now be clearly seen to be squares, indicating that a thin sample did indeed result in more even desorption. These results show that the imaging of lines as opposed to squares by the apparatus is an artefact of the laser irradiation and sample preparation, and not the technique of imaging itself. However, a bright edge at the top of each square may still be observed in the ion image, showing greater signal at the edge facing the incoming radiation. This suggests that the effects of incident angle are still present. In order to remove this artefact, an improved incident angle is required. Currently, the incidence angle is limited by the geometric limitations of the apparatus: there is a maximum angle through the window in the chamber and between the repeller and extractor plate that the laser path may take. Possible solutions to this may be back illumination, such as that used for LAMMA [135], or by the introduction of holes in the ion optics for an improved laser incidence angle. Simulations involving the latter approach for the apparatus have so far determined no detrimental effects on the spatial imaging conditions [86].

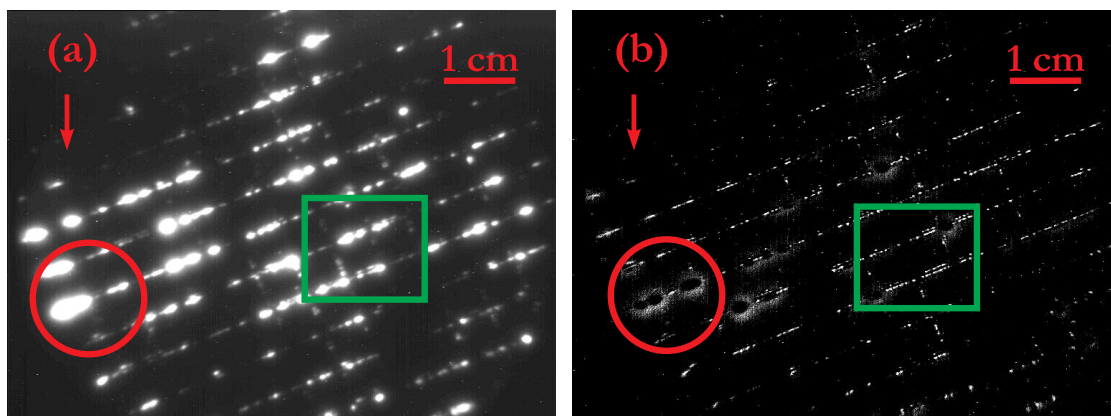


Figure 5.14: Effect of centroiding and ion counting. (a) shows an ion image acquired of the $500\mu\text{m}$ -pitch matrix squares acquired without ion-counting, while (b) shows the same grid acquired using ion counting. The centroided image is discernibly sharper, with signal spread over an area in (a) located in a point in (b). Areas of saturated signal become dark in ion-counted images, such as one indicated by the red circle. The red arrows indicate the direction of the incident laser.

Again, the intensity profile of the laser is clear from the intensity of signal over the sample.

5.3.3 Event Counting

A method of improving the spatial resolution in pixel imaging is that of centroiding, discussed in Sec. 3.2.3. The method described by Chang *et al.* [97] was used for on-the-fly centroiding and event-counting with the conventional CCD camera in order to determine its effect on the spatial ion images. For the centroided images, a larger number of shots were required to acquire a converged image, and so the images were acquired over 5000 laser shots.

Fig. 5.14 shows the effects of ion counting on the ion image acquisition. Fig. 5.14(a) shows the image obtained without centroiding, while Fig. 5.14(b) shows the image obtained using centroiding and ion counting. The sharpness of the image in (b) is clearly better than that of (a). Signal that shows up as large spots and blurring the image in (a) has been reduced to a pixel in the centre of the spot in (b), so that it no longer obscures other lower intensity signal in the image. Event counting records a

pixel for each event (ion arrival), without paying heed to its intensity. The intensity of the spot from the arrival of each individual ion may vary with a number of factors, such as the ion arriving slightly early or late at the edges of the detection window. As these factors are not related to the concentration of the analyte on the surface, the event-counted image is perhaps a more accurate indication of concentration on the sample, as higher intensity areas in the recorded ion image are then points in which a large number of ions had arrived, not intensity.

A striking effect of the improvement is marked by the green box in Fig. 5.14. In (a), the repeating pattern appears to be a grid made up of lines. There is a suggestion of structure, but this is unclear due to the blurriness of the image. However, with the centroided image (b), it is clear that the signal is actually made up of parallel lines. The green box encompasses two sets of these double lines.

However, a disadvantage of using centroiding in the situation is also evident in Fig. 5.14(b). In areas with a high amount of signal above the threshold, there is some degree of saturation in the detector. This results in areas of pixels with the same intensity, that of the highest value in the camera's dynamic range. The centroiding algorithm then identifies this plateau of signal as one event, as there is no way to distinguish multiple ions striking the saturated area. Effectively, this masks any other signal in the area, leaving areas lacking signal visible in the image. An example of such areas is indicated by the red circle in Fig. 5.14. However, in a sense, there is no loss of information in using centroiding, as the areas in which this occurs are already saturated, and no further information could have been gleaned from the uncentroided ion images.

A method of reducing this saturation is the lowering of the produced signal, which is controlled by the incident laser power. However, there are two main challenges to this. The first is the "threshold" requirement for desorption of MALDI, as described in Sec. 5.1.3. Thus, a minimum laser fluence is necessary for signal to be observed. In addition to this is the uneven laser profile that has been observed in previous ion images. Reducing the laser power to prevent saturation from the high-signal

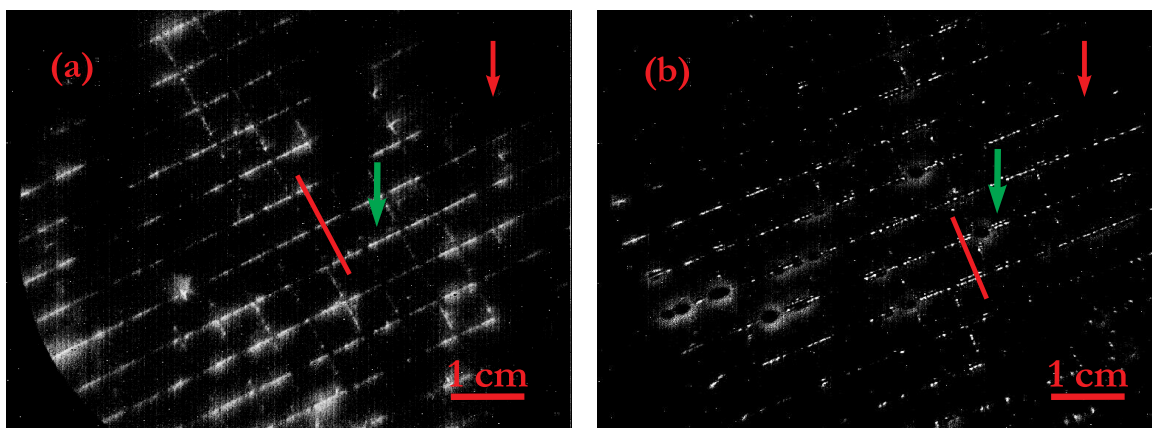


Figure 5.15: Comparison of images taken with repeller voltages at (a) 2 kV and (b) 10 kV. The images were acquired with on-the-fly centroiding. In the regions indicated with the green arrows, a double line structure can be seen in the 10 kV image that is not visible in the 2 kV images, demonstrating the improved resolution at higher extraction voltages. The red lines on each image represent the lines along which the intensity profiles were taken for determination of spatial resolution. The red arrows indicate the direction of the incident laser.

areas leads loss of signal in the lower intensity areas. A potential solution would be to produce a smoother beam profile by passing the beam through a beam diffuser. Experiments involving the use of such a diffuser are currently under way in the group [85].

5.3.4 Accelerating Potential and Spatial Resolution

In the field simulations of trajectories in Sec. 2.2.4, it was predicted that there would be an improvement in spatial resolution as the accelerating potentials increased. This effect has been investigated for accelerating potentials of 2 kV and 10 kV, the limit attainable with the apparatus. Fig. 5.15 shows two ion images collected at different accelerating potentials. (a) shows an ion image at 2 kV, while (b) shows an image acquired at 10 kV. A sharpening of the image is observed in the higher extraction potential. Also, the double line structure that has been observed in Fig. 5.14 is visible in the 10 kV image in Fig. 5.15(b), but not in the 2 kV image in Fig. 5.15(a).

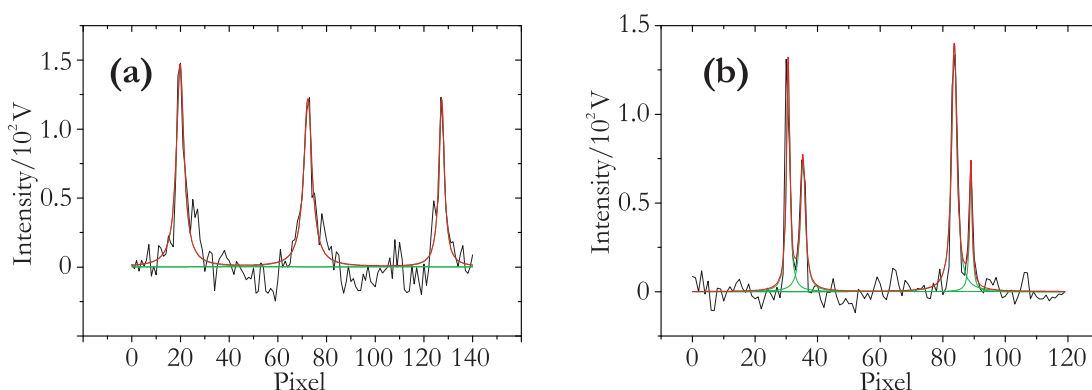


Figure 5.16: Intensity profiles for the determination of spatial resolution. **(a)** shows the intensity along the red line in Fig. 5.15(a), while **(b)** shows the intensity along the line in Fig. 5.15(b). The peaks have been fitted with Lorentzian functions. The individual fitted peaks are shown in green, while the red line shows the sum of the fits. The fitted peaks in **(a)** have a FWHM of $32.7\ \mu\text{m}$, while those of **(b)** have a FWHM of $14.4\ \mu\text{m}$.

In order to obtain a more quantitative measure of spatial resolution, intensities were acquired along the red lines indicated in Fig. 5.15(a) and (b). The intensity profiles for the images acquired at 2 kV and 10 kV are shown in Fig. 5.16(a) and (b), respectively. The peaks have been fit with the sum of Lorentzian functions. To determine the lower limit to the resolution, the signal is assumed to originate from a point source on the sample and hence any broadening of the lines to originate from the apparatus. The green lines show the individual peaks, while the red line shows the sum of the fits. The full width half maximum of the peaks for the line profile obtained at 2 kV, and hence the spatial resolution, was found to be $32.7\ \mu\text{m}$. The corresponding FWHM at 10 kV was $14.4\ \mu\text{m}$.

The increase in spatial resolution is in agreement with the predictions of the simulations in Sec. 2.2.4. However, the experimentally observed $14.4\ \mu\text{m}$ is smaller than the predicted value of $26\ \mu\text{m}$. This indicates that the aberrations in the simulation were greater than those of the experiment, likely stemming from the use of $n = 2$ in the $\cos^n$ term of Eqn. 2.29. It is expected that the experimental distributions have more directionality, with a higher value of n in $\cos^n \theta$. Assuming that the speeds of the ions (the $g(v)$ term in Eqn. 2.28) are in line with those described in the litera-

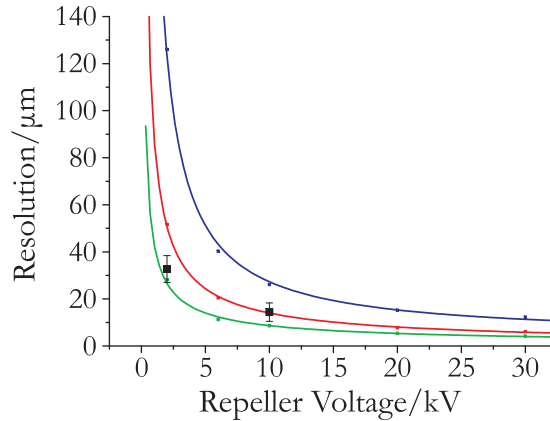


Figure 5.17: Comparison of experimental and theoretical dependence of spatial resolution on accelerating voltages. The colours of the lines represent different simulated values of n in the $\cos^n$ recoil velocity distribution of the ions. $n = 2, 12$, and 28 are represented by the blue, red and green curves respectively. The experimental results are shown in black squares with errors. From the acquired resolutions, a value of $12 < n < 28$ can be obtained.

ture, comparison of the experimentally observed resolutions with those predicted by theory may reveal information about the directionality of the ion plume (the $h(\theta)$ term of Eqn. 2.28) upon desorption from the sample.

In addition to the simulations for $n = 2$, further simulations using $n = 12$ and $n = 28$ were performed, and the resolution curves plotted in Fig. 5.17. The computational and time cost of the simulations limited the number of values that were calculated. The blue, red and green curves represent simulated spatial resolutions for $n = 2$, 12 , and 28 respectively. The experimentally observed resolutions using 2 kV and 10 kV accelerating potentials are plotted with black squares. It can be seen that the experimental values fall between the red and green curves, suggesting that the desorption plume has an angular distribution of $\cos^n \theta$, with $12 < n < 28$.

5.4 Summary and Future Directions

This chapter has served to characterise the capabilities of the experimental apparatus. The mass resolution for the current apparatus is $m/\Delta m = 60$, and appears to be limited by the decay time of the phosphor screen. A solution to this would be

the elimination of the phosphor screen, and use detectors capable of direct electron detection in-vacuum directly behind the MCPs. Timepix [95, 96] is capable of such direct electron detection, as will future versions of PimMS.

The apparatus has been shown to reliably produce spatially-resolved ion images. The magnification of the apparatus has been determined to be $\times 10.8$, in good agreement with simulations. This may be improved by the extension of the flight tube. However, the desirability of this is uncertain: a larger magnification leads to a smaller field-of-view for a given detector size. The relatively large size of the field-of-view available with this apparatus in comparison to the majority of microscope-mode imaging mass spectrometers is a distinct advantage in the imaging of biological samples, maximising on the advantages of fast-detection systems for fast analysis and throughput. This is discussed further in Chapter 7.

Event counting and raising of the extraction voltages have both been demonstrated to improve the spatial resolution, with a maximum resolution of $14.4\ \mu\text{m}$ attainable. Potential improvements to the spatial resolution could be achieved in a number of ways. The two most effective of simple modifications that can be made are the introduction of a contrast aperture and the further increase of extraction potential. Attempts to implement contrast apertures, however, have been limited technically with the challenge of accurately placing the aperture on the time-of-flight axis, as any misalignment causes the blocking of the incorrect trajectories and instead worsens the resolution. Improved mounts for the ion optics are being designed and constructed to ensure the accurate positioning of the aperture. The effect of increasing the accelerating potentials in the ion optics have already been described in Fig. 5.17, with the expected spatial resolution decreasing with the increasing potential. Improvements to the electrical insulation and feedthroughs allowing potentials up to and beyond 20 kV are expected to allow sub- $10\ \mu\text{m}$ resolution.

Chapter 6

Imaging Using Fast Detection Systems

6.1 Introduction

Fast imaging detectors have a wide variety of uses and great potential to broaden the range of imaging applications. The main advantage of fast imaging detectors lies in the ability to observe real-time events. In principle, a reliably repeatable event would not require a fast camera for observation, as the experiment can be repeated with varying delays between the event and detection. A classic example of this is the studies of the splashes of a drop of liquid by Worthington in 1876 [224], in which the author uses a spark triggered at various delays after impact to illuminate an open-shutter detector (the eye). However, for the recording of non-repeatable events, fast detectors capable of capturing the entire window of interest are required.

One of the current uses of fast cameras in fluid dynamics, in which a variety of phenomena such as fragmentation, ligaments, jets and plumes are studied [225, 226]. The real-time nature of the detectors are here used to probe particularly unrepeatable events: instabilities causing the characteristics within the fragmentation and ligament formation may not be identical in each experimental cycle even with the same initial conditions. It is therefore important to follow each event as it progresses. Another field in which the similar challenge of unrepeatable events can be overcome by fast detectors events is in the passage of shock waves through a variety of mediums, in which techniques such as Schlieren visualisation may be used together with

the fast cameras [227]. Finally, fast cameras have been extensively applied to the field of animal movement. For example, the phenomena of cavitation bubbles have been shown to be produced by both snapping [228] and mantis shrimps [229]. The appeal of fast imaging of animal movement have led to the appearance of such work in popular science recently [230–232].

It is important to note, however, the frame rates of the detection systems in the studies mentioned. These range from 500 frames per second (fps) to 100 kfps (kiloframes per second). Even for the highest frame rate of these cameras, this would correspond to a time resolution of $10\ \mu\text{s}$. With timescales of time-of-flight mass spectrometry typically in the range of less than $100\ \mu\text{s}$, with multiple peaks within that time period potentially having separation of $< 1\ \mu\text{s}$, these detectors are unsuitable for the application to ion imaging mass spectrometry.

6.1.1 Delay Line Detectors

There are a number of different types of two-dimensionally resolved detection systems. The majority of the detectors are pixel-based, but delay lines should be briefly mentioned. This was developed into a position sensitive detector by Eland in the early 1990s [233, 234]. The delay line is coupled with an MCP: the ion hits are converted into an electron cloud, which then impact on the delay line, consisting of one or more long anodes. The time difference between the arrival of an electronic pulse at either end of the anode is measured used to determine the distance of the impact from each end, providing positional information of the impact.

Their main advantage lies in a very short deadtime and high temporal resolution. These detectors are still regularly used in coincidence experiments and ion imaging experiments. However, a fundamental limitation to the delay line detector is its failure upon detecting multiple hits: the pulses from these events are difficult to distinguish from each other, and thus the time differences along the lines cannot be determined. Recent improvements such as in those described in Ref. [235], have gone some way to improve this, but the detectors remain limited in number of events

to single figures. This limitation is not serious in conventional coincidence imaging, where very low event rates per cycle are desired and maintained, with high repetition rates used to compensate for the low event count. However, in systems in which the rate is limited, such as by pulsed molecular beams this becomes impractical: if fewer than 5 ions may be detected simultaneously, the amount of time required for sufficient ion hits, typically in the range of 2×10^5 , to be acquired would be prohibitive. Additionally, the high number of laser cycles required would rapidly degrade the sample. For this reason, delay lines are unsuitable for the apparatus used in this work.

6.1.2 Pixel Detectors

Fast pixel detectors generally use one of a two methods in order to acquire at rates higher than conventional CCD devices [73], parallel/partial readout and *in situ* storage. There are advantages and disadvantages to both schemes. Time-stamping detectors are also of potential use in ion imaging experiments. Streaking techniques also offer a method of improving the frame rate of the acquired images.

Parallel readout detectors

Parallel readout detectors improve frame rate by having multiple readout taps, each of which reads out part of the image. These are used in parallel to reduce the data transfer time, and may be used alternately in order to run the process of reading and acquiring in parallel. These are the types of detectors usually used in commercial fast cameras. The main advantage to these detectors is that there is virtually no limit to the number of consecutive frames that can be taken, as the data is constantly read out from the sensor. Currently, commercial parallel readout detectors are available which can attain up to 1 Mfps [236], but at a dramatic cost in pixel resolution: the detector at this frame rate is restricted to 64×16 pixels, out of an initial 1024×1024 pixels at 1 kfps. This is the main disadvantage of parallel readout detector systems: the bottleneck of data readout necessitates a trade-off between the frame rate and

pixel resolution.

***In situ* storage detectors**

In situ storage detectors avoid the impact of the data transfer on the frame rate by storing the signal on-chip during the acquisition cycle [75, 237]. The frame rate is then determined by the speed at which the acquired charge can be moved into the storage sites. Theoretical predictions based on simulations by Son *et al.* [73] estimate this limit to be 100 Mfps. Each pixel may have its own set of data storage buffers and hence the frame rate should remain largely independent of size. However, the finite number of storage sites on-chip means that the number of consecutive acquisitions is limited, and the data must still be read out at some dead-time during the cycle. With pulsed TOF systems, this is not a significant disadvantage: the ions will generally complete their flight through the drift tube well within $100\ \mu\text{s}$, and after this window of interest there is a long period of time during which the data can be read out. The fast-framing CCD camera used in this work [90] is based on this principle. This detector has so far had the highest frame rate of up to 100 Mfps, or 10 ns time resolution. However, the number of consecutive frames is limited to 16, and the pixel resolution 64×64 .

Time-stamping detectors

Time-stamping detectors function in a different way from conventional pixel detectors. While conventional detectors store an intensity value for each pixel in a frame exposure, the detectors await signal which exceeds a pre-set threshold. The time of this signal is then recorded. The data are read out as a series of events with values of (x, y, t) , the pixel coordinates and the time of impact, from which the images can be constructed post-acquisition. In situations where there is a relatively low amount of signal against a dark background (such as in ion imaging experiments), this results in a much lower amount of data which must be stored and read out, as no data are collected for the low-intensity pixels. The TimePix detectors [95] are

an example of such time-stamping detectors, with a time resolution of 10 ns and pixel resolution of 265×265 . However, a limitation to these detectors is the number of events that may be stored in each experimental cycle: in their time-stamping mode, they are capable of only storing one event. This is disadvantageous for use with imaging mass spectrometry, as ions that arrive later in the same area within the same experimental cycle will not be detected. Another time-stamping detector, PImMS [91, 238], is a recently developed detector, which may store up to four hits on-chip before the buffers are filled. The prototype of this detector, used in this work, has a pixel resolution of 70×70 pixels, with up to 25 ns time resolution.

Streaking Camera Systems

A technique that may be used in conjunction with two dimensional detectors is streaking [239]. This involves using a time-based deflection system to spread the image along one dimension of the detector, much like the principle of an oscilloscope. In this way, this axis of the detector becomes a time dimension, the scale of which is the dependent on the speed of the deflector. Commercially available streaking cameras are available with down to two picosecond time resolutions [240], and results claiming reconstructed frame rates of $5 \times 10^{11} \text{ s}^{-1}$ [241] have been presented. Also, the application of picosecond streaking detectors has been proposed in conjunction with time-of-flight mass spectrometry [242]. However, the use of a spatial dimension on the camera for streaking means that, effectively, only one-dimensional images are collected. Many acquisitions must be performed on the event, collecting multiple one-dimensional images, before a two dimensional image may be reconstructed. This requires the event to be repeatable, thus defeating the main advantage fast detectors have been chosen for the application in the field of microscope imaging mass spectrometry.

6.1.3 In Spatially-Resolved Imaging Mass Spectrometry

The combination of fast detectors with TOF-MS imaging apparatus should allow the acquisition of images for multiple mass peaks in each experimental cycle. This would produce a number of advantages. The most immediate of these would be the reduction of collection time: separate runs are no longer necessary for each mass of interest, with the collection time reduced by a factor equalling the number of masses under investigation. Additionally, the real-time collection of fast-frame detectors complements the label-free nature of spatial imaging mass spectrometry: no prior knowledge of the masses or nature of the sample is required: the fast detectors can be used to collect distributions of all the masses desorbed from a surface simultaneously. In this chapter, experiments designed to test the viability of using fast detectors in conjunction with spatially-resolved imaging mass spectrometry are presented. The aim is the creation and imaging of a number of mass-dependent spatial distribution on the sample surface, all of which can be imaged within the period of one experimental cycle.

6.2 Sample Preparation

For experiments involving the fast detectors, two main types of samples were used. The matrix CHCA was used on its own as an analyte as described in Sec. 5.2. For the samples prepared using CHCA, a large spatial distribution was prepared in order to ensure that it could be distinguished on the lower resolutions of the fast detectors. A washer of 6 mm outer diameter and 3 mm inner diameter was placed on the sample and solution of 15 mg/ml CHCA applied over it with an airbrush, then air-dried. The washer was removed after the matrix was dried, leaving a negative image of the washer on the sample plate. An optical image of the sample is shown in Fig. 6.1. However, a disadvantage of this method of preparation was that it could create only one spatial distribution for all masses.

To achieve spatially distinct mass spectra with multiple masses, an effective and



Figure 6.1: Optical image of CHCA sample prepared for imaging with a fast detector.

convenient technique described by Klerk *et al.* [243] was used. Permanent markers (Staedtler Lumocolor) of different colours were used, which were known to contain organic dyes of different masses. Investigation of these dyes in a commercial ESI mass spectrometer gave strong mass peaks for the green pigment at 385.25 Da, red pigment at 443.24 Da, and blue pigment at 478.32 Da. The mass spectra of each of these pigments from the commercial mass spectrometer are shown in Fig. 6.2-Fig. 6.5. Fig. 6.2, Fig. 6.3, and Fig. 6.4 show the mass spectra acquired of the green, red and blue pigment respectively, while Fig. 6.5 shows the mass spectrum of a sample with the three dyes mixed together, to give an indication of the relative mass positions.

The markers were applied directly to the stainless steel plates used in the MALDI sample plates in the desired pattern and allowed to air dry. For additional spatial resolution, the electro-formed mesh (Goodfellows NI008720/9) was fixed to the sample plate over the pigment in a similar manner as described in section 5.2.

6.3 Results and Discussion

Three sets of representative results will be presented in this chapter, two using distributions created with marker pigment and one using CHCA. The two distributions

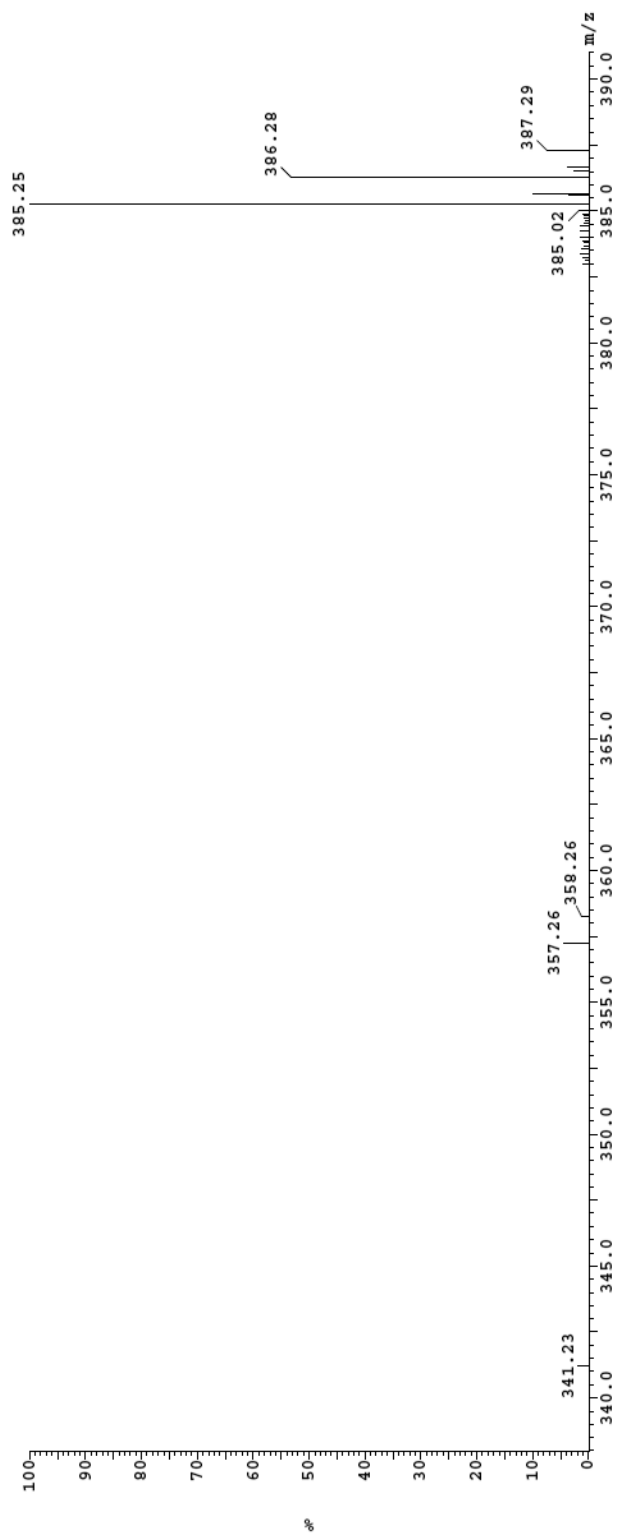


Figure 6.2: Mass spectra of green permanent marker ink. The mass spectrum was acquired on a commercial ESI mass spectrometer in positive ion mode.

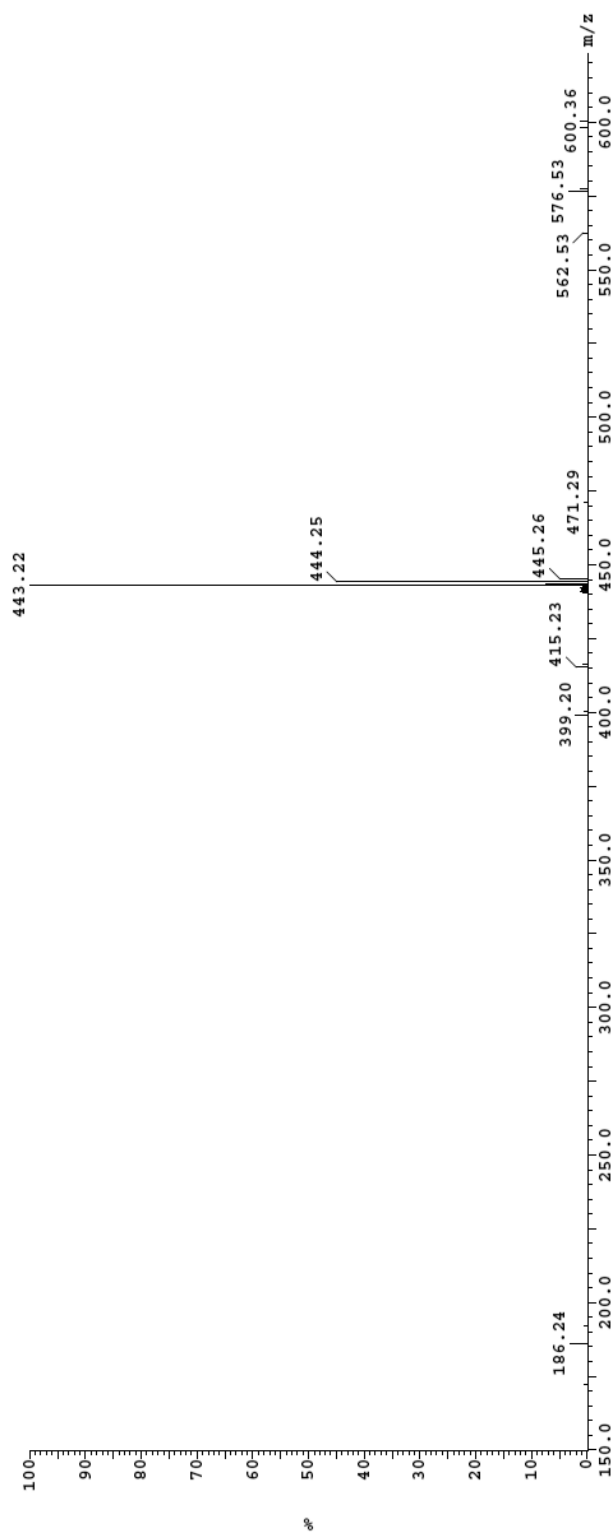


Figure 6.3: Mass spectra of red permanent marker ink. The mass spectrum was acquired on a commercial ESI mass spectrometer in positive ion mode.

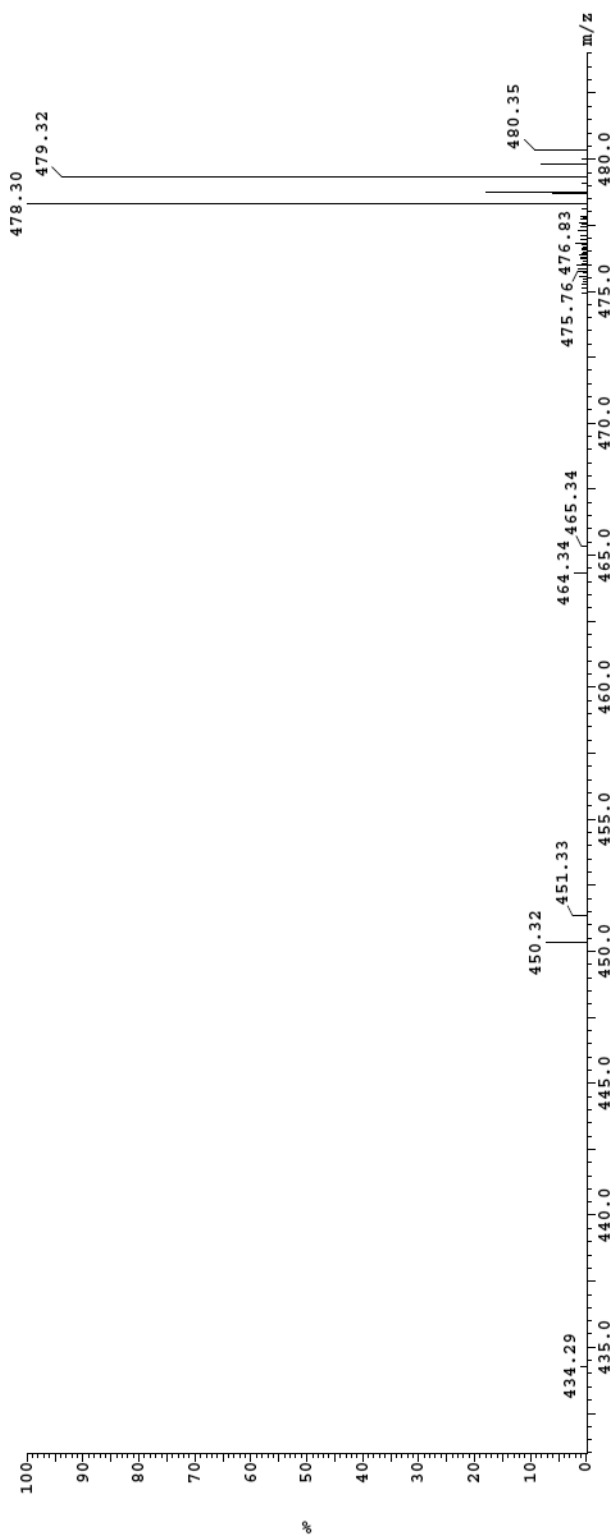


Figure 6.4: Mass spectra of blue permanent marker ink. The mass spectrum was acquired on a commercial ESI mass spectrometer in positive ion mode.

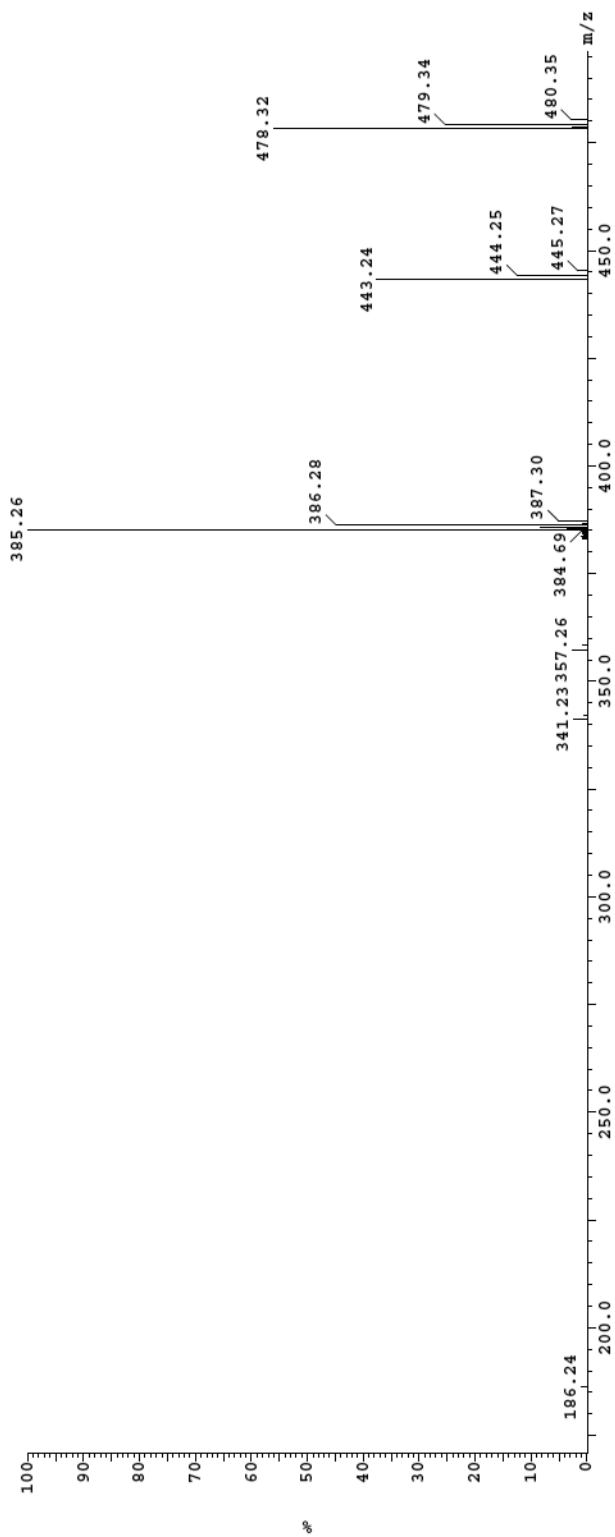


Figure 6.5: Mass spectra of green, red and blue permanent marker ink mixed together for an indication of relative mass differences. The mass spectrum was acquired on a commercial ESI mass spectrometer in positive ion mode.

from pigment were imaged with the fast-framing CCD camera, while that of the matrix was used with the PImMS detector. Each of these distributions was also imaged using the conventional CCD camera to produce images to which the data from the fast detectors could be compared.

6.3.1 Acquisition with Fast-Framing CCD Camera

Image of Spots

Fig. 6.6 shows the first of the samples created using marker dye. A line of green, blue and red spots was laid onto the sample surface, together with a block of black dye. The sample has been overlaid with the $500\text{ }\mu\text{m}$ pitch nickel grid (see Tab. 5.1) for additional spatial distribution. The optical image of this is shown in Fig. 6.6(a). (b) and (c) shows ion images acquired with the conventional CCD camera over 1000 laser shots, with accelerating potentials of 2 kV. At the time of the acquisition of these results, the high-voltage feedthroughs allowing extraction voltages up to 10 kV were not yet available. These ion images were acquired at times of flight $26.9\text{ }\mu\text{s}$ and $30.5\text{ }\mu\text{s}$ respectively. By comparing the spatial distributions of the figures with the optical image in (a), it can be seen that the image (c), acquired at $30.5\text{ }\mu\text{s}$, corresponds to ions from the central blue spot. Image (b) appears to have contributions from both the green and red dyes, although they have differing parent ion masses. A possible cause is that some fragments from the heavier red dye have masses similar to the green. This may be due to fragmentation of the pigment due to the conditions of the direct laser desorption, which is significantly “harder” than the method of electrospray ionisation (see Sec. 5.1.1).

Fig. 6.7 shows the corresponding time-of-flight spectrum acquired from the phosphor response in the detector array for the sample in Fig. 6.6. Inspection of the time of flight spectrum shows three peaks in the region: two closely-spaced peaks at around $30\text{ }\mu\text{s}$ and $31\text{ }\mu\text{s}$, and a larger broad peak near $28\text{ }\mu\text{s}$. Comparing the order of the mass peaks in the acquisition for Fig. 6.6 identifies the peak near $31\text{ }\mu\text{s}$ as originating from the blue dye, and hence should have a mass of around 478 Da

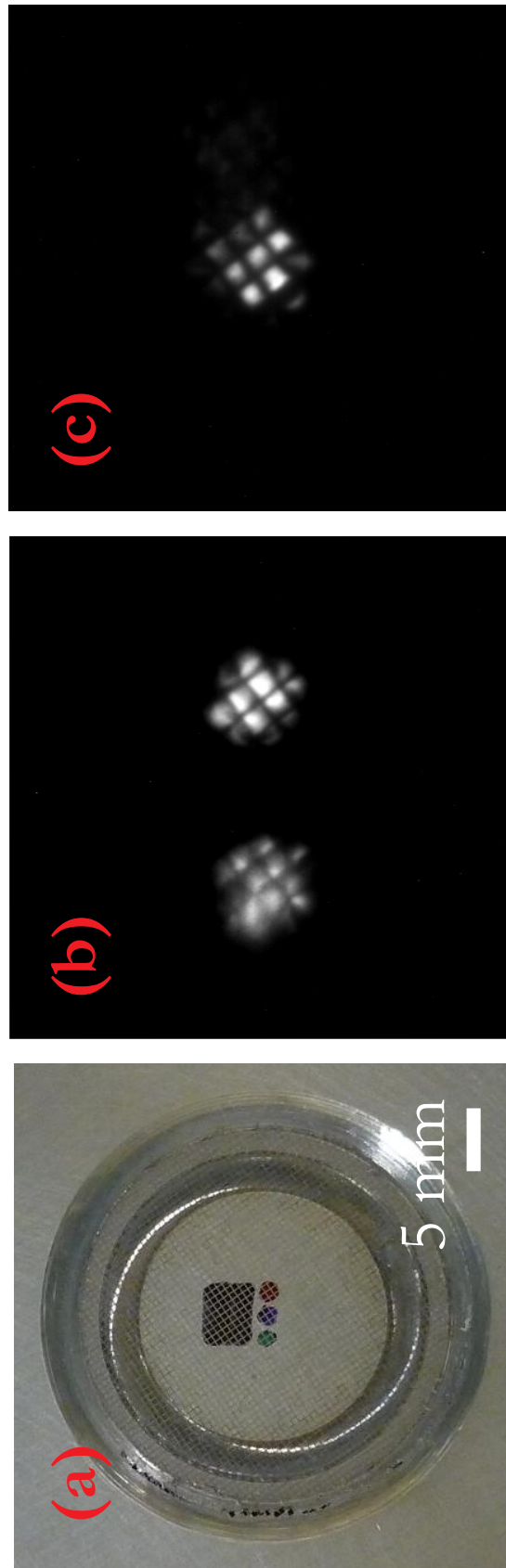


Figure 6.6: Optical image (a) of the first sample used with the fast-framing camera. (b) and (c) shows ion images collected with the conventional CCD camera at $26.9 \mu\text{s}$ and $30.5 \mu\text{s}$ respectively. There are different spatial distributions at the different masses. The masking pattern of the grid is clearly distinguishable.

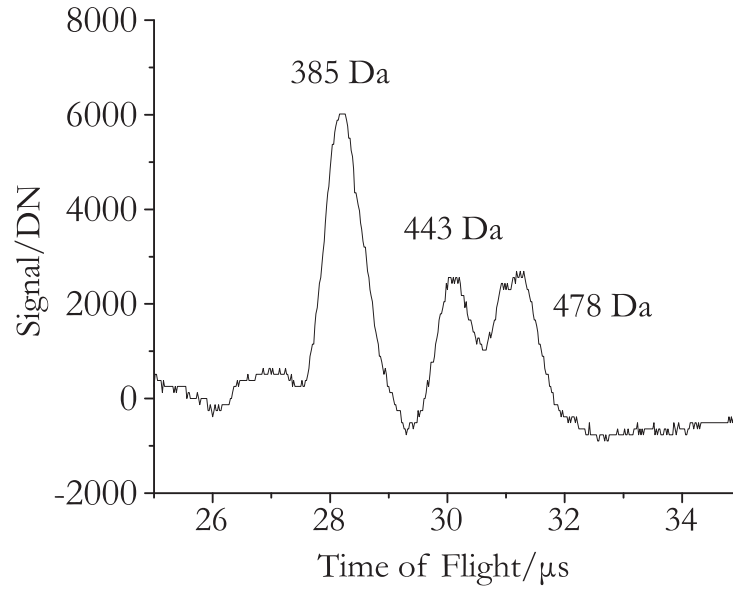


Figure 6.7: Time-of-flight spectrum acquired from the voltage response of the phosphor screen. The masses have been identified by comparison with Fig. 6.5, and the spatial distributions of the ion images at the times of flight.

according to Fig. 6.4. Similarly, the peak at about $30\ \mu\text{s}$ should be that of the red pen, using the mass spectrum in Fig. 6.5. Finally, the image in Fig. 6.6(b) shows the broad peak to have contributions from both the green and red peaks, so should be approximately 390 Da. Approximate masses have been calculated for the three peaks using the mass calibration of Sec. 5.3.1 and corrected for accelerating voltages. These give masses of 410 Da, 458 Da, and 483 Da for the green, red and blue mass peaks respectively, slightly higher than those given by the commercial mass spectrometer. A cause for this disparity may be the lower acceleration potential of 2 kV used here, leading to deviation from the first-order approximations used in the calculations. The ability to identify the origin of the ions even with the mass accuracy and resolution limitations on the apparatus by using the spatial distributions of the ion images serves to highlight the potential of this imaging technique.

Using the time-of-flight spectrum in Fig. 6.7, the frames of the fast-framing CCD camera were programmed such that each mass peak was just bounded by an exposure period. As all frames were acquired consecutively, sacrificial frames were programmed to acquire during the periods in between the peaks in which there was no

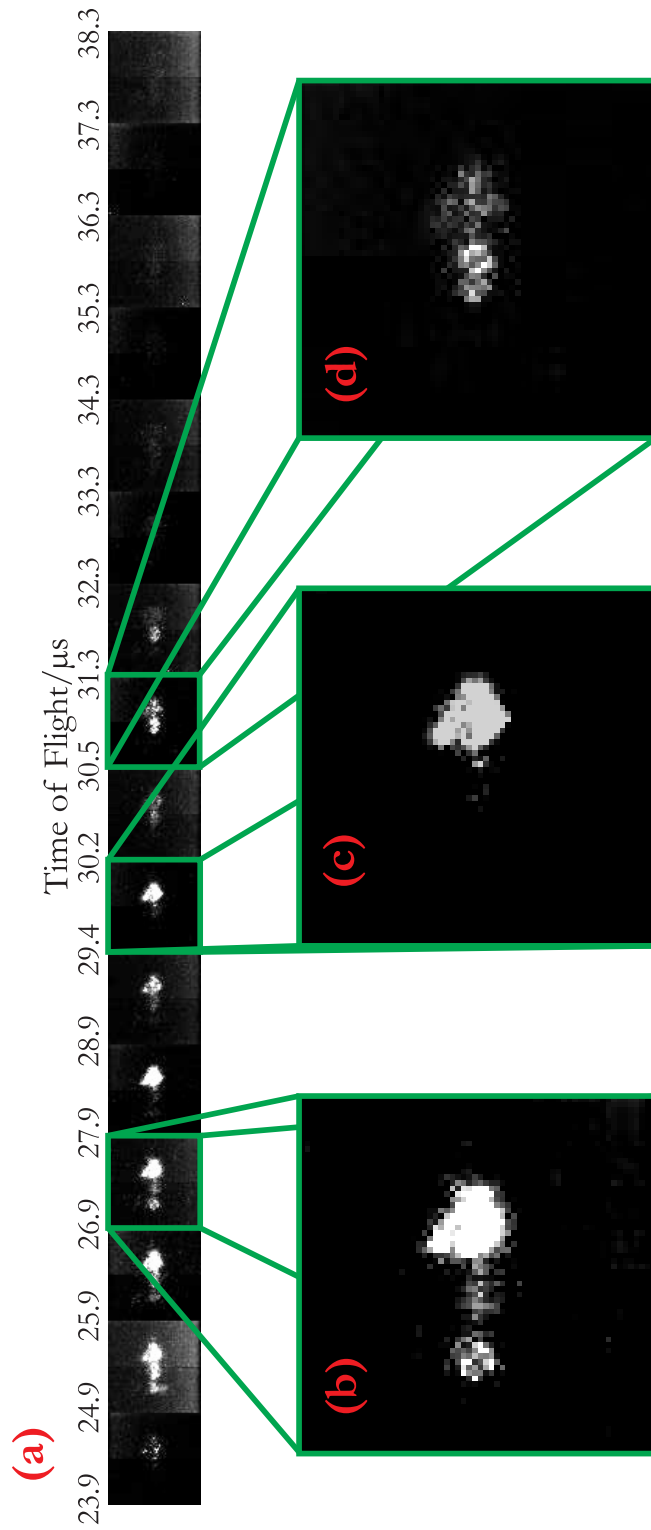


Figure 6.8: Ion images acquired with the fast-framing CCD camera. A series of 16 frames was acquired with each laser shot. The corresponding frames were summed over 200 laser shots. The entire series is shown in (a). The times above the frames show the time-of-flight at the beginning of each frame. The mass distributions of each frame can be seen to be time-dependent. Panels (b), (c) and (d) show expansion of frames in the series corresponding to the mass peaks in Fig. 6.7. (a) and (c) can be seen to correspond to Fig. 6.6(b) and Fig. 6.6(c), while the distribution of (c) can be identified as originating from the red dye.

signal in order to reduce the noise [77]. In each cycle, a series of consecutive images were acquired over a period of $\sim 25 \mu\text{s}$ with each laser shot. Fig. 6.8(a) shows the series of sixteen frames acquired over the time-of-flight window. The times above each frame indicate the time between irradiation and the beginning of that exposure. The corresponding frames of each series were summed over 200 acquisition cycles. As the ions from the black pigment arrived at much shorter times of flight ($\sim 11 \mu\text{s}$), the collection window did not cover a sufficiently long period to acquire the black distributions in the same cycle as the red, green and blue pigments.

Figs. 6.8(b), (c) and (d) show enlargements of the frames indicated in Fig. 6.8(a). Comparison of the enlarged frames acquired with the fast-framing CCD camera with the corresponding higher-resolution images collected with the conventional CCD camera in Fig. 6.6 shows that even though the pixel resolution is significantly lower, the spatial distributions are clearly recognisable and similar to the higher-resolution images. This is in agreement with previous work with the fast-framing CCD [77]. Brouard *et al.* found that the lowered pixel resolution of the camera still allowed the same Fourier moment analysis of the velocity-mapped ion images as a higher-pixel resolution camera, with similar results. However, in these images, the pattern of the grid is only barely discernible.

Image of Bars

From the images acquired with the fast framing camera in Fig. 6.6, the patterning of the grid cannot be clearly seen. Another sample with a larger area of pigment was prepared, in which a more extended repeating pattern may be more obvious. This consisted of long bars of pigment. An optical image of this sample is shown in Fig. 6.9(a). The corresponding ion image acquired with the conventional CCD camera is shown in Fig. 6.9(b). The grid pattern is once again clearly visible in the ion image with the higher pixel resolution of the conventional CCD. The time-of-flight spectrum acquired for this sample from the phosphor response is shown in Fig. 6.10. Once again, the masses have been identified by comparison of the order

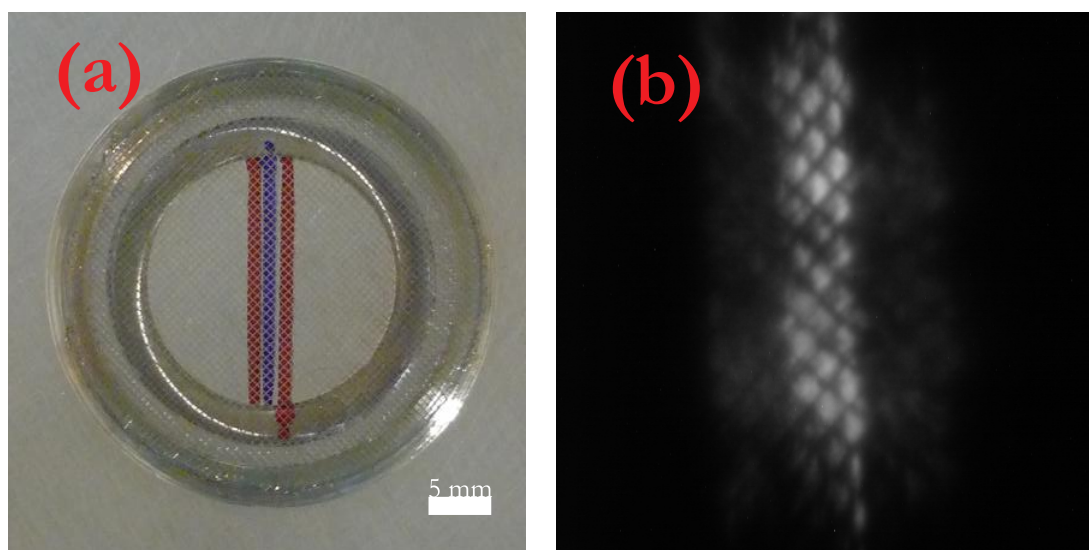


Figure 6.9: Optical (a) and ion (b) image of sample with wider sample region. The ion image was acquired with the conventional CCD camera over 1000 laser shots at time of flight of $30.5 \mu\text{s}$. The extended grid pattern can be clearly seen in (b).

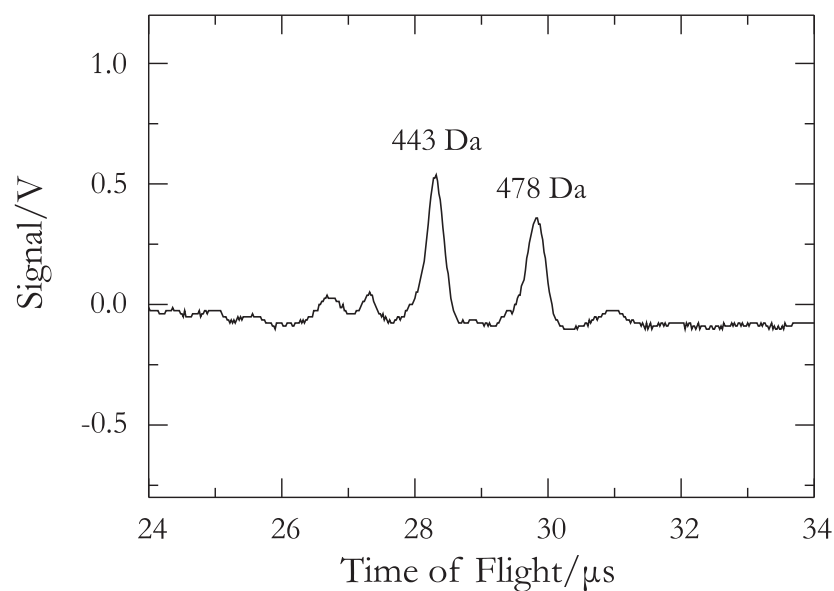


Figure 6.10: Time-of-flight mass spectrum acquired from MCP response of sample shown in Fig. 6.9. The masses have been identified by comparison to the mass spectra of the same sample acquired with a commercial mass spectrometer, as well as the comparison of the images acquired at the corresponding times of flight.

of arrival with the mass spectrum in Fig. 6.5, together with the spatial distributions at the times of flight.

The fast-framing camera was again used to acquire spatial-mapped ion images of the sample in Fig. 6.9. Fig. 6.11(a) is the series of frames acquired with the fast-framing CCD. Panels (b) and (c) are the enlargements of the frames indicated in (a), which correspond to the ion images from the red and blue mass peaks respectively. Once again, the red and blue distributions can be easily identified from their spatial distributions and their order in the time of flight. The spatial distribution of the image at $30.5\ \mu\text{s}$, with its single bar, can be readily identified as corresponding to the blue pigment. This is confirmed by the mass peak being the last one to arrive on the time of flight, in agreement with the commercial mass spectrum in Fig. 6.5. The double bar spatial distribution at $29.4\ \mu\text{s}$ also clearly corresponds to the red pigment. The earlier peaks in Fig. 6.10 can once again be identified from their corresponding spatial distributions in Fig. 6.9(c) ($25.9\ \mu\text{s}$ and $27.9\ \mu\text{s}$) as fragments originating from the red pigment.

The pattern is now large enough that the effect of laser profile is evident: areas with similar amounts of sample (along the “bars”) produce different amounts of signal. The centre of the sample appears to be less intensely illuminated and hence produces less ions. This would be a significant issue in potential applications of spatial imaging mass spectrometry, in which the spatial dependence of the concentration of analyte is of importance. There are two possible solutions to this: the homogenisation of the laser profile using methods such as multi-mode fibre-optic cables or beam diffusers, or the deconvolution of the intensity from the laser profile. The deconvolution method could be achieved with a homogeneous sample over the field of view, which would be irradiated with the laser. The intensity profile could be mapped and further acquired images weighted with this profile. However, this method requires that all experimental parameters such as laser alignment and sample position are identical. The creation of a homogeneous sample is investigated in Chapter 5.

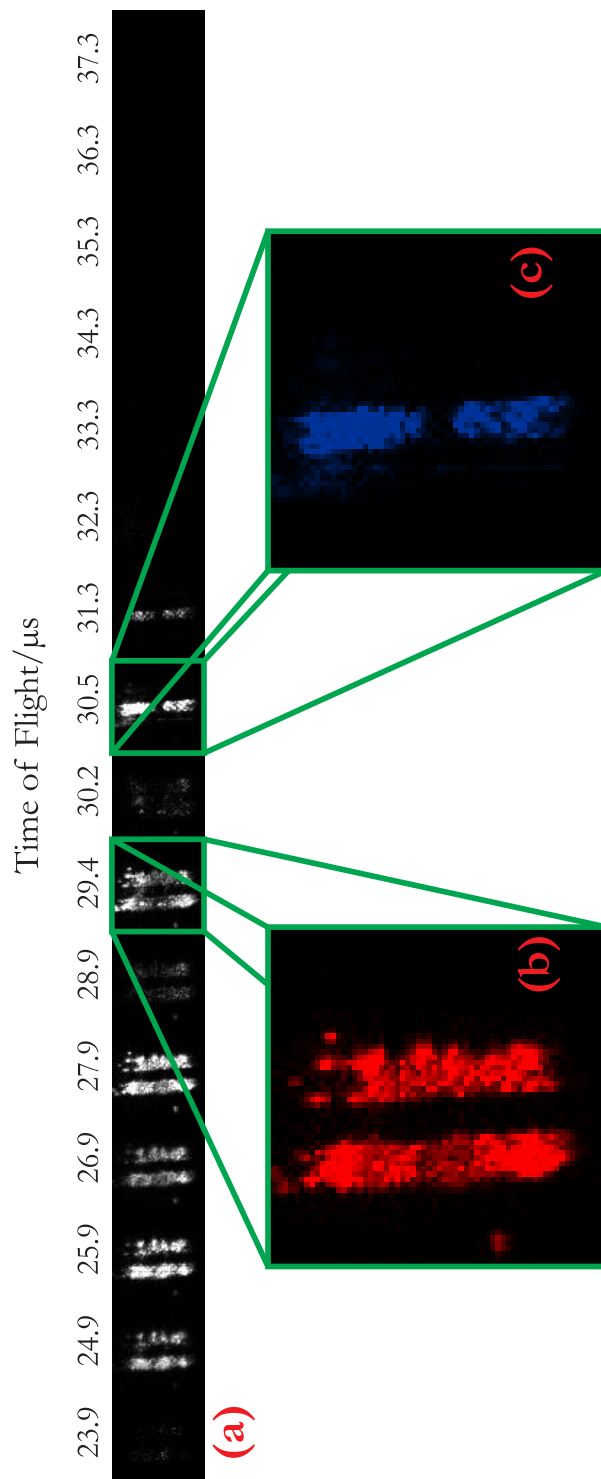


Figure 6.11: Ion images of the sample in Fig. 6.9. (a) shows the series of 16 consecutive images acquired per laser cycle. Corresponding frames from each series were summed over 200 laser shots. The same exposure times were used as in Fig. 6.8. Panels (b) and (c) show expanded frames corresponding to the mass peaks of the red and blue fragment, respectively.

The grid pattern from the electro-formed mesh over the sample is just discernible in some of the frames, such as at $31.3\ \mu\text{s}$. However, this is not clear in every frame of the acquisition and the pattern is not clearly evident. The spatial resolution for this fast detector does appear to be dictated by the pixel count. From the point-to-point distance on the grid, the field-of-view can be determined to be about $6.5\ \text{mm}$ on the sample. Each pixel on the 64×64 would then cover $100\ \mu\text{m} \times 100\ \mu\text{m}$. Since the wires of the grid used (Goodfellows NI008720/9, table 5.1) are of $70\ \mu\text{m}$ width, it is perhaps unsurprising that the lines can barely be observed in the ion images, and the limitation to the spatial resolution in this case is the pixel count of the detector.

6.3.2 Acquisition with PImMS

Imaging Capabilities

The PImMS camera has also been used to acquire data from multiple mass peaks per experimental cycle. A preliminary ion image has been compared with one taken using the conventional camera in Fig. 6.12. The image was acquired with an uncalibrated detector, with the thresholds of each pixel yet to be adjusted to respond to the same levels of signal as each other. The ion image presented in panel (a) was acquired using the conventional CCD camera, and shows the sample of CHCA on a stainless steel plate (Sec. 6.2). The optical image of the sample is shown in Fig. 6.12(b). The distribution can be seen to clearly correspond to that on the sample. The red square in (a) indicates the area of the phosphor collected by the PImMS camera, with an enlarged image in (c). (d) shows the image reconstructed from data acquired by the PImMS detector. The image was created by plotting the x and y values falling within a certain window of time onto a two-dimensional plot. The similarity in distribution between the ion images taken by a conventional camera (c) and time-stamping (d) are clear, despite the much lower pixel resolution of the PImMS camera.

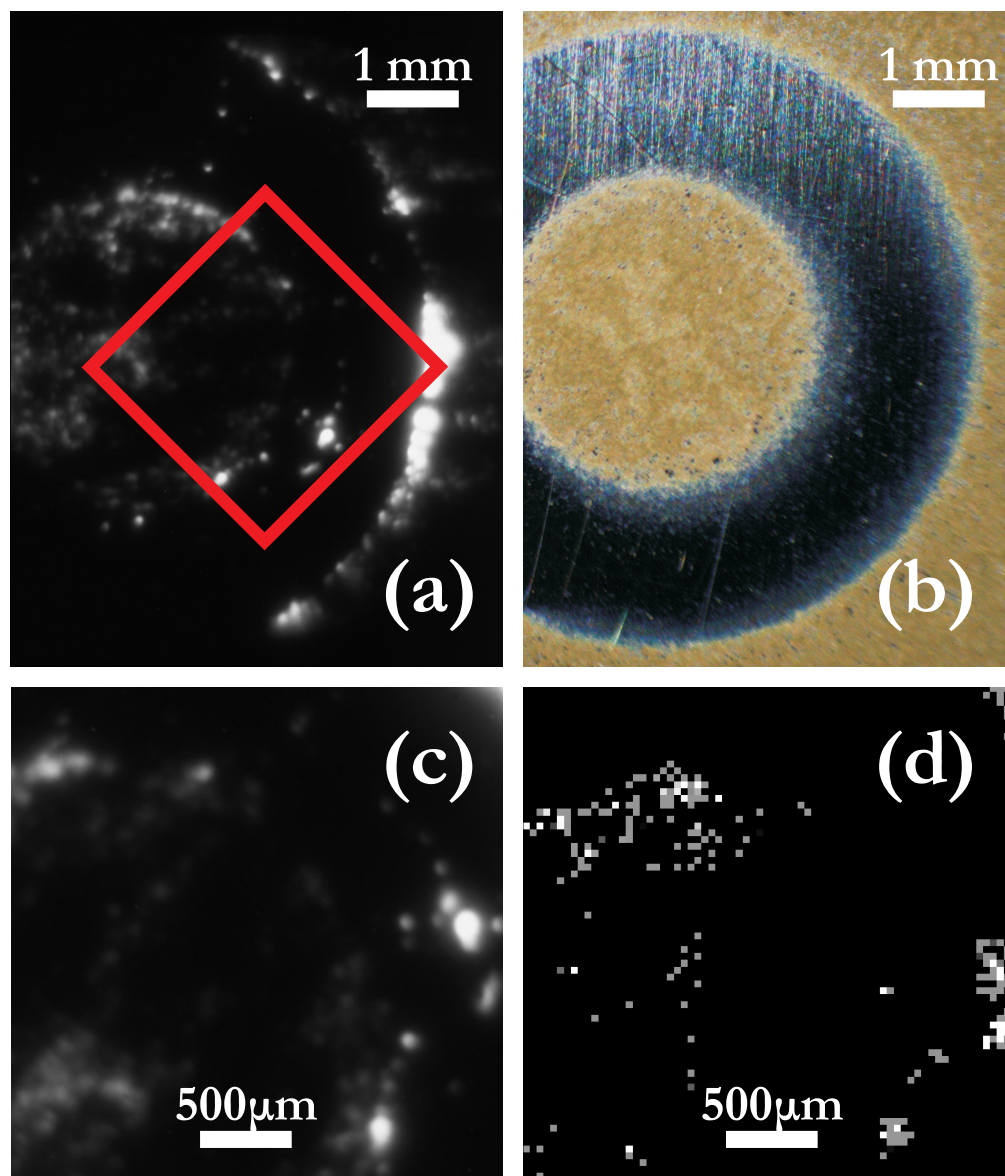


Figure 6.12: Optical and ion images of CHCA acquired with both conventional CCD and PImMS. (a) Ion image of sample shown in optical image (b). The red square indicates field-of-view of PImMS. (c) Enlarged image of area indicated by red square. (d) Ion image acquired with PImMS.

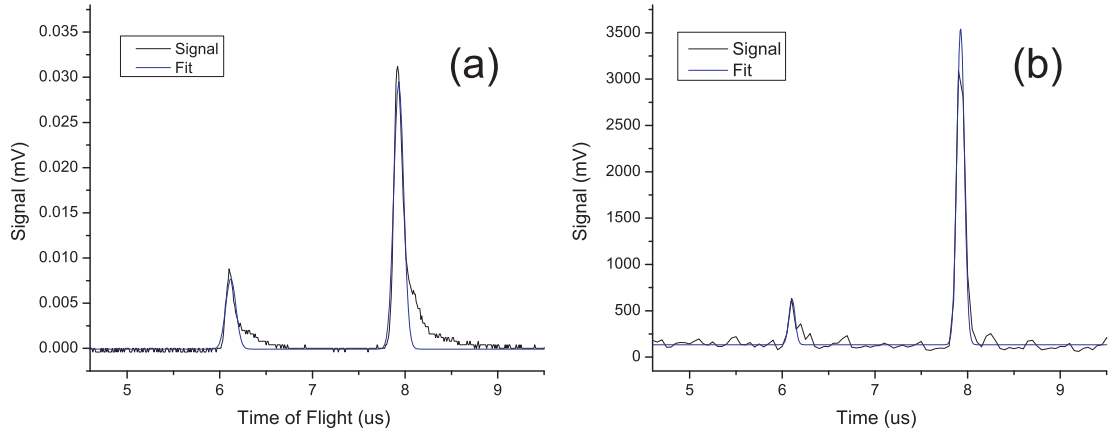


Figure 6.13: Times of flight acquired for the sample shown in Fig.6.12. **(a)** Time-of-flight acquired with photomultiplier tube *via* fibre-optic link. **(b)** Time of flight reconstructed from PImMS time-stamping data. The similarity is clear. The peaks have been fit with a Lorentzian function. Peaks in **(a)** have a FWHM of 130 ns and 123 ns, while **(b)** have FWHM of 95 ns and 79 ns. The increased time resolution is a result of the higher threshold of the PImMS detector.

Time Resolution

Fig.6.13 compares the time-of-flight spectra acquired using (a) a photomultiplier and (b) extracted from the data collected by PImMS. The strong similarity of the peaks suggests that the mass resolution of the system is not limited by the cameras but by the phosphor screen, with a 100%-10% decay of 60 ns. This decay is clearly visible in the tail of a peak in (b). A fit of the peaks in (a) gives a FWHM of less than 95 ns, while (b) returns about 123 ns. The resolution in (a) was improved by the higher thresholding of the PImMS detector removing the tail of the spectrum.

PImMS Registers

Time-of-flight distributions reconstructed with data from each of the four registers of the PImMS camera are shown in Fig.6.14. The sample was the same as that used in Fig.6.12 and Fig.6.13. Each plot shows the number of hits against arrival time stored in every pixel's first register on the detector; (b) shows the same for the second registers on the detector, and so forth. These can be compared with the time-of-flight spectra shown in Fig.6.13. However, the time-of-flight spectra in Fig.6.14

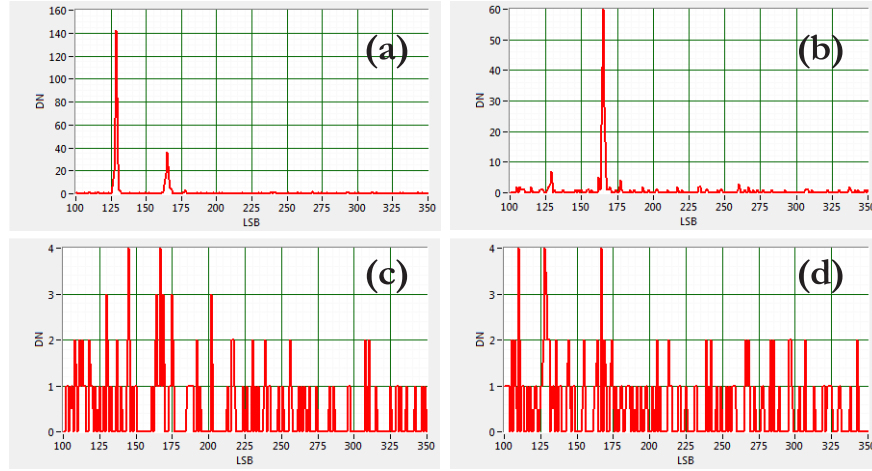


Figure 6.14: Occupancy of each register in PImMS. The plots show the frequency of arrival times of the hits in each register, over all pixels on the detector. The sample is the same as that used to obtain the time-of-flight spectra in Fig. 6.13. (a)-(d) show registers 1-4 respectively. It can be seen that the large majority of ions arriving at around $6.1 \mu\text{s}$ are recorded in the first register, while the second consists mainly of the peak at $7.9 \mu\text{s}$. The third and fourth registers are still available for recording and have only background noise. The x -axis labels the time bin of the register, with each bin corresponding to 50 ns.

were acquired a number of laser shots before that of Fig. 6.13. The difference in conditions, such as surface morphology, ablation and degradation, as well as laser power, results in different relative intensities of the mass peaks. It can be seen that the large signal at $6.1 \mu\text{s}$ is almost entirely recorded in the first register (Fig. 6.14(a)), as it represents the first major amount of signal that the detector receives. As the first and second ($7.9 \mu\text{s}$) peaks both originate from the same spatial position, it might be expected that the heavier of the two masses should mainly hit the detector where the first peak has already been recorded. This is shown to be the case in that the majority of the signal at $7.9 \mu\text{s}$ is in the second register. A small amount of the signal from the second peak may also be recorded in the first register if it strikes at a place where the first ions were not recorded. This may be because the signal from the first peak was not high enough to be recorded, or that the fragments were not ionised. Also, a small amount of the first peak appears in the second register when a pixel first registered background noise. The third and fourth registers can be seen

to only have background noise, potentially free to record further signal from other analytes. These results show that the time-stamping detector successfully records signal within its registers and can be used effectively to obtain data from which images at different masses can be extracted within the same duty cycle.

Potential Spatial Resolution

In the case of the PImMS detector, the pixel resolution was 72×72 . This results in the magnified image on the detector being binned into a 72×72 grid and the resolution limited by the coarseness of said grid. In this case, the field of the view on the PImMS camera is $2.68 \text{ mm} \times 2.68 \text{ mm}$, and so the resolution is limited by the pixels to $\sim 34 \mu\text{m}$. This is in a similar range to the limits on the experimental setup found by using the high-resolution camera (Sec. 5.3.4). Hence, improvements to spatial resolution require addressing both the pixel resolution of the detector on top of the aforementioned improvements to the aberrations of the experiment. However, it is worth noting that the field-of-view is significantly reduced, and if a greater field of view together with the same spatial resolution is desired, a higher pixel resolution is required. Future versions of the PImMS camera, currently being manufactured, will have in excess of 300×300 pixels and will greatly improve the resolution of the experiment. Additionally, they will be capable of direct electron detection, thus removing the need for a phosphor and greatly improving the mass resolution.

6.4 Summary and Future Directions

Proof-of-concept experiments with fast detectors have been performed, demonstrating the ability to detect and acquire images of multiple mass peaks within each laser cycle. Mass-dependent spatial distributions were found using a fast framing CCD camera. The origins of the fragments imaged were identified by comparing the time-of-flight spectra together with the spatial distributions of the ion and optical images.

This highlights an advantage of the combination of spatial imaging with mass spectrometry: the two methods provide complementary information which may be used together in the analysis of the sample.

The uncalibrated PImMS detector was also used to acquire data on multiple peaks. Ion images and time-of-flight spectra were reconstructed from the data. These were in good agreement with those acquired using more conventional methods. A higher threshold and lower sensitivity for the PImMS detector was suggested by results obtained in this chapter. The spatial resolution appears to currently be limited by both the pixel resolution of the detector and the characteristics of the apparatus.

There are a few areas of potential improvements suggested by the result of this chapter. The pixel count of future versions of PImMS will be significantly higher. The improvements in spatial resolution in the apparatus have been discussed in Chapter 5. The lower sensitivity and noisy acquisition PImMS may be improved by its calibration. The calibrated PImMS detector has been used for later experiments in this work, and the results of its application to the fast imaging of tissue samples will be presented in the final chapter.

Chapter 7

Imaging of Biological Samples

7.1 Introduction

This chapter will examine the application of spatial-mapped imaging mass spectrometry in a variety of biological fields. A brief overview of techniques currently used in biological imaging will be presented, and their strengths and weaknesses considered. Current applications of imaging mass spectrometry to the life sciences are examined, and the potential advantages that fast imaging techniques may provide in these applications are discussed. Finally, preliminary imaging experiments conducted with both conventional and fast detectors will be presented, in order to demonstrate the feasibility of fast microscope-mode imaging in biological fields of study.

7.1.1 Optical Imaging in the Life Sciences

The use of imaging has always been an integral part of the life sciences, from the very origins of microscopy in the 17th century. The method of imaging for the majority of the time since then has been that of optical imaging: the projection of a real light image onto either the observer's eye or camera. A large variety of methods and improvements have developed over the last three centuries. There are a number of imaging methods currently employed in modern research which may be

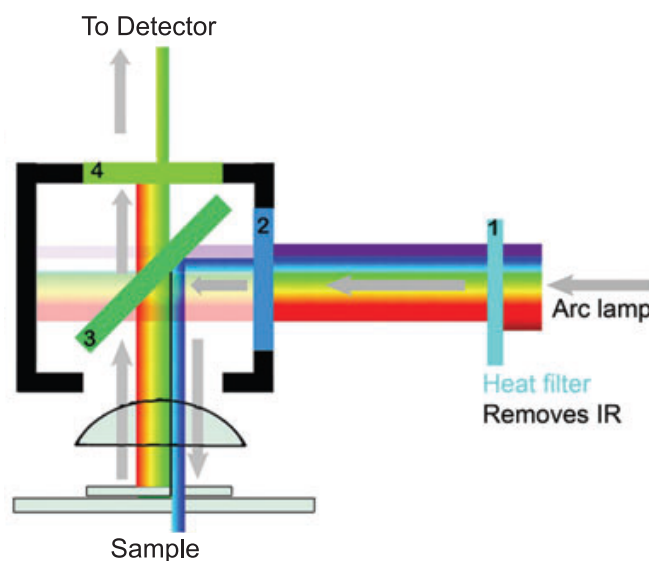


Figure 7.1: Schematic showing the principles of fluorescence microscopy. Broad-band light is filtered to remove infrared radiation (1) and non-excitation wavelengths (2). A dichroic (3) selectively reflects the pump light onto the sample tagged with known fluorophores. The emitted light passes through the dichroic and all light other than the fluorescence wavelength is removed by the filter cube (4). Adapted from Ref. [244].

used separately, or in conjunction with each other. A brief overview of the principles involved with the most commonly used ones is presented here, in order to facilitate a comparison between the currently used (optical) techniques and mass spectrometric imaging.

Fluorescence Microscopy

Fluorescence microscopy can be described as the imaging of the light emitted by certain markers attached to the analyte of interest [244]. Fluorescence imaging is, in its applications, the most similar to imaging mass spectrometry: it is used to provide analyte-specific images over a two-dimensional sample. Fluorescent probe molecules are either attached to or genetically engineered into the proteins of interest. The sample is field illuminated with the excitation wavelength of the chosen probe, which then emits at a slightly longer, lower energy, wavelength. By filtering out all light but the emission wavelength, the positions of the markers can be identified against

a dark background. This reduction in background noise results in a very powerful improvement in signal-to-noise ratio, providing sensitive detection capabilities. A schematic of fluorescence microscopy is shown in Fig. 7.1. In excess of three thousand fluorescence probes are available commercially, with differing absorption and emission wavelengths and specificities [245]. With a careful choice of probes, multiple analytes may be tagged in a single sample, each with non-overlapping emission wavelengths. These may be excited by either the same or different wavelengths. Differing emission wavelengths in combination with appropriate filters allows the imaging of one particular analyte at a time while filtering out the fluorescence from the other probes. This then allows the determination of the location of different proteins on the same sample, as well as their relative concentrations. This simultaneous location of different analytes makes fluorescence microscopy particularly powerful.

In practice, the number of fluorescent probes that may be used simultaneously is limited due to their finite (and usually broad) absorption and emission bands. The probes must be chosen to be well separated in at least one of these wavelengths so that they can be either selectively excited or observed. Overlapping of these bands leads to “crosstalk” and “bleed-through”, in which signal is detected from multiple probes on different analytes in the same acquisition on a particular filter, and are indistinguishable without further knowledge or investigation. Additionally, as the concentration of fluorophores within a volume of interest is generally constant [246], the improvement of resolution and magnification leads to the light produced dropping in proportion to r^3 , where r is the diameter of the volume of interest. This leads to a significant reduction in brightness and signal. Compensation for this may be done in the form of increased illumination intensity, but at the cost of increased sample damage. Finally, fluorescence microscopy is not a surface-specific technique: irradiation of the sample results in all fluorophores in the sample emitting. Emission from probes further below the surface may confuse the resulting data and obscure the surface distribution. Light from deeper within the sample will be scattered as it makes its way out, and will appear to originate from the last point from which it

was scattered [247].

Optical Sectioning Microscopy

Optical sectioning microscopy is used to describe microscopy that is capable of characterising samples by their depth (z) in addition to their position (x, y). The challenge of distinguishing emission from different depths of a sample in fluorescence microscopy may be alleviated with the use of such techniques. There are a number of approaches to optical sectioning microscopy, including two-photon laser-scanning microscopy, computational optical sectioning, and confocal microscopy.

Two-photon laser scanning microscopy (2PLSM) [248] uses the same principles of excitation and emission of fluorophores as in fluorescence imaging. However, a lower wavelength is chosen such that the excitation step becomes a two-photon process. In this way, the excitation becomes only viable in the small volume in which the laser is sufficiently focussed that multi-photon processes are possible. This volume is typically in the order of femtolitres. By varying the position of the focus point, the depth from which the sample is imaged may be controlled, and 2PLSM has been performed on live mouse grey matter in the brain at depths of up to 1 mm [249]. This ability to localise excitation also reduces the photodamage in other parts of the sample. These two advantages make this technique particularly suitable for *in vivo* experiments. An additional advantage of the multi-photon excitation process is that the emission wavelength is well separated from the excitation, reducing the chances of crosstalk and allowing easier light separation.

A disadvantage to 2PLSM is the requirement of powerful tunable lasers, in order to produce multi-photon excitation at specific wavelengths required for the range of fluorescent probes used. Additionally, since this technique relies on scanning the sample with a very small volume, high repetition rates are also desirable. Typically, Titanium-sapphire lasers are employed, with ranges of 670-1070 nm and rates of ~ 100 MHz [250]. Also, the technique is affected by scattering as the light passes through the sample, with the loss of resolution becoming more acute at shorter

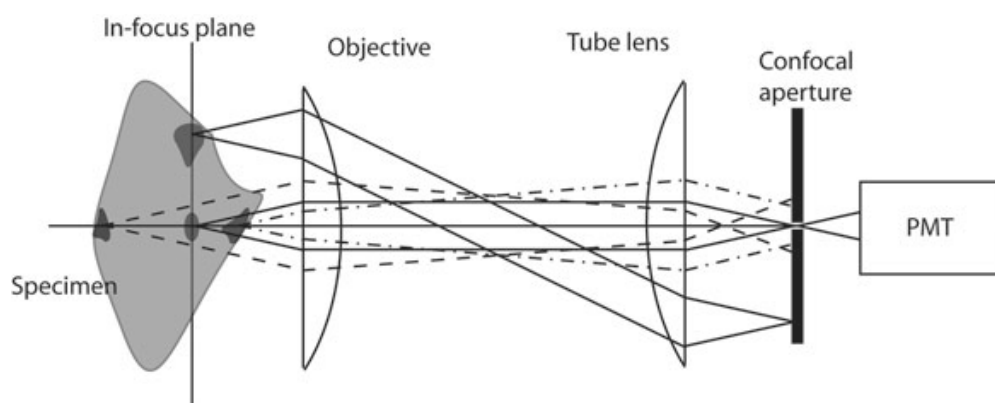


Figure 7.2: Schematic showing the principles of confocal microscopy. Light from the in-focus plane is focussed at the plane of the confocal aperture. Only light from the centre of the in-focus plane passes through the aperture to be detected by the photomultiplier (PMT). Off-centre light from the in-focus plane is cut out by the aperture. Light from in front of (----), or behind the focus plane (---), does not come into focus and is diffuse at the aperture plane. Hence, the majority of the light is also removed by the aperture. Adapted from Ref. [247].

wavelengths. The increased scattering also adds the requirement of wide field-of-view detection [251, 252], limiting the maximum magnification of the image.

Another approach to controlling the depth from which the image is acquired is confocal imaging [247]. A schematic of the principles of a confocal microscope is shown in Fig. 7.2. A pinhole (the confocal aperture) is introduced at the real image plane in front of the detector (PMT). Light originating from anywhere other than the in-focus plane is diffuse at the plane of the confocal aperture, resulting in a large proportion of the light being removed from the detected image. The paths of these rays are shown by the dashed (light from beyond the in-focus plane) and dot-dashed (light from before the in-focus plane) lines of Fig. 7.2. Another effect of this pinhole is the masking of all light originating from off-centre in the in-focus plane: only the small area allowed by the aperture size is imaged. Consequently, confocal microscopy is necessarily a scanning technique. By scanning over the sample with a focussed laser beam, a “section” at the desired depth may be imaged. Changing the geometry of the optics controls the depth of the section in the sample.

Confocal microscopy reduces the background noise even further from wide-field

fluorescence imaging by removing the fluorescence from the sample outside the plane of interest: the contrast is hence greatly improved. Theoretically, the resolution attainable from confocal microscopy is below the diffraction limit. With a perfect lens of nominal aperture D and focal length f , the smallest waist that light of wavelength λ may be focussed down to is dictated by the Airy disc [84, 253]:

$$q_1 \approx 1.22 \frac{f\lambda}{D}, \quad (7.1)$$

where q_1 is the radius of the waist. By stopping down the aperture to smaller than this radius, spatial resolution below the diffraction limit is achieved (and the thickness of the plane observed is reduced). However, in practice, this results in a great attenuation of the signal [254, 255], limiting its application.

Finally, in addition to the modifications in the apparatus described above, there are post-acquisition analysis techniques for the determination of the three-dimensional distribution of the fluorescent probes within the sample. The aim of computational optical sectioning microscopy (COSM) is to remove the out-of-focus light from wide-field microscopy [256, 257]. A series of two-dimensional images is acquired from the sample at different focus depths. Each optical section contains both in- and out-of-focus information. By using models for the propagation and scattering of light through the sample, the contribution of the out-of-focus light to each section may be calculated and removed from each image. There are a large number of models with varying degrees of rigour, such as nearest neighbours subtraction (NNS), Moore-Penrose pseudo-inverse (MPPI), Wiener-Helstrom filter (WHF), and Jansson van Cittert (JvC). The discussion of these methods is beyond the scope of this work and the reader is directed to the reviews in Ref. [247] and Ref. [258]. However, an important point to note is that the computational power and time required increases with the accuracy of the model used.

“Super-Resolution” Fluorescence Microscopy

The technologies based on light microscopy described so far have been limited in their spatial resolution due to the diffraction limit of light, with the exception of the use of a strongly-stoppered aperture in confocal imaging. With the (near-)visible wavelengths of light used in optical microscopy, this is a resolution of around 200-350 nm [246]. Some methods of deconvolution COSM by examining the spread of light (point spread function) and calculating the ideal origin of the emission [256, 259]. This is used as the location for the detected fluorophore. However, this method is effective only for sparse objects and low-noise images [260], as a large number of sources in close proximity makes the point spread function difficult to unambiguously assign.

A number of novel methods have been developed to improve this resolution below the diffraction limit. The term “super-resolution” is applied to these techniques, recently reviewed by Schermelleh *et al.* [246]. Near-field methods involve the measurement of evanescent waves. Total internal reflection microscopy (TIRFM) [261] uses the evanescent waves from total internal reflection to ensure low depth penetration (~ 100 -200 nm) bringing axial resolution below the diffraction limit. The lateral resolution in the plane of the sample remains the same as conventional microscopy. Near-field scanning optical microscopy (NSOM/SNOM) utilises a sharp tip with a sub-wavelength aperture, thus limiting the area of probing axially and laterally on the sample. A resolution below 20 nm was attained with this method by Betzig *et al.* [262].

A number of ingenious far-field methods have also been devised to circumvent the diffraction limit. Structured illumination microscopy (SIM) makes use of a excitation light in a pre-defined pattern, such as from through a grating [263, 264]. This results in a Moiré pattern from the interference between the structures in the sample and the structured illumination. By taking multiple images with different structures of illumination and heavy post-processing, a resolution of twice the diffraction limit may be obtained. The technique may be extended into the axial

plane giving 3D-SIM [265, 266]. A modification of this technique, non-linear saturated structured illumination microscopy (SSIM) has demonstrated a resolution of better than 50 nm [267]. Stimulated emission depletion (STED) excites the entirety of the sample, then stimulates emission using a depletion beam [268]. The depletion beam is shaped with a phase plate to produce a zero-intensity section of smaller than the diffraction radius. Subsequent fluorescence from this undepleted region of the sample is recorded, giving resolutions in the range of 30-80 nm [246]. However, the requirement for a large number of non-overlapping excitation, emission, and depletion wavelengths has limited the number of analytes that may be simultaneously investigated, with two-colour images the current limit [269].

By combining the localisation from point spread functions of a fluorophore over multiple acquisitions, a single molecule may be accurately determined. In single molecule localisation and composition, the density of fluorophores is greatly decreased in order to unambiguously identify the source of the fluorescence. A number of non-overlapping probes are used. Each fluorophore is repeatedly excited and observed in turn until it is bleached, and the large number of acquired images reconstructed to produce an image with resolution in the order of 1–100 nm [262]. This principle has been applied under a variety of acronyms, such as (fluorescence) photoactivation localisation microscopy, (F)PALM, and stochastic optical reconstruction microscopy, STORM. Once again, these variations are beyond the scope of this work, but have been recently reviewed by Schermelleh *et al.* [246].

7.1.2 Mass Spectrometric Imaging in the Life Sciences

Imaging mass spectrometry has received in the last fifteen years a great amount of interest in the biological sciences, with applications in biology, pathology, medicine, and pharmacology, to name but a few [270, 271]. These applications have recently been described in detail by Chughtai and Heeren [56]. The principles, methods, and applications used in mass spectrometric imaging for biological imaging have already been described in Chapter 1. Hence, only a discussion of the advantages

and disadvantages of the use of mass spectrometric imaging in comparison with the optical imaging methods will be presented here.

Advantages of Imaging Mass Spectrometry

There are a number of characteristics of mass-spectrometric imaging which prove particularly advantageous in comparison to conventional imaging techniques when applied to the life sciences. An important one to begin with is its label-free nature. As mentioned in Sec. 1.3.3, the majority of optical imaging techniques requires the staining or marking of the features of interest. The techniques described above in Sec. 7.1.1 all require the attachment of fluorescent markers to the protein of interest. The tagging of proteins and peptide sequences is a non-trivial process, requiring the combination of fluorophores and specific antibodies for the protein. Alternatively, genetic modification is used such that the target protein carries the fluorophore. Consequently, prior knowledge of the analytes is required: one must choose specific analytes to be investigated when preparing a sample. Using imaging mass spectrometry, the markers are an intrinsic property of all molecules: their mass. Ideally, it is possible to then prepare a sample for IMS without any knowledge of its nature, and after analysis, determine both the components and their spatial distribution.

Mass spectrometry is also theoretically limitless in its mass range, and the number of peaks imaged limited only by the mass resolution of the analysing apparatus. Modern mass spectrometers have achieved an $m/\Delta m$ in excess of 10^5 [56]. Thus, the restraint imposed on the number of analytes that may be investigated simultaneously by excitation and emission wavelength overlap is lifted. This effect is complementary to the label-free nature of IMS, allowing the identification of a large number of unknown analytes and their spatial distributions. The identification of the analytes can be further refined using sequential fragmentation and mass separation of the products (*eg.*, MS/MS) or ion mobility experiments [272].

Another advantage of mass spectrometric imaging is its high sensitivity. While optical imaging requires good contrast against background light and a minimum

fluorescence, concentrations as low as 500 attomol have been detected using IMS techniques [273]. The spatial resolution that may be attained using IMS is also on par with super-resolution microscopy, with microprobe-mode SIMS attaining 50 nm spatial resolution [274].

Finally, as the analysis may be carried without requiring any sample- or analyte-specific information, the process is easily automated. This ameliorates somewhat the long times required to acquire high spatial resolution images with microprobe mode imaging mass spectrometry. The sample preparation may be carried out with a robotic spotter or sprayer. The analysis itself is performed at a high laser rate, with mechanical steppers rastering the sample with relation to the ionisation area.

Disadvantages of Imaging Mass Spectrometry

There are, of course, a number of challenges to using imaging mass spectrometry in the analysis of biological samples. The first is the requirement for the detectors to be maintained at room temperature and in vacuum. This temperature requirement leads to the rapid degradation of some fragile samples [275] during the analysis. Apparatus with cryogenic sample mounts have been developed in order to reduce the effect of this requirement during analysis [69], but are not in widespread use. The sample preparation must be carried out as quickly as possible, but degradation will still be unavoidable during the time taken for sample preparation and, if used, the matrix deposition process.

Preparation of the sample for imaging may also be detrimental to the spatial resolution, in particular the washing and matrix deposition. Solvents that are used in this step may lift and transport analytes across the surface, blurring the resolution and distorting the results. In the case of matrix deposition, a trade-off is required between the droplet size and spatial resolution. The larger the droplets used, the slower the drying time and the better the signal as more analyte is incorporated into the crystals. However, the resolution is poorer as the droplet absorbs analytes from a larger area into itself (Fig. 7.3). Additionally, the crystals of matrix, typically larger

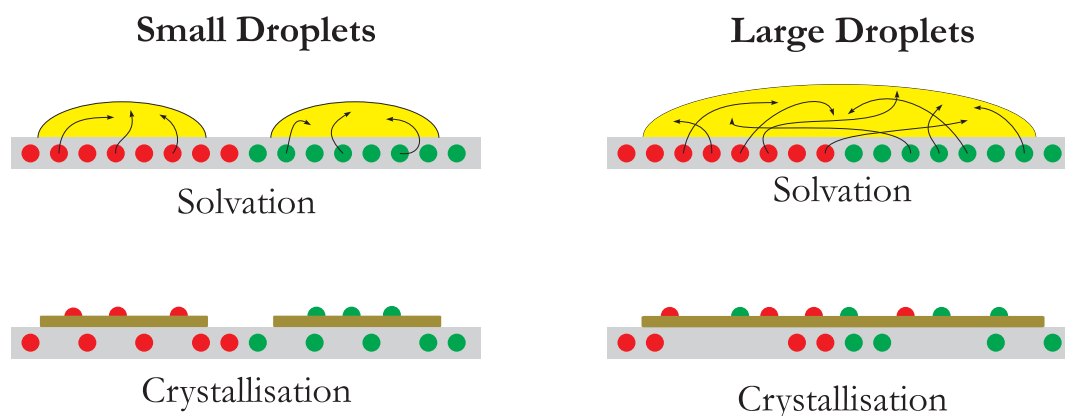


Figure 7.3: Analyte incorporation into matrix crystals. Matrix solution (yellow) on the surface of the sample solvates the analyte (red, green), which is mixed with the matrix within the droplet. Upon crystallisation, the matrix crystals (brown) incorporate the analyte. The larger the droplet, the more analyte is solvated and the better the signal but the lower the spatial resolution.

than $5\text{ }\mu\text{m}$ [276] will limit the spatial resolution in these cases. The use of SIMS circumvents this problem as it does not require a matrix covering, but produces highly-fragmented ions, limiting its use to ions of $< 1000\text{ Da}$ [60].

Another problem arising from the use of MALDI is the presence of the matrix within the mass spectra. In addition to what would be otherwise easily identifiable parent ion (or $[\text{M} + \text{H}]^+ / [\text{M} - \text{H}]^-$) the matrix tends to fragment or form clusters, leading to a large amount of signal at low masses which would saturate the detector. Thus, most mass spectrometers use either blanking or pulsing techniques to remove these early masses, potentially losing information in this mass range. Another problem with MALDI is the ion suppression effect [175], described in Sec. 5.1.2. Although this effect may be used to reduce the matrix signal seen in the mass spectrum, it may also suppress other useful analyte signal [277].

To summarise, optical imaging methods in the life sciences are undeniably very powerful tools. Differing methods can provide very good spatial resolution ($< 20\text{ nm}$), high speed analysis, non-destructiveness, and ease of use. A distinct advantage over mass spectrometric imaging is the ability (for some methods) to observe specimens

in vivo. However, no one technique in optical imaging is capable of providing all of these advantages, and the method most suitable for the immediate must be chosen. Similarly, imaging mass spectrometry has a variety of methods with differing advantages and disadvantages, providing an additional tool for the analysis of a biological sample. Depending on the desired information and applications, imaging mass spectrometry may be the optimum technique for analysis.

The majority of imaging mass spectrometry in the life sciences is performed with microprobe-mode imaging, due to its favourable mass and spatial resolution. However, microscope-mode imaging with fast detection systems can provide a much shorter analysis time when a large field of view is required. In particular, whole organ sections may be imaged with the wide field-of-view of the apparatus described in this work. In these cases, the spatial resolution may not be of such importance, as one may be more interested in the localisation of analytes within a certain region, not at sub-cellular levels. In short, wide-field microscope-mode imaging mass spectrometry provides the fastest method of performing a complete analysis of a large sample, providing a reasonable spatial and mass resolution while minimising sample damage. The remainder of this chapter will present results obtained from mouse brain sections in order to demonstrate the feasibility of technique. The images have been acquired using this wide-field microscope-mode mass spectrometry with both conventional and fast detectors.

7.2 Sample Preparation

As described in the previous section, the preparation of a sample is an extremely important factor in the imaging of biological materials. The samples used in this chapter were kindly provided by the Sibson Group of the Gray Institute for Radiation Oncology & Biology, Oxford, and Dr. Daniel Antoni of Oxford Pharmacology. Detailed protocols for sample preparation have been developed in order to minimise sample contamination, damage and degradation. Three procedures were used to apply matrix to the brain sections in preparation for imaging. The matrix was applied

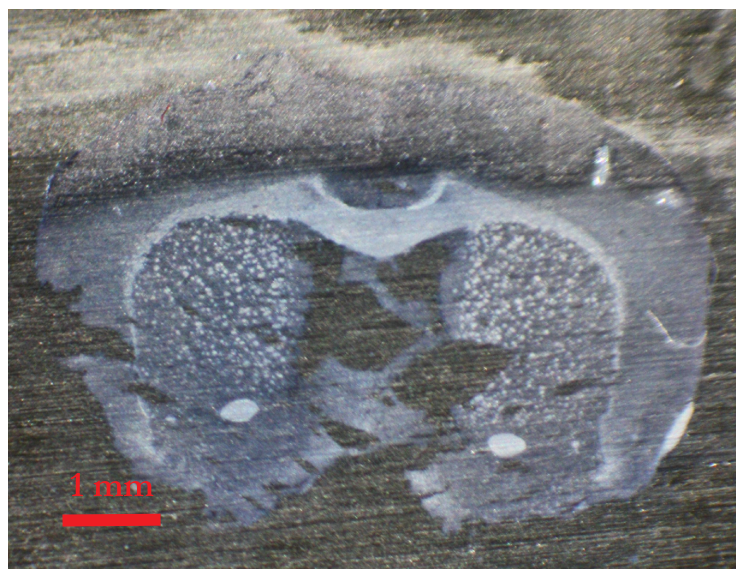


Figure 7.4: Optical imaging of a coronal mouse brain section thaw-mounted onto a stainless steel sample plate. The sample has been stained with trypan blue to improve visibility. The stain shows the regions of higher cell density in darker blue.

by airbrush, with the electrosprayer described in Sec. 5.2, or by direct staining.

In the first preparation method, the extracted brain was embedded in optimal cutting temperature (OCT) polymer. This was frozen on dry ice and a cryostatic microtome used to produce coronal sections of $10\text{ }\mu\text{m}$ thickness. These sections were then thaw mounted onto stainless steel plates like those used in the MALDI samples (Sec. 5.2). These sections were stored at $-20\text{ }^{\circ}\text{C}$ until required. When the samples were to be imaged, CHCA was applied with an airbrush. The solution of CHCA was 15 mg/ml in a $20 : 80$ mixture of 0.1% TFA and methanol. This was air-dried (without the use of a hotplate) and mounted in the apparatus for imaging. A photograph of a mounted coronal section is shown in Fig. 7.4. The section has been stained with trypan blue to improve visibility of the tissue structures. The stain is absorbed by cells with broken membranes [278]. In the case of a brain section, the cutting of the tissue breaks these cell membranes, and so trypan blue is absorbed in all the cells present. Hence, the areas of high cell density are darker blue, while the white matter, with low cell density, is visible as the white regions in the figure.

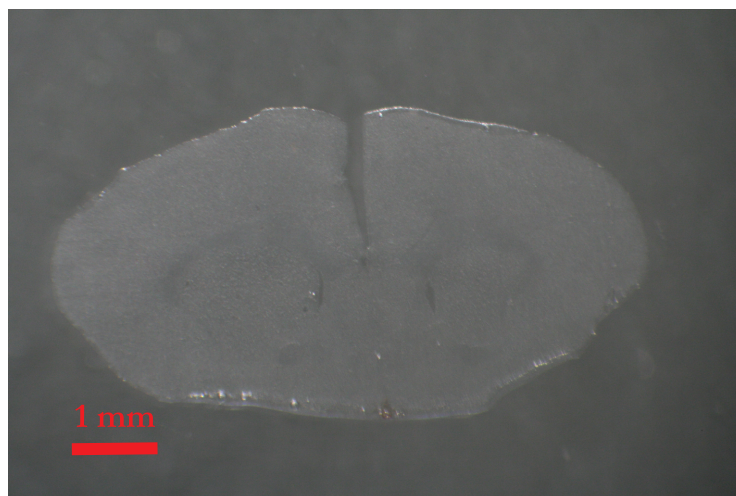


Figure 7.5: Optical image of the prepared mouse brain section without OCT and mounted on an ITO plate.

An improved preparation method was used in conjunction with the electrosprayer, in order to minimise the possibility of contamination and analyte diffusion over the sample. The extracted brain was immediately frozen in liquid isopentane, then cooled on dry ice. This avoided the use of OCT polymer, which has been reported to selectively suppress the signal of certain mass peaks [217]. The frozen sample is again sectioned to $10\ \mu\text{m}$ thickness in a cryotome and thaw-mounted. Indium tin oxide (ITO) coated glass plates were used as a mount in order to provide a clear surface on which to place the sample. This allowed the improved centring of the sample in the field of view. The conductive surface of the ITO was required in order to facilitate the ionisation mechanism of MALDI (see Sec. 5.1.2). The sectioned samples were stored in sealed petri dishes at -80°C until required. When they were to be imaged, the ITO plates were placed in the electrospraying device to coat in matrix. The CHCA solution used was $13.6\ \text{mg/ml}$, and was applied at $35\ \text{nl/hr}$ for 15 min. After this point, the entire sample was coated with an even layer of fine matrix crystals. The ITO plate could then be mounted on a stainless steel plate (see Sec. 5.2). A photograph of the prepared sample is shown in Fig. 7.5.

In order to observe additional masses, staining methods common in the life sciences were also used. The application of crystal violet (mass $407.2\ \text{Da}$), added

a mass peak which could be observed in conjunction with the matrix and tissue analytes. The irradiation of crystal violet leads to the detachment of Cl^- , giving a positive ion of mass 372.2 Da [38]. Imaging of multiple masses could be performed with this staining. A solution of 0.29 mg/ml crystal violet and 0.48 mg/ml CHCA was made up in 48:52 Ethanol:Water. The solution was dropped directly onto the ITO plate such that the entire sample was covered. This was left for 5 minutes, following which the solution was washed off with water. The sample was washed until the water no longer removed crystal violet from the sample. The sample was then dried under a flow of nitrogen, then mounted onto the sample mount with the recession for ITO plates.

7.3 Results and Discussion

Ion images acquired from mouse brain tissue samples are presented in this section, and compared with optical images to confirm their spatial imaging quality. Results from each of the sample preparation methods described are shown and the quality of images compared. Finally, the fast detector PImMS has been used to acquire images of different mass peaks within each experimental cycle.

The average laser power per pulse was in the range of 25 mJ to 34 mJ. Repeller/extractor potentials of 7025V/6729V were used. These potentials were sufficient to produce converged and identifiable spatial images. With the biological samples, it was found that higher potentials resulted in occasional voltage breakdown, perhaps due to out-gassing from the sample. Achieving a lower pressure allowed the use of the full 10 kV accelerating potential, but required the apparatus to be pumped for in excess of twelve hours, while only one hour was required to reach pressures sufficient for 7 kV.

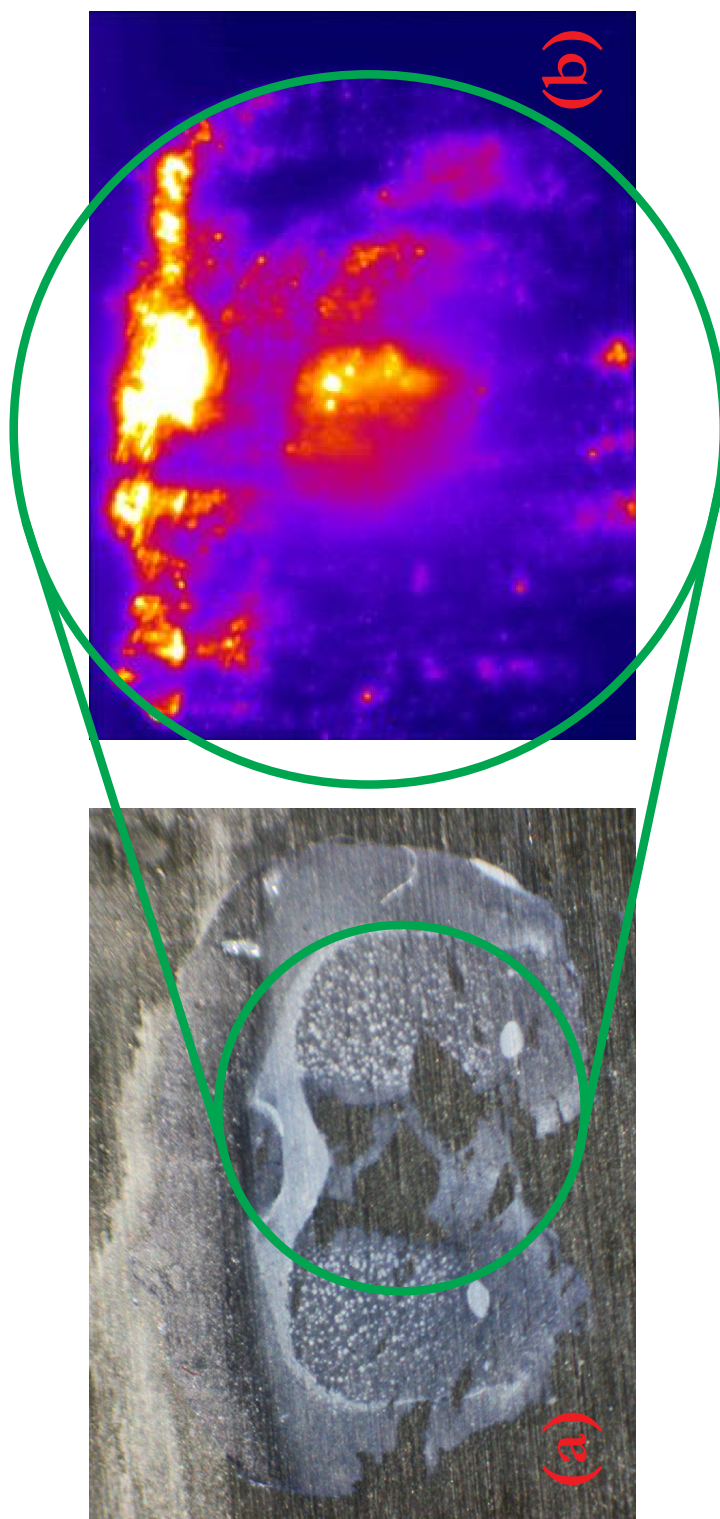


Figure 7.6: (a) Optical and (b) Ion image of brain section using the airbrush matrix preparation method. The optical image (a) is reproduced from Fig. 7.4 for ease of comparison. The field of view of the ion image is circled in green, together with the corresponding area of the optical image. The spatial distribution of the white matter in the optical image corresponds closely with that of the ion image.

7.3.1 Airbrush Matrix Deposition

An ion image of a sample prepared using the airbrush matrix application method is shown in Fig. 7.6. The image was acquired using the conventional CCD camera over 1000 shots. The optical image (a) is reproduced from Fig. 7.4 for ease of comparison. The area of the detector in the ion image (b) is indicated with a green circle. This corresponds to the field of view on the sample also circled in green in the optical image. It can be seen that the ion distribution observed closely corresponds to the white matter of the brain seen in the optical image. The arc of white is obvious on the top of the ion image. A point to note is the graininess of the image. A possible cause of this is variable sizes of the matrix crystals on the sample: the airbrush application may have resulted in different droplet sizes. The larger droplets would have adsorbed more analyte and upon irradiation produce larger amounts of signal, leading to the brighter and uneven areas within the structure. However, some of this irregularity may not be an effect of the sample preparation: it can be seen from the optical image that there are areas in which the white matter is distributed in spots on the sample.

The ions were acquired at a mass of approximately 800 Da. However, in the acquired mass spectrum, only a very broad band was observed apart from the matrix peaks. This indicates that there are a large number of peaks too closely-spaced to be resolved by the mass resolution available to the measurement technique. It is clear from the inability to distinguish these mass peaks that a greater mass resolution is required for a useful analysis of the sample. This should be achieved by the post-acceleration techniques described in Sec. 5.4, which would greatly improve the mass resolution to the order of $m/\Delta m = 2500$. Additionally, the lack of distinct peaks may be an effect of degradation during the sample preparation or storage. Hence, the improved preparation process described in Sec. 7.2 was developed, using the sprayer and colder storage.

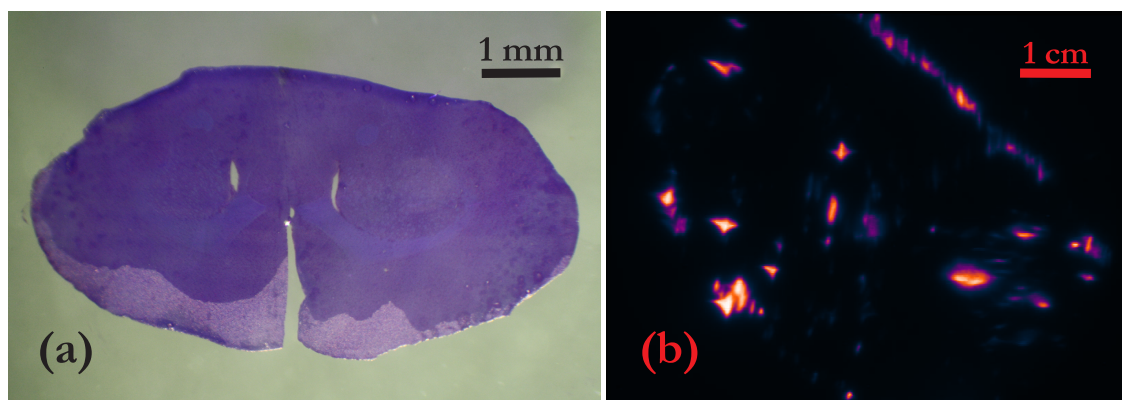


Figure 7.7: Images acquired of mouse brain prepared without OCT polymer. (a) shows an optical image of an adjacent section, stained for clarity. (b) shows the ion image of the sample acquired at the mass of ~ 800 Da, as in Fig. 7.6. The “ ω ” shape of the white matter can again be clearly observed in both images.

7.3.2 Electrospray Matrix Deposition

Images of the brain section prepared avoiding the use of OCT polymer are shown in Fig. 7.7. Fig. 7.7(a) shows an optical image of an adjacent section to that used for ion imaging analysis. This has been stained with crystal violet to improve optical visibility. Fig. 7.7(b) shows the ion image acquired from the prepared sample. The image has been acquired at the same mass region as that in Fig. 7.6. This image was also acquired with the conventional CCD camera.

An improvement in the quality of the image from Fig. 7.6(b) may be seen. The “ ω ” shape in the spatial distribution of the ion image (b) corresponding to the white matter in the brain can now be clearly identified. However, the effects of the lower sensitivity described by Altelaar *et al.* [216] for samples with matrix applied by electrospray can be seen in this image. The image shows the effect of lower amounts of signal: the image is not as well converged.

7.3.3 Direct Staining and Matrix Application

Staining is a commonly used technique in biological optical imaging, as described in Sec. 7.1.1. In order to introduce additional peaks with differing spatial distributions,

a stain commonly used in the life sciences was added to the tissue sections. Crystal violet, with a molecular mass of 407.98 Da, is commonly used in Gram staining [279]. The Gram staining is commonly used to differentiate between bacteria types by the selective absorption through the cell membranes. When applied to tissue samples, crystal violet is absorbed by the cells nuclei [280, 281]. Hence, areas with higher cell densities are of a darker colour. Crystal violet was used together with methylene blue by Hazama *et al.* [38] to image the hippocampus of a mouse brain using microscope-mode mass spectrometry. However, the field of view of the apparatus was much smaller than that of the apparatus used here, requiring the stitching of 78 images to image a region of $3.33 \text{ mm} \times 1.53 \text{ mm}$.

Images of a sample prepared by the direct staining method described in Sec. 7.2 are shown in Fig. 7.8. (a) shows an optical image of the mouse brain section after staining with the matrix and crystal violet solution. Regions of a higher cell density absorb more crystal violet, and appear darker on the sample. The differences of dye absorption in different regions is clear on the sample. Additionally, note the higher concentrations of stain at the edges of the sample. This may be due to the region of the sample being particularly susceptible to staining, or a build-up of matrix/stain at the edge of the sample during crystallisation.

Fig. 7.8(b) shows an ion image acquired from the sample in (a). The image was acquired using the conventional CCD camera, with 5000 laser shots, at the mass of $\sim 370 \text{ Da}$, corresponding to that of crystal violet. The shape of the sample is extremely clear, with the structure within the section visible. The high concentration of crystal violet visible at the edges of the sample in (a) can be seen as the outline of the brain in (b). The centroiding of high intensity signal in this area has created the dark areas artefact seen in Sec. 5.3.3. This is indicated by the green arrow in the figure. It can be seen that areas corresponding to the white matter show lower amounts of signal in this image. During the preparation of the sample, after the matrix and crystal violet solution has been applied, the sample was washed with water. This removes any excess matrix and stain. This means that, unlike in the

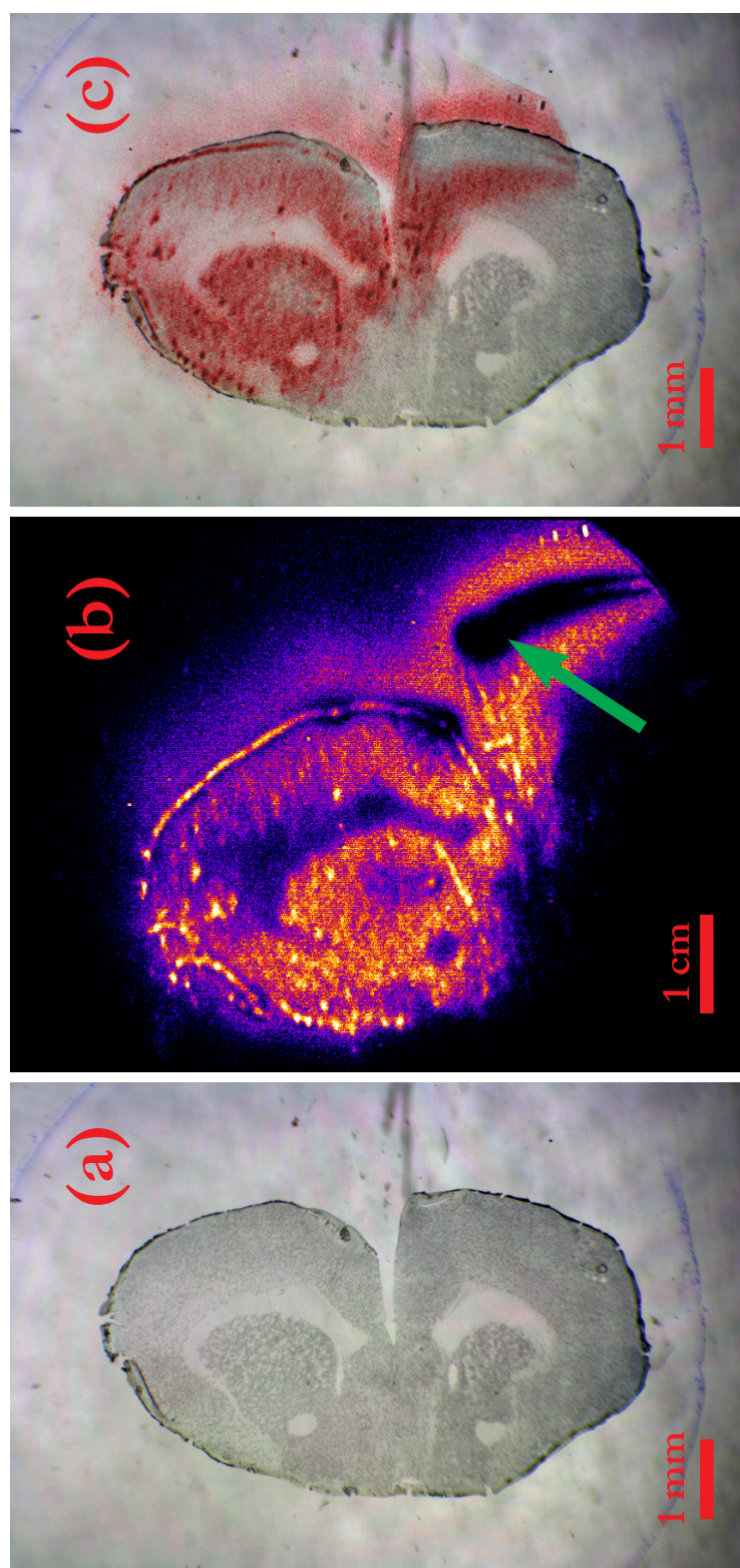


Figure 7.8: Images of mouse brain sections with matrix and crystal violet applied by direct staining method. (a) shows an optical image of the section prepared. Note the higher concentrations of matrix/crystal violet at the edges. (b) shows an ion image acquired of the same sample at mass ~ 372 Da. The structure of the brain is extremely clear and corresponds closely to that of the optical image. The green arrow indicates a dark region artefact from centroiding. (c) shows an overlay of the ion image on the optical image. The origins of the high intensity spots may be identified as darker regions of crystal violet. The distortion of the lens on the CCD camera results in a slightly stretched ion image. The image was acquired with event-counting over 5000 laser shots.

airbrush and electro-spray methods, there is no matrix left on the surface of the sample. Any matrix and crystal violet present in the sample will be that which had been absorbed into the cells. Judging from the colour of the sample in that area, there is a low concentration of the solution in the white matter. Therefore, in contrast to Figs. 7.6 and 7.7, these regions now show up as dark areas on the ion images, as matrix is not present on the surface to desorb the masses seen in the previous ion images. Finally, it can be seen that once again the laser profile limits the areas of the image which are visible with the apparatus, with the region of the brain on the bottom right of the image not illuminated.

Fig. 7.8(c) shows the overlay of the ion image on the optical image, as an additional aid to the comparison of distributions. The ion image is shown in red. The two images can be seen to fit together closely, and the dark regions on the ion image correspond to the white parts of the optical image. The distortion of the CCD camera lens can be seen, with the ion image slightly stretched in comparison to the optical image. The origin of the higher intensity spots seen along the outline of the brain can each be identified as one of the darker spots corresponding to higher concentrations of crystal violet. It can thus be concluded that the ion image acquired of the tissue sample is an accurate representation of the concentrations of the analyte.

7.3.4 Imaging with PImMS

Finally, in order to demonstrate the application of fast pixel imaging to multi-mass microscope-mode imaging mass spectrometry, the same sample as in Sec. 7.3.3 has been imaged using the PImMS detector. As the sample has been treated with CHCA and crystal violet, multiple mass peaks corresponding to these should be expected, in addition to those originating from the sample itself. Thus, the application of this fast detector should produce separate mass-dependent images from the mouse brain section for every experimental cycle.

Time-of-flight spectra acquired for the mouse brain section prepared by direct staining with crystal violet and CHCA are shown in Fig. 7.9. The data was acquired

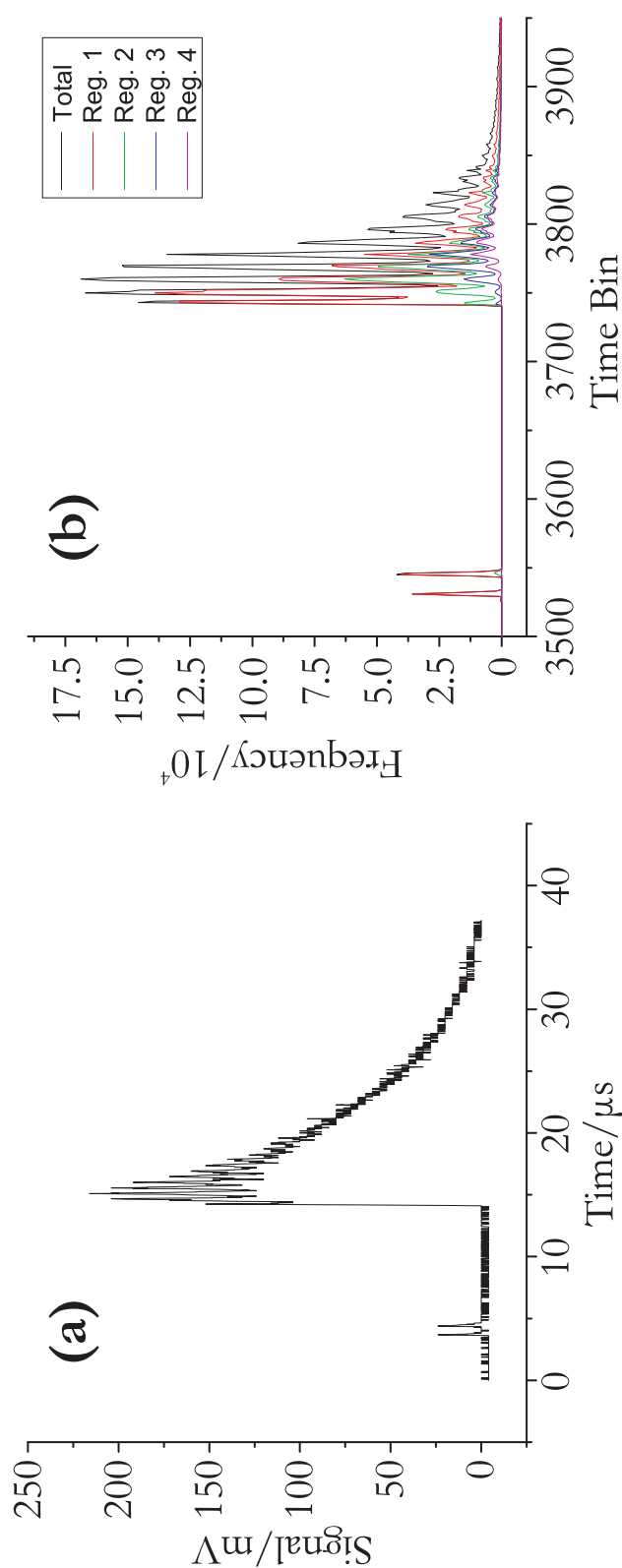


Figure 7.9: Time-of-flight spectra of mouse brain section stained with crystal violet in matrix. (a) shows a time-of-flight recorded using the PMT. The detector has been pulsed to reduce signal up to 14 μ s. The high intensity of the two matrix peaks allows them to appear at about 3 μ s and 4 μ s, even through the signal suppression. The long tail of desorbed molecules appears after signal suppression. (b) shows a reconstructed time-of-flight from acquired PImMS data. The x-axis is the time bin from triggering. The black curve shows the total signal, while the curves labelled Reg. 1 to Reg. 4 show the mass spectra reconstructed from memory registers one to four respectively.

over 5000 laser shots, with an average of ~ 1000 events per experimental cycle. As the matrix peaks produced a large amount of signal, potentially saturating or damaging the detector, the front face of the MCPs were initially held at 250 V. This reduced the gain of the first MCP, limiting the signal from the matrix peaks while still allowing some signal to be observed. The matrix peaks are evident at about $3\ \mu\text{s}$ and $4\ \mu\text{s}$ in Fig. 7.9(a). At just before $14\ \mu\text{s}$, well away from both the matrix and crystal violet mass peaks, the front face of the MCP was brought down to 0 V, allowing full normal operation of the MCPs. This point may be seen in Fig. 7.9(a) by the sharp increase in signal followed by a decaying tail. This is the signal from the large number of unresolvable desorbed masses.

Fig. 7.9(b) shows the time-of-flight spectra reconstructed from the PImMS data. The time-of-flight is plotted with time bins of the PImMS detector. Each of these bins is 50 ns, and the value on the x -axis is the number of bins from the triggering time of the experiment. The spectra reconstructed from each memory register of the pixels has been plotted separately. For example, the frequency of hits at each time bin in the first register has been plotted in the red curve. The curves for registers one to four are labelled Reg. 1 to Reg. 4 respectively. The sum of these curves is plotted in the black curve. The total curve is in good agreement with that obtained from the PMT. From the population of the registers, it is possible to determine if the four registers available per pixel are sufficient to record all the events from the multiple masses that are arriving per experimental cycle. It can be seen that on the time-of-flight spectra, the population at each time is always greater in the first register than the second, the second more than the third, and so forth. This suggests that the registers in each pixel are never being saturated: if this were the case, there would be a higher population of later registers at high masses. Thus, it can be concluded that four registers are sufficient under these operating conditions to image the large number of masses present here. The duration of the broad peaks observed on the reconstructed PImMS TOF spectrum (b) may be seen to be in the region of about 100 time bins, corresponding to a time of $5\ \mu\text{s}$. The arrival of

this about 200 time bins ($10\ \mu\text{s}$) after the matrix peaks shows that the PImMS is detecting only the top of the broad peak, a result of the higher threshold or lower sensitivity predicted in Sec. 6.3. The time-of-flight spectra are in good agreement on the oscillations in this region.

Fig. 7.10 shows the images reconstructed from the data acquired by PImMS. From the $x/y/t$ data volume, data within values of t corresponding to the mass peaks seen in Fig. 7.9(b) was extracted. The x/y position of the pixels for each of these events was plotted in two dimensions, and giving the total images shown in Fig. 7.10. Each of these images corresponds to a different time window. (a) shows the image from time bins 3529-3534, (b) from 3542-3550, and (c) from 3739-3842. The time bins in (a) and (b) correspond to those of the matrix and fragment, while (c) is signal from the broad mass peaks. The smaller field of view due to the lens focal distance described in Sec. 6.3 is again evident in the images.

It can be seen that the spatial distributions for each of these mass peaks are significantly different from each other. The image of the first mass peak, (a), has a large proportion of intensity around the edges of the sample. This may be rationalised by the large concentrations of the staining solution observed at the edges of the sample (Fig. 7.8(a)). The matrix fragments are also observed from the rest of the sample, although the image is slightly blurred. This may be due to charge repulsion effects from high densities of ions, or high recoil velocities from matrix fragmentation. In contrast, the image of the matrix parent ion (b) is sharper but has much less signal, with almost all of it concentrated at the edges. This suggests that unfragmented matrix is mainly desorbed from the high-concentration region at the edges. This may be due to high concentrations of matrix being required to allow the soft desorption mechanisms normally observed in MALDI. A possible cause of the improvement in sharpness may be the lack of fragmentation, limiting the initial recoil velocity.

Finally, the image of the broad peak in (c) shows an even distribution of signal over the stained region, without the high intensity at the edges. This indicates that

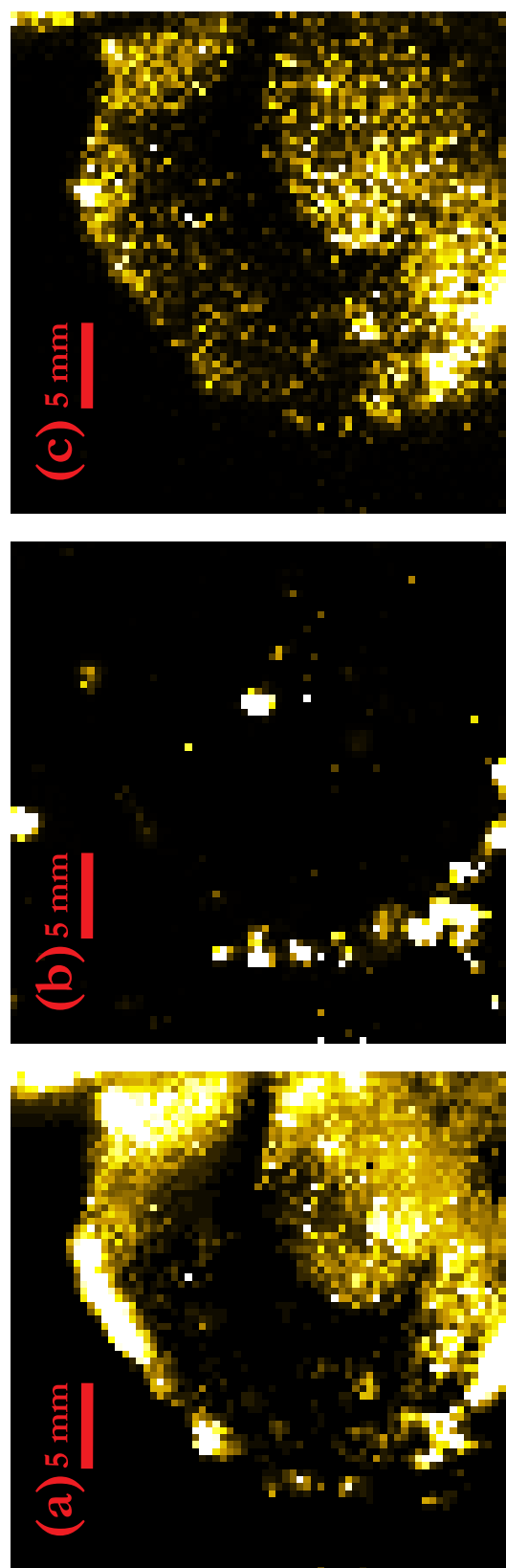


Figure 7.10: Images acquired with PImMS. Each image has been reconstructed by plotting the x/y positions of each pixel within a window of time bins. Image (a) is from the signal between time bins 3529-3534, corresponding to the first matrix peak. (b) is the data in bins 3542-3550, the second matrix peak. (c) is from 3739-3842, from the signal in the broad peak. The spatial distributions of each image can be seen to be different.

it is not the crystal violet masses being observed here, as the higher concentration spots in Fig. 7.8(a) are not visible. It is perhaps unsurprising that the crystal violet is not observed in this image: it was found that although crystal violet was easily ionised and gave a good amount of observable signal, this signal was only present for relatively small number of laser shots, and faded after about 10,000 experimental cycles. As this sample had already been imaged for the conventional CCD analysis in Sec. 7.3.3, the supply of crystal violet on the sample may have already been exhausted. The masses observed in Fig. 7.10(c) must then originate from the sample itself, similar to in Sec. 7.3.1 and Sec. 7.3.2. Again, higher mass resolution would be required to accurately characterise the mass fragments.

7.4 Summary and Future Direction

In this chapter, the imaging of tissue samples, namely of mouse brain sections, has been presented. Samples were prepared with a variety of methods, with differing qualities of images acquired. Acquisition of spatial distributions of different masses desorbed from these samples for the matrix, crystal violet, and ions from the brain sample was demonstrated. However, the masses from the brain sample would require a higher mass resolution in order to unambiguously identify the analytes. The PImMS detector has been used to acquire multiple mass-dependent spatial distributions from the tissue samples during each experimental cycle. This demonstrates the applicability of fast detection systems to microscope-mode spatial-mapped ion imaging. The number of registers in PImMS has also been demonstrated to be sufficient for collecting the large numbers of masses observed under the experimental conditions used. Finally, the images were collected over, at most, 5000 shots with a 10 Hz experimental cycle. This corresponds to a time of slightly less than $8\frac{1}{2}$ minutes, a good speed for images acquired with this field-of-view.

Again, the low sensitivity of samples prepared by electrospray application of the matrix may be improved by the sputter-coating of the sample with a metal such as gold. This technique has been successfully employed by Altelaar *et al.* [223] and

Hazama *et al.* [38] on biological samples. The narrower field of view for the fast detector could be improved by the use of a lens with a longer focus length. Experiments using such a lens with a larger aperture, resulting in improved sensitivity, are currently underway [85, 86]. Other areas of improvement have been discussed in previous chapters. The mass resolution may be improved with modifications to the ion optics and direct in-vacuum electron detection after the MCPs; the pixel resolution of the fast detectors will be greatly improved in future versions of the PImMS detectors. The laser beam profile may be improved with a beam diffuser, allowing even irradiation of the sample. These improvements will make the microscope-mode imaging mass spectrometry with fast two-dimensional detectors an extremely useful method in the life sciences.

Bibliography

- [1] R. Castaing and G. Slodzian, *Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences*, **255**, 1893 (1962).
- [2] R. M. Caprioli, T. B. Farmer, and J. Gile, *Anal. Chem.*, **69**, 4751 (1997).
- [3] C. A. Evans and R. J. Blattner, *Ann. Rev. Mat. Sci.*, **8**, 181 (1978).
- [4] S. S. Rubakhin and J. V. Sweedler In *Mass Spectrometry Imaging: Principles and Protocols*, S. S. Rubakhin and J. V. Sweedler, Eds., Humana Press, 2010.
- [5] R. W. Odom, *App. Spec. Rev.*, **29**, 67 (1994).
- [6] P. Williams, *App. Surf. Sci.*, **13**, 241 (1982).
- [7] D. Willingham, A. Kucher, and N. Winograd, *App. Surf. Sci.*, **255**(4), 831 (2008).
- [8] P. Malmberg, H. Nygren, K. Richter, Y. Chen, F. Dangardt, P. Friberg, and Y. Magnusson, *Microscopy Research and Technique*, **70**, 828 (2007).
- [9] I. Lanekoff, P. Sjobvall, and A. G. Ewing, *Anal. Chem.*, **83**, 5337 (2011).
- [10] D. Touboul, S. Roy, D. P. Germain, P. Chaminade, A. Brunelle, and O. Laprevote, *Int. J. Mass Spec*, **260**, 158 (2007).
- [11] C. W. Magee, W. L. Harrington, and R. E. Honig, *Rev. Sci. Inst.*, **49**, 477 (1978).
- [12] J. Maul and K. Wittmaack, *Surface Science*, **47**, 358 (1975).
- [13] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, *Science*, **246**, 64 (1989).
- [14] Z. Takats, J. M. Wiseman, B. Gologan, and R. G. Cooks, *Science*, **306**, 471 (2004).
- [15] G. J. van Berkel, V. Kertesz, K. A. Koeplinger, M. Vavrek, and A.-N. T. Kong, *J. Mass Spec.*, **43**, 500 (2008).

- [16] J. S. Becker, *Inorganic Mass Spectrometry: Principles and Applications*, John Wiley & Sons, 2007.
- [17] M. A. Posthumus, P. G. Kistemaker, H. L. C. Meuzelaar, and M. C. Ten Noever de Brauw, *Anal. Chem.*, **50**, 985 (1978).
- [18] A. Górecka-Drzazga, S. Bargiel, R. Walczak, J. A. Dziuban, A. Kraj, T. Dylag, and J. Silberring, *Sensors and Actuators B*, **103**, 206 (2004).
- [19] J. Wei, J. M. Buriak, and G. Siuzdak, *Nature*, **399**, 243 (1999).
- [20] K. Shibamoto, K. Sakata, K. Nagoshi, and T. Korenaga, *J. Phys. Chem.*, **113**, 17774 (2009).
- [21] E. P. Go, J. V. Apon, G. Luo, A. Saghatelian, R. H. Daniels, V. Sahi, R. Dubrow, B. F. Cravatt, A. Vertes, and G. Siuzdak, *Anal. Chem.*, **77**, 1641 (2005).
- [22] G. Luo, Y. Chen, H. Daniels, R. Dubrow, and A. Vertes, *J. Phys. Chem.*, **110**, 13381 (2006).
- [23] Y. Coffinier, S. Janel, A. Addad, R. Blossey, L. Gengembre, E. Payen, and R. Boukherroub, *Langmuir*, **23**, 1608 (2007).
- [24] Thomas W. Burgoyne and Gary M. Hieftje, *Mass Spec. Rev.*, **15**, 241 (1996).
- [25] W. Paul and H. Steinwedel, *Zeitschrift Fur Naturforschung Section A-A Journal of Physical Sciences*, **8**, 448 (1953).
- [26] A. Makarov, *Anal. Chem.*, **72**, 1156 (2000).
- [27] Q. Hu, R. J. Noll, H. Li, A. Makarov, M. Hardman, and R. Graham Cooks, *J. of Mass Spec.*, **40**, 430–443 (2005).
- [28] A. G. Marshall, C. L. Hendrickson, and G. S. Jackson, *Mass Spec. Rev.*, **17**, 1 (1998).
- [29] P. Arpino, *Mass Spec. Rev.*, **8**, 35 (1989).
- [30] P. Arpino, *Mass Spec. Rev.*, **9**, 631 (1990).
- [31] P. Arpino, *Mass Spec. Rev.*, **11**, 3 (1992).
- [32] W. C. Wiley and I. H. McLaren, *Rev. Sci. Inst.*, **26**, 1150 (1955).
- [33] B. A. Mamyrin, V. I. Karataev, D. V. Shmikk, and V. A. Zagulin, *Zhurnal Eksperimentalnoi i Teoreticheskoi Fiziki*, **64**, 82 (1973).
- [34] B. D. Leskiw, M. H. Kim, G. E. Hall, and A. G. Suits, *Rev. Sci. Inst.*, **76** (2005).

- [35] B. Schueler, P. Sander, and D. A. Reed, *Vacuum*, **41**, 1661 (1990).
- [36] M. Toyoda, M. Ishihara, S. Yamaguchi, H. Ito, T. Matsuo, R. Roll, and H. Rosenbauer, *J. Mass Spec.*, **35**, 163 (2000).
- [37] M. Toyoda, *European Journal of Mass Spec.*, **16**, 397 (2010).
- [38] H. Hazama, H. Yoshimura, J. Aoki, H. Nagao, M. Toyoda, K. Masuda, K. Fujii, T. Tashima, Y. Naito, and K. Awazu, *J. Bio. Optics*, **16**, 046007 (2011).
- [39] J. L. Wiza, *Nuc. Inst. and Methods*, **162**, 587 (1979).
- [40] P. B. O'Connor and F. Hillenkamp In *MALDI MS: A Practical Guide to Instrumentation, Methods and Applications*, F. Hillenkamp and J. Peter-Ktalinić, Eds., Wiley-VCH, 2007.
- [41] J. H. Jungmann, L. MacAleese, R. Buijs, F. Giskes, A. de Snaijer, J. Visser, J. Visschers, M. J. J. Vrakking, and R. M. A. Heeren, *J. Am. Soc. Mass Spectrom.*, **21**, 2023 (2010).
- [42] B. Spengler In *MALDI MS: A Practical Guide to Instrumentation, Methods and Applications*, F. Hillenkamp and J. Peter-Ktalinić, Eds., Wiley-VCH, 2007.
- [43] C. J. Davisson and C. J. Calbick, *Phys. Rev.*, **38**, 585 (1931).
- [44] R. Castaing, B. Jouffrey, and G. Slodzian, *Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences*, **251**, 1010 (1960).
- [45] G. Slodzian, *Journal de Microscopie et de Spectroscopie Electroniques*, **3**, 447 (1978).
- [46] D. W. Chandler and P. L. Houston, *J. Chem. Phys.*, **87**, 1445 (1987).
- [47] A. T. J. B. Eppink and D. H. Parker, *Rev. Sci. Inst.*, **68**, 3477 (1997).
- [48] A. J. R. Heck and D. W. Chandler, *Annu. Rev. Phys. Chem.*, **46**, 335 (1995).
- [49] M. N. R. Ashfold and J. D. Howe, *Annu. Rev. Phys. Chem.*, **45**, 57 (1994).
- [50] T. Suzuki and B. J. Whitaker, *Int. Rev. Phys. Chem.*, **20**, 313 (2001).
- [51] V. J. Allan, Ed., *Protein Localization by Fluorescence Microscopy: A Practical Approach*, Oxford University Press, 2000.
- [52] S. L. Luxembourg, T. H. Mize, L. A. McDonnell, and R. M. A. Heeren, *Anal. Chem.*, **76**, 5339 (2004).
- [53] J. C. Jurchen, S. S. Rubakhin, and J. V. Sweedler, *J. Am. Soc. Mass Spectrom.*, **16**, 1654 (2005).

- [54] Y. Li, B. Shrestha, and A. Vertes, *Anal. Chem.*, **79**, 523 (2007).
- [55] S. Khatib-Shahidi, M. Andersson, J. L. Herman, T. A. Gillespie, and R. M. Caprioli, *Anal. Chem.*, **78**, 6448 (2006).
- [56] K. Chughtai and R. Heeren, *Chem. Rev.*, **110**, 3237 (2010).
- [57] I. Klinkert, L. A. McDonnell, S. L. Luxembourg, A. F. M. Altelaar, E. R. Amstalden, S. R. Piersma, and R. M. A. Heeren, *Rev. Sci. Instr.*, **78**, 053716 (2007).
- [58] S. L. Luxembourg, L. A. McDonnell, T. H. Mize, and R. M. A. Heeren, *J. Proteome Res.*, **4**, 671 (2005).
- [59] <http://www.phil.com/surface-analysis-products/nanoTOF/overview.html>.
- [60] L. A. McDonnell and R. M. A. Heeren, *Mass Spec. Rev.*, **26**, 606 (2007).
- [61] B. Spengler and M. Hubert, *J. Am. Soc. Mass Spectrom.*, **13**, 735 (2002).
- [62] M. Stoeckli, P. Chaurand, D. E. Hallahan, and R. M. Caprioli, *Nat. Med.*, **7**, 493 (2001).
- [63] I. Fournier, M. Wisztorski, and M. Salzert, *Expert Rev. Proteomics*, **5**, 413 (2008).
- [64] M. Wisztorski, R. Lemaire, J. Stauber, S. A. Menguelet, D. Croix, O. J. Mathe, R. Day, M. Salzert, and I. Fournier, *Current Pharmaceutical Design*, **13**, 3317 (2007).
- [65] M. R. Groseclose, M. Andersson, W. M. Hardesty, and R. M. Caprioli, *J. Mass Spectrom.*, **42**, 254 (2006).
- [66] T. Gemoll, U. J. Roblick, and J. K. Habermann, *Molec. Med. Reports*, **4**, 1045 (2011).
- [67] M. Stoeckli, D. Staab, and A. Schweitzer, *Int. J. Mass Spec.*, **260**, 195 (2007).
- [68] Y. Sugiura and M. Setou, *J. Immune Pharmacol.*, **5**, 31 (2010).
- [69] R. M. Braun, P. Blenkinsopp, S. J. Mullock, C. Corlett, K. F. Willey, J. C. Vickerman, and N. Winograd, *Rap. Comm. Mass Spec.*, **12**, 1246 (1998).
- [70] J. S. Fletcher, N. P. Lockyer, S. Vaidyanathan, and J. C. Vickerman, *Anal. Chem.*, **79**, 2199 (2007).
- [71] A. Römpf, S. Guenther, Z. Takats, and B. Spengler, *Anal. Bioanal. Chem.*, **401**, 65 (2011).

- [72] E. R. A. van Hove, D. F. Smith, and R. M. A. Heeren, *J. Chromatography A*, **1217**, 3946 (2010).
- [73] D. V. T. Son, T. G. Etoh, M. Tanaka, N. H. Dung, V. Le Cuong, K. Takehara, T. Akino, K. Nishi, H. Aoki, and J. Nakai, *Sensors*, **10**, 16 (2010).
- [74] G. T. Etoh, D. Poggemann, A. Ruckelshausen, A. Theuwissen, G. Kreider, H.-O. Folkerts, H. Mutoh, Y. Kondo, H. Maruno, K. Takubo, H. Soya, K. Takehara, T. Okinaka, Y. Takano, T. Reisinger, and C. Lohmann In *2002 IEEE International Solid-State Circuits Conference. Digest of Technical Papers*, number vol.1, page 46. IEEE Solid-State Circuits Soc.; IEEE San Francisco Sect.; Bay Area Council; Univ. PA.
- [75] T. G. Etoh, D. Poggemann, G. Kreider, H. Mutoh, A. J. P. Theuwissen, A. Ruckelshausen, Y. Kondo, H. Maruno, K. Takubo, H. Soya, K. Takehara, T. Okinaka, and Y. Takano, *IEEE Transactions on Electron Devices*, **50**, 144 (2003).
- [76] H. Ohtake, T. Hayashida, K. Kitamura, T. Arai, J. Yonai, K. Tanioka, H. Maruyama, T. Goji Etoh, D. Poggemann, A. Ruckelshausen, H. Van Kuijk, and Jan T. Bosiers In Cohen, MJ and Dereniak, EL, Ed., *Semiconductor Photodetectors III*, Vol. 6119 of *Proceedings of the Society of Photo-Optical Instrumentation Engineers (SPIE)*, page E1190, 2006.
- [77] M. Brouard, E. K. Campbell, A. J. Johnsen, C. Vallance, W. H. Yuen, and A. Nomerotski, *Rev. Sci. Inst.*, **79**, 123115 (2008).
- [78] H. Liebl, *Applied Charged Particle Optics*, Springer, 2008.
- [79] www.simion.com.
- [80] C. J. Davisson and C. J. Calbick, *Phys. Rev.*, **42**, 580 (1932).
- [81] E. Halford, Part II thesis, University of Oxford (2011).
- [82] R. C. Beavis and B. T. Chait, *Chem. Phys. Lett.*, **181**, 479 (1991).
- [83] W. Zhang and B.T. Chait, *Int. J. Mass Spec. and Ion Proc.*, **160**, 259 (1997).
- [84] E. Hecht, *Optics*, Addison Wesley, fourth ed., 2002.
- [85] B. Winter, Personal communication (2012).
- [86] E. Halford, Personal communication (2012).
- [87] J. Aoki, H. Hazama, and M. Toyoda, *J. Mass. Spec. Soc. Jpn.*, **59**, 57 (2011).
- [88] F. Quadrini PhD thesis, University of Oxford, (2007).
- [89] www.dalsa.com.

- [90] L. Lazovsky, D. Cismas, G. Allan, and D. Given, *Proc. SPIE*, **5787**, 184 (2005).
- [91] A. Nomerotski, M. Brouard, E. Campbell, A. Clark, J. Crooks, J. Fopma, J. J. John, A. J. Johnsen, C. Slater, R. Turchetta, C. Vallance, E. Wilman, and W. H. Yuen, *J. Inst.*, **5** (2010).
- [92] J.P. Crooks, J.A. Ballin, P.D. Dauncey, A.-M. Magnan, Y. Mikami, O. Miller, M. Noy, V. Rajovic, M. Stanitzki, K.D. Stefanov, R. Turchetta, M. Tyndel, E.G. Villani, N.K. Watson, and J.A. Wilson In *Nuclear Science Symposium Conference Record, 2007. NSS '07. IEEE*, Vol. 2, page 931, 2007.
- [93] J. W. L. Lee, Personal communication (2012).
- [94] C. S. Slater, Personal communication (2012).
- [95] X. Llopart, R. Ballabriga, M. Campbell, L. Tlustos, and W. Wong, *Nuc. Inst. and Meth. in Phys. Res.*, **581**, 485 (2007).
- [96] E. Wilman, Personal communication (2012).
- [97] B. Y. Chang, R. C. Hoetzlein, J. A. Mueller, J. D. Geiser, and P. L. Houston, *Rev. Sci. Inst.*, **69**, 1665 (1998).
- [98] <http://www.aspect-sys.com/>.
- [99] S. E. Bohndiek, C. D. Arvantis, G. J. Royle, R. D. Speller, A. T. Clark, J. Chooks, M. L. Prydderch, R. Turchetta, A. Blue, and V. O'Shea, *Opt. Eng.*, **46**, 124003 (2007).
- [100] L.D.A. Siebbeles, M. Glass-Maujean, O. Vasyutinskii, J.A. Beswick, and O. Roncero, *J. Chem. Phys.*, **100**, 3610 (1994).
- [101] A.S. Bracker, E.R. Wouters, A.G. Suits, and O.S. Vasyutinskii, *J. Chem. Phys.*, **110**, 6749 (1999).
- [102] G.G. Balint-Kurti, A.J. Orr-Ewing, J.A. Beswick, A. Brown, and O.S. Vasyutinskii, *J. Chem. Phys.*, **116**, 3610 (2002).
- [103] K. Blum, *Density Matrix Theory and Applications*, Plenum, New York, 2nd ed., 1996.
- [104] R.N. Zare, *Angular Momentum, Understanding Spatial Aspects in Chemistry and Physics*, John Wiley and Sons, New York, 1988.
- [105] M. Brouard, R. Cireasa, A.P. Clark, G.C. Groenenboom, G. Hancock, S.J. Horrocks, F. Quadrini, G.A.D. Ritchie, and C. Vallance, *J. Chem. Phys.*, **125**, 133308 (2006).

- [106] A.G. Smolin, O.S. Vasyutinskii, E.R. Wouters, and A.G. Suits, *J. Chem. Phys.*, **121**, 6759 (2004).
- [107] A.G. Smolin, O.S. Vasyutinskii, O.P.J. Vieuxmaire, M.N.R. Ashfold, G.G. Balint-Kurti, and A.J. Orr-Ewing, *J. Chem. Phys.*, **124**, 094305 (2006).
- [108] M. J. Bass, M. Brouard, A. P. Clark, and C. Vallance, *J. Chem. Phys.*, **117**, 8723 (2002).
- [109] J.W. Rabalais, J.M. McDonald, V. Scherr, and S.P. McGlynn, *Chem. Rev.*, **71**, 73 (1971).
- [110] R.J. Hemley, D.G. Leopold, J.L. Roebber, and V. Vaida, *J. Chem. Phys.*, **79**, 5219 (1983).
- [111] A. Douglas and I. Zanon, *Can. J. Phys.*, **42**, 627 (1963).
- [112] D.J. Donaldson, *J. Chem. Phys.*, **91**, 7455 (1989).
- [113] C. Cossart-Magos and M. Horani, *J. Chem. Phys.*, **104**, 7412 (1996).
- [114] I.M. Waller and J.W. Hepburn, *J. Chem. Phys.*, **87**, 3261 (1987).
- [115] S.C. Yang, A. Freedman, M. Kawasaki, and R. Bersohn, *J. Chem. Phys.*, **72**, 4058 (1980).
- [116] M.C. Addison, R.J. Donovan, and C. Fotakis, *Chem. Phys. Lett.*, **74**, 58 (1980).
- [117] D. Dornhofer, W. Hack, and W. Langel, *J. Phys. Chem.*, **88**, 3060 (1984).
- [118] V.R. McCrary, R. Lu, D. Zakheim, J.A. Russell, J.B. Halpern, and W.M. Jackson, *J. Chem. Phys.*, **83**, 3481 (1985).
- [119] G. Black and L.E. Jusinski, *Chem. Phys. Lett.*, **124**, 90 (1986).
- [120] H. Kanamori and E. Hirota, *J. Chem. Phys.*, **86**, 3901 (1987).
- [121] W.S. McGivern, O. Sorkhabi, A.H. Rizvi, and A.G. Suits, *J. Chem. Phys.*, **112**, 5301 (2000).
- [122] W.-B. Tzeng, H.-M. Yin, W.-Y. Leung, J.-Y. Luo, S. Nourbakhsh, G.D. Flesch, and C.Y. Ng, *J. Chem. Phys.*, **88**, 1658 (1988).
- [123] D. Xu, J. Huang, and W. M. Jackson, *J. Chem. Phys.*, **120**, 3051 (2004).
- [124] D.C. Tseng and R.D. Poshusta, *J. Chem. Phys.*, **100**, 7481 (1994).
- [125] Q. Zhang and P.F. Vaccaro, *J. Phys. Chem.*, **99**, 1799 (1995).

- [126] A. Mank, C. Starrs, M.N. Jago, and J.W. Hepburn, *J. Chem. Phys.*, **104**, 3609 (1996).
- [127] C. Z. Bisgaard, O. J. Clarkin, G. Wu, A. M. D. Lee, O. Gessner, C. C. Hayden, and A. Stolow, *Science*, **323**, 1464 (2009).
- [128] T.N. Kitsopoulos, C.R. Gebhardt, and T.P. Rakitzis, *J. Chem. Phys.*, **115**, 9727 (2001).
- [129] B. Jones, J. Zhou, L. Yang, and C.Y. Ng, *Rev. Sci. Inst.*, **79**, 123106 (2008).
- [130] C.-W. Hsu, C.-L. Liao, Z.-X. Ma, P. J. H. Tjossem, and C. Y. Ng, *J. Chem. Phys.*, **97**, 6283 (1992).
- [131] E. K. Campbell PhD thesis, University of Oxford, (2011).
- [132] J.G. Frey and P. Felder, *Chem. Phys.*, **202**, 397 (1996).
- [133] M. Brouard, A.P. Clark, and C. Vallance, *J. Chem. Phys.*, **119**, 771 (2003).
- [134] F. J. Vastola, R. O. Mumma, and A. J. Pirone, *Org. Mass Spectrom.*, **3**, 101 (1970).
- [135] F. Hillenkamp, E. Unsöld, R. Kaufmann, and R. Nitsche, *Nature*, **256**, 119 (1975).
- [136] H. D. Beckey and H. R. Schulten, *Bioorganicheskaya Khimiya*, **3**, 437 (1977).
- [137] R. D. Macfarlane and D. F. Torgerson, *Science*, **191**(4230), 920–925 (1976).
- [138] M. Barber, R. S. Bordoli, R. D. Sedgwick, and A. N. Tyler, *Nature*, **293**, 270 (1981).
- [139] M. Karas, D. Bachmann, and F. Hillenkamp, *Anal. Chem.*, **57**, 2935 (1985).
- [140] M. Karas, D. Bachmann, U. Bahr, and F. Hillenkamp, *Int. J. Mass Spec. and Ion Proc.*, **78**, 53 (1987).
- [141] M. Karas and F. Hillenkamp, *Anal. Chem.*, **60**, 2299 (1988).
- [142] G. L. Glish and R. W. Vachet, *Nat. Rev. Drug Discovery*, **2**, 140 (2003).
- [143] F. Hillenkamp and M. Karas In *MALDI MS: A Practical Guide to Instrumentation, Methods and Applications*, F. Hillenkamp and J. Peter-Katalinić, Eds., Wiley-VCH, 2007.
- [144] Q. Zhan, S. J. Wright, and R. Zenobi, *J. Am. Soc. Mass Spectrom.*, **8**, 525 (1997).
- [145] D. M. Hrubowchak, M. H. Ervin, M. C. Wood, and N. Winograd, *Anal. Chem.*, **63**, 1947 (1991).

- [146] V. N. Varakin, V. A. Lunchev, and A. P. Simonov, *High Energy Chemistry*, **28**, 406 (1994).
- [147] S. L. Wang, K. W. D. Ledingham, W. J. Jia, and R. P. Singhal, *App. Surf. Sci.*, **93**, 205 (1996).
- [148] Z. Shen, J. J. Thomas, C. Averbuj, K. M. Broo, M. Engelhard, J. E. Crowell, M. G. Finn, and G. Siuzdak, *Anal. Chem.*, **73**, 612 (2001).
- [149] www.waters.com.
- [150] S. Alimpiev, S. Nikiforov, V. Karavanskii, T. Minton, and J. Sunner, *J. Chem. Phys.*, **115**, 1891 (2001).
- [151] P. Kraft, S. Alimpiev, E. Dratz, and J. Sunner, *J. Am. Soc. Mass Spectrom.*, **9**, 912 (1998).
- [152] R. L. Garrell, *Anal. Chem.*, **61**, 401A (1989).
- [153] K. Nagoshi, K. Sakata, K. Shibamoto, and T. Korenaga, *e-J. Surf. Sci. Nanotech.*, **7**, 93 (2009).
- [154] K. P. Law and J. R. Larkin, *Anal. Bioanal. Chem.*, **399**, 2597 (2011).
- [155] L. A. Klerk, A. F. Maarten Altelaar, M. Froesch, L. A. McDonnell, and R. M. A. Heeren, *Int. J. of Mass Spec.*, **285**, 29 (2009).
- [156] G. K. Agrawal, J. Bourguignon, N. Rolland, G. Ephritikhine, M. Ferro, M. Jaquinod, K. G. Alexiou, T. Chardot, N. Chakraborty, P. Jolivet, J. H. Doonan, and R. Rakwal, *Mass Spec. Rev.*, **30**, 772 (2011).
- [157] M. L. Vestal, *J. Am. Soc. Mass Spectrom.*, **22**, 953 (2011).
- [158] R. Stoll and F. W. Rllgen, *Organic Mass Spectrometry*, **14**, 642 (1979).
- [159] R. J. Cotter, *Anal. Chem.*, **53**, 719 (1981).
- [160] E. D. Hardin and M. L. Vestal, *Anal. Chem.*, **53**, 1492 (1981).
- [161] F. Hillenkamp, E. Unsöld, R. Kaufmann, and R. Nitsche, *Appl. Phys. A: Materials Science & Processing*, **8**, 341 (1975).
- [162] R. B. van Breemen, M. Snow, and R. J. Cotter, *Int. J. Mass Spectrom. and Ion Proc.*, **49**, 35 (1983).
- [163] D. M. Lubman, Ed., *Lasers and Mass Spectrometry*, Oxford University Press, 1990.
- [164] B. Spengler, M. Karas, U. Bahr, and F. Hillenkamp, *J. Phys. Chem.*, **91**, 6502 (1987).

- [165] B. Spengler, U. Bahr, M. Karas, and F. Hillenkamp, *Anal. Instrum.*, **17**, 173 (1988).
- [166] M. Barber, R. S. Bordoli, R. D. Sedgwick, and A. N. Tyler, *J. Chem. Soc.-Chem. Commun.*, page 325 (1981).
- [167] E. Depauw, *Mass Spectrom. Rev.*, **5**, 191 (1986).
- [168] M. Karas and U. Bahr, *TRAC-Trends in Analytical Chemistry*, **5**, 90 (1986).
- [169] K. Tanaka, H. Waki, Y. Ido, S. Akita, Y. Yoshida, and T. Yoshida, *Rap. Comm. Mass Spec.*, **2**, 151 (1988).
- [170] R. C. Beavis, B. T. Chait, and H. M. Fales, *Rap. Comm. Mass Spec.*, **3**, 432 (1989).
- [171] K. Strupat, M. Karas, and F. Hillenkamp, *Int. J. Mass Spectrom. and Ion Proc.*, **111**, 89 (1991).
- [172] R. C. Beavis, T. Chaudhary, and B. T. Chait, *Org. Mass Spectrom.*, **27**, 156 (1992).
- [173] T. W. Jaskolla, W.-D. Lehmann, and M. Karas, *Proc. Nat. Acad. Sci. USA*, **105**, 12200 (2008).
- [174] M. L. Vestal, P. Juhasz, and S. A. Martin, *Rap. Comm. Mass Spec.*, **9**, 1044 (1995).
- [175] R. Knochenmuss, F. Dubois, M. J. Dale, and R. Zenobi, *Rap. Comm. Mass Spec.*, **10**, 871 (1996).
- [176] A. J. Kubis, K. V. Somayajula, A. G. Sharkey, and D. M. Hercules, *Anal. Chem.*, **61**, 2516 (1989).
- [177] W. J. Henzel, T. M. Billeci, J. T. Stults, S. C. Wong, C. Grimley, and C. Watanabe, *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 5011 (1993).
- [178] K. B. Tomer, A. M. Moseley, L. J. Deterding, and C. E. Parker, *Mass Spectrom. Rev.*, **13**, 431 (1994).
- [179] R. Knochenmuss, *Analyst*, **131**, 966 (2006).
- [180] M. Karas, M. Gluckmann, and J. Schafer, *J. Mass Spectrom.*, **35**, 1 (2000).
- [181] M. Karas and R. Kruger, *Chem. Rev.*, **103**, 427 (2003).
- [182] A. Benninghoven, *Int. J. Mass Spectrom. and Ion Proc.*, **53**, 85 (1983).

- [183] K. L. Busch, S. E. Unger, A. Vincze, R. G. Cooks, and T. Keough, *J. Am. Chem. Soc.*, **104**, 1507 (1982).
- [184] S. J. Pachuta and R. G. Cooks, *ACS Symposium Series*, **291**, 1 (1985).
- [185] A. Benninghoven, Ed., *Ion Formation from Organic Solids*, Springer-Verlag, 1983.
- [186] B. D. Musselman, J. T. Watson, and C. K. Chang, *Org. Mass Spectrom.*, **21**, 215 (1986).
- [187] Q. W. Huang, G. L. Wu, and H. T. Tang, *Int. J. Mass Spectrom. and Ion Proc.*, **70**, 145 (1986).
- [188] D. A. Kidwell, M. M. Ross, and R. J. Colton, *Int. J. Mass Spectrom. and Ion Proc.*, **78**, 315 (1987).
- [189] J. Sunner, A. Morales, and P. Kebarle, *Int. J. Mass Spectrom. and Ion Proc.*, **86**, 169. (1988).
- [190] R. Krüger, A. Pfenninger, I. Fournier, M. Glückmann, and M. Karas, *Anal. Chem.*, **73**, 5812 (2001).
- [191] K. Dreisewerd, *Chem. Rev.*, **103**, 395 (2003).
- [192] H. Ehring, M. Karas, and F. Hillenkamp, *Org. Mass Spectrom.*, **27**, 472 (1992).
- [193] V. Karbach and R. Knochenmuss, *Rap. Comm. Mass Spec.*, **12**, 968 (1998).
- [194] J. B. Birks, *Photophysics of Aromatic Compounds*, Wiley Interscience, 1970.
- [195] J. B. Birks, *Organic Molecular Photophysics*, Wiley, 1973.
- [196] H. Ehring and B. U. R. Sundqvist, *J. Mass Spectrom.*, **30**, 1303 (1995).
- [197] H. Ehring and B. U. R. Sundqvist, *Appl. Surf. Sci.*, **96**, 577 (1996).
- [198] P. D. Setz and R. Knochenmuss, *J. Phys. Chem. A*, **109**, 4030 (2005).
- [199] L. V. Zhigilei, E. Leveugle, B. J. Garrison, Y. G. Yingling, and M. I. Zeifman, *Chem. Rev.*, **103**, 321 (2003).
- [200] V. Horneffer, K. Dreisewerd, H. C. Ludemann, F. Hillenkamp, M. Lage, and K. Strupat, *Int. J. of Mass Spectrom.*, **185**, 859 (1999).
- [201] A. A. Puretzky and D. B. Geohegan, *Appl. Surf. Sci.*, **127**, 248 (1998).
- [202] A. Overberg, M. Karas, and F. Hillenkamp, *Rap. Comm. Mass Spec.*, **5**, 128 (1991).

- [203] C. Menzel, S. Berkenkamp, and F. Hillenkamp, *Rap. Comm. Mass Spec.*, **13**, 26 (1999).
- [204] K. Dreisewerd, M. Schurenberg, M. Karas, and F. Hillenkamp, *Int. J. Mass Spec.*, **141**, 127 (1995).
- [205] W. Ens, Y. Mao, F. Mayer, and K. G. Standing, *Rap. Comm. Mass Spec.*, **5**, 117 (1991).
- [206] R. E. Johnson, *Int. J. Mass Spectrom. and Ion Proc.*, **139**, 25 (1994).
- [207] A. Ingendoh, M. Karas, F. Hillenkamp, and U. Giessmann, *Int. J. of Mass Spectrom. and Ion Proc.*, **131**, 345 (1994).
- [208] L. V. Zhigilei and B. J. Garrison, *J. App. Phys.*, **88**, 1281 (2000).
- [209] A. Vertes, R. Gijbels, and R. D. Levine, *Rap. Comm. Mass Spec.*, **4**, 228 (1990).
- [210] R. Knochenmuss and L. V. Zhigilei, *J. Phys. Chem. B*, **109**, 22947 (2005).
- [211] M. Glückmann and M. Karas, *J. Mass Spectrom.*, **34**, 467 (1999).
- [212] B. Spengler and V. Bokelmann, *Nucl. Inst. & Meth. in Phys. Res. B -Beam Interactions With Materials and Atoms*, **82**, 379 (1993).
- [213] R. R. Hensel, R. C. King, and K. G. Owens, *Rap. Comm. Mass Spec.*, **11**, 1785 ((1997)).
- [214] J. Axelsson, A. M. Hoberg, C. Waterson, P. Myatt, G. L. Shield, J. Varney, D. M. Haddleton, and P. J. Derrick, *Rap. Comm. Mass Spec.*, **11**, 209 (1997).
- [215] L. A. McDonnell, T. H. Mize, S. L. Luxembourg, S. Koster, G. B. Eijkel, E. Verpoorte, N. F. de Rooij, and R. M. A. Heeren, *Anal. Chem.*, **75**, 4373 (2003).
- [216] A. F. M. Altelaar, J. van Minnen, C. R. Jimenez, R. M. A. Heeren, and S. R. Piersma, *Anal. Chem.*, **77**, 735 (2005).
- [217] S. A. Schwartz, M. L. Reyzer, and R. M. Caprioli, *J. Mass Spectrom.*, **38**, 699 (2003).
- [218] M. C. Fitzgerald, L. Zhu, and L. M. Smith, *Rap. Comm. Mass Spec.*, **7**, 895 (1993).
- [219] X. Y. Chen, M. S. Westphall, and L. M. Smith, *Anal. Chem.*, **75**, 5944 (2003).
- [220] J. B. Greenwood, O. Kelly, C. R. Calvert, M. J. Duffy, R. B. King, L. Belshaw, L. Graham, J. D. Alexander, I. D. Williams, W. A. Bryan, I. C. E. Turcu, C. M. Cacho, and E. Springate, *Rev. Sci. Inst.*, **82** (2011).

- [221] S. F. Wong, C. K. Meng, and J. B. Fenn, *J. Phys. Chem.*, page 546 (1988).
- [222] G. McCombie and R. Knochenmuss, *J. Am. Soc. Mass Spectrom.*, **17**, 737 (2006).
- [223] A. F. M. Altelaar, I. Klinkert, K. Jalink, R. P. J. de Lange, R. A. H. Adan, R. M. A. Heeren, and S. R. Piersma, *Anal. Chem.*, **78**, 734 (2006).
- [224] A. M. Worthington, *Proc. of the Royal Soc. of London*, **25**, 261 (1876).
- [225] P. Marmottant and E. Villiermaux, *J. Fluid Mech.*, **498**, 73 (2004).
- [226] E. Villiermaux, *Annu. Rev. Fluid Mech.*, **39**, 419 (2007).
- [227] H. Kleine, K. Hiraki, H. Maruyama, J. Yonai, T. Hayashida, J. Yonai, K. Kitamura, Y. Kondo, and T. G. Etoh, *Shock Waves*, **14**, 333 (2005).
- [228] M. Versluis, B. Schmitz, A. v. d. Heydt, and D. Lohse, *Science*, **289**, 2114 (2000).
- [229] S. N. Patek and R. L. Caldwell, *J. Exp. Bio.*, **208**, 3655 (2005).
- [230] R. L. Essner, Jr., D. J. Suffian, P. J. Bishop, and S. M. Reilly, *Naturwissenschaften*, **97**, 935 (2010).
- [231] P. M. Reis, S. Jung, J. M. Aristoff, and R. Stocker, *Science*, **330**, 1231 (2010).
- [232] A. W. Crompton and C. Musinsky, *Biol. Lett.*, **7**, 882 (2011).
- [233] J. H. D. Eland, *Meas. Sci. Technol.*, **1**, 36 (1990).
- [234] J. H. D. Eland, *Meas. Sci. Technol.*, **5**, 1501 (1994).
- [235] O. Jagutzki, A. Cerezo, A. Czasch, R. Dorner, M. Hattass, M. Huang, V. Mergel, U. Spillmann, K. Ullmann-Pfleger, T. Weber, H. Schmidt-Bocking, and G. D. W. Smith, *IEEE Trans. Nucl. Sci.*, **49**, 2477 (2002).
- [236] http://www.photron.com/index.php?cmd=product_general&product_id=7&product_name=FASTCAM+SA5.
- [237] W. F. Kosonocky, G. Yang, R. K. Kabra, C. Ye, Z. Pektas, J. L. Lowrance, V. J. Mastrocola, F. V. Shallcross, and V. Patel, *IEEE Trans. Electron Devices*, **44**, 1617 (1997).
- [238] A. Nomerotski, S. Adigun-Boaye, M. Brouard, E. Campbell, A. Clark, J. Crooks, J. J. John, A. J. Johnsen, C. Slater, R. Turchetta, C. Vallance, E. Wilman, and W. H. Yuen, *Nucl. Inst. & Meth. in Phys. Res. A-Accelerators Spectrometers Detectors and Associated Equipment*, **633**, S243 (2011).

- [239] A. J. Campillo and S. L. Shapiro, *IEEE Journal of Quantum Electronics*, **19**, 585 (1983).
- [240] <http://sales.hamamatsu.com/en/products/system-division/ultra-fast/streak-systems/part-c5680.php>.
- [241] A. Velten, E. Lawson, A. Bardagjy, M. Bawendi, and R. Raskar In *ACM SIGGRAPH 2011 Posters*, SIGGRAPH 2011, page 13:1, New York, NY, USA, 2011. ACM.
- [242] A. Velten, R. Raskar, and M. Bawendi In *Imaging Systems Applications, OSA Technical Digest (CD)*. Optical Society of America.
- [243] L. A. Klerk, N. P. Lockyer, A. Kharchenko, L. MacAleese, P. Y. W. Dankers, J. C. Vickerman, and R. M. A. Heeren, *Anal. Chem.*, **82**, 801 (2010).
- [244] J. W. Lichtman and J. A. Conchello, *Nature Methods*, **2**, 910 (2005).
- [245] <http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html>.
- [246] L. Schermelleh, R. Heintzmann, and H. Leonhardt, *J. Cell. Biol.*, **190**, 165 (2010).
- [247] J. A. Conchello and J. W. Lichtman, *Nature Methods*, **2**, 920 (2005).
- [248] W. Denk, J. H. Strickler, and W. W. Webb, *Science*, **248**, 73 (1990).
- [249] P. Theer, M. T. Hasan, and W. Denk, *Optics Letters*, **28**, 1022 (2003).
- [250] F. Helmchen and W. Denk, *Nature Methods*, **2**, 932 (2005).
- [251] M. Oheim, E. Beaurepaire, E. Chaigneau, J. Mertz, and S. Charpak, *J. Neurosci. Meth.*, **111**, 29 (2001).
- [252] E. Beaurepaire and J. Mertz, *Appl. Opt.*, **41**, 5376 (2002).
- [253] E. Abbe, *Archiv fr Mikroskopische Anatomie*, **9**, 413 (1873).
- [254] A. J. North, *J. Cell Biol.*, Jan 2 (2006).
- [255] R. Heintzmann, *Micron*, **34**, 283 (2003).
- [256] D. A. Agard, Y. Hiraoka, P. Shaw, and J. W. Sedat, *Meth. Cell Biol.*, **30**, 353 (1989).
- [257] D. A. Agard, *Annu. Rev. Biophys. Bioeng.*, **13**, 191 (1984).
- [258] J. G. McNally, T. Karpova, J. Cooper, and J. A. Conchello, *Methods-A Companion to Methods in Enzymology*, **19**, 373 (1999).

- [259] D. A. Agard and J. W. Sedat, *Nature*, **302**, 676 (1983).
- [260] R. Heintzmann, *Micron*, **38**, 136 (2007).
- [261] D. Axelrod, *J. Cell Biol.*, **89**, 141 (1981).
- [262] E. Betzig and J. K. Trautman, *Science*, **257**, 189 (1992).
- [263] R. Heintzmann and C. G. Cremer Vol. 3568, page 185. SPIE: Opt. Biopsies and Micros. Tech. III, 1999.
- [264] M. G. L. Gustafsson, *J. Microscopy*, **198**, 82 (2000).
- [265] M. G. L. Gustafsson, L. Shao, P. M. Carlton, C. J. R. Wang, I. N. Golubovskaya, W. Z. Cande, D. A. Agard, and J. W. Sedat, *Biophys. Journal*, **94**, 4957 (2008).
- [266] L. Schermelleh, P. M. Carlton, S. Haase, L. Shao, L. Winoto, P. Kner, B. Burke, M. C. Cardoso, D. A. Agard, M. G. L. Gustafsson, H. Leonhardt, and J. W. Sedat, *Science*, **320**, 1332 (2008).
- [267] M. G. L. Gustafsson, *Proc. Nat. Acad. Sci. USA*, **102**, 13081 (2005).
- [268] S. W. Hell and J. Wichmann, *Opt. Lett.*, **19**, 780 (1994).
- [269] G. Donnert, J. Keller, C. A. Wurm, S. O. Rizzoli, V. Westphal, A. Schoenle, R. Jahn, S. Jakobs, C. Eggeling, and S. W. Hell, *Biophys. Journal*, **92**, L67 (2007).
- [270] M. Wisztorski, D. Croix, E. Macagno, I. Fournier, and M. Salzter, *Developmental Neurobiology*, **68**, 845 (2008).
- [271] M. L. Reyzer and R. M. Caprioli, *Current Opinion in Chem. Biol.*, **11**, 29 (2007).
- [272] P. J. Trim, C. M. Henson, J. L. Avery, A. McEwen, M. F. Snel, E. Claude, P. S. Marshall, A. West, A. P. Princivalle, and M. R. Clench, *Anal. Chem.*, **80**, 8628 (2008).
- [273] T. R. Northen, O. Yanes, M. T. Northen, D. Marrinucci, W. Uritboonthai, J. Apon, S. L. Golledge, A. Nordstrom, and G. Siuzdak, *Nature*, **449**, 1033 (2007).
- [274] V. I. Slaveykova, C. Guignard, T. Eybe, H.-N. Migeon, and L. Hoffmann, *Anal. Bioanal. Chem.*, **393**, 583 (2009).
- [275] R. J. A. Goodwin, S. R. Pennington, and A. R. Pitt, *Proteomics*, **8**, 3785 (2008).
- [276] H. R. Aerni, D. S. Cornett, and R. M. Caprioli, *Anal. Chem.*, **78**, 827 (2006).

- [277] P. Chaurand, J. L. Norris, D. S. Cornett, J. A. Mobley, and R. M. Caprioli, *J. Proteome Res.*, **5**, 2889–2900 (2006).
- [278] J. R. Tennant, *Transplantation*, **2**, 685 (1964).
- [279] H. C. Gram, *Fortschritte der Medizin*, **2**, 185189 (1884).
- [280] R. J. Gillies and D. W. Deamer, *Curr. Top. Bioenerget.*, **9**, 37 (1979).
- [281] R. J. Gillies, N. Didier, and M. Denton, *Anal. Biochem.*, **159**, 109 (1986).