Applications of nanospray desorption electrospray ionization mass spectrometry

In situ lipid and metabolite analysis from cells to tissue

HILDE-MARLÉNE BERGMAN
Ambient mass spectrometry (MS) has proved to be an important addition to the bioanalytical toolbox. These methods perform analyte sampling and ionization under atmospheric pressure, and require very little sample preparation other than the sampling process in front of the machine. Nanospray desorption electrospray ionization (nano-DESI) is an ambient MS technique developed in 2010 that utilizes localized liquid extraction for surface sampling. The aim of this thesis was to explore the possibilities of this technique, and identify areas in which nano-DESI MS could further contribute to the community of MS-based surface analysis.

One such area was found to be mass spectrometry imaging (MSI) of small-molecule neurotransmitters. By the use of deuterated standards of acetylcholine, γ-aminobutyric acid and glutamate, the respective endogenous compounds were successfully imaged in coronal sections of rat brain. The use of internal standards was shown to be essential to compensate for matrix effects in different regions of the brain. In a second imaging study, nano-DESI MSI was used to compare the chemical profiles of diabetic rat kidney tissue and control. Analysis was performed on kidney two weeks after diabetic onset, before any pathohistological changes relating to diabetic nephropathy can be seen in a microscope. In our study, it was shown that a large number of chemical species related to energy metabolism were detected with altered signal intensity in diabetic kidney tissue.

To push the limits of nano-DESI analysis, its use for single-cell analysis was evaluated. By placing buccal epithelial cells in contact with the nano-DESI probe, it was possible to identify 46 endogenous compounds and detect differences between cells from three human donors. In addition, it was shown that molecules from single cells on a surface could be detected by scanning the surface with the nano-DESI probe, which opens up for development of an automated analysis with higher throughput.

The last study in this thesis was concerned with method development rather than application, as it presented a setup for pneumatically assisted nano-DESI. Evaluation showed that the setup provided improved sensitivity in the analysis of small metabolites, and provided the possibility of using pure water as nano-DESI solvent.

Keywords: Mass spectrometry, mass spectrometry imaging (MSI), nanospray desorption electrospray ionization (nano-DESI), single-cell analysis, neurotransmitter imaging, diabetic nephropathy, pneumatic nebulization, lipidomics, metabolomics

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"You know the greatest danger facing us is ourselves, and irrational fear of the unknown. There is no such thing as the unknown. Only things temporarily hidden, temporarily not understood."

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II *Bergman, H.-M.,* Lindfors, L., Palm, F, Kihlberg, J., Lanekoff, I., Increased levels of acylcarnitines, fatty acids and glycerolipids in diabetic kidney sections from STZ-treated rats. (Manuscript in preparation)


*The authors contributed equally to the paper

Papers I, III and IV are reproduced with permission from The Royal Society of Chemistry
Contribution report

The author wishes to clarify her contribution to the research presented in papers I-IV.

I Took part in performing the experiments and analyzed the data. Responsible for writing the paper.

II Took part in planning the study. Performed nano-DESI MS and MSMS analysis as well as parts of the data analysis. Responsible for writing the paper.

III Responsible for planning the study. Performed experiments and data analysis. Responsible for writing the paper.

IV Took part in performing the experiments and writing the paper.

The author also wishes to point out that parts of this thesis are based on her licentiate thesis from 2016. Updated content has been largely rewritten, expanded with current results and adapted to the form of a doctoral thesis.

Papers not included in this thesis


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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>DESI</td>
<td>Desorption electrospray ionization</td>
</tr>
<tr>
<td>DG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
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<tr>
<td>FT</td>
<td>Fourier transform</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>ICR</td>
<td>Ion cyclotron resonance</td>
</tr>
<tr>
<td>IM</td>
<td>Inner medulla</td>
</tr>
<tr>
<td>IS</td>
<td>Inner strip or outer medulla</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>MG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MSI</td>
<td>Mass spectrometry imaging</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>Nano-DESI</td>
<td>Nanospray desorption electrospray ionization</td>
</tr>
<tr>
<td>OS</td>
<td>Outer strip of outer medulla</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SIMS</td>
<td>Secondary ion mass spectrometry</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion current</td>
</tr>
</tbody>
</table>
Introduction

Chemistry as a scientific field is an enormous playground for people with a fascination for atoms and molecules. One specialization within this field is analytical chemistry, which aims at answering questions such as “what molecules?” and “how many?”. One important tool for analytical chemists to answer these questions is mass spectrometry (MS), a technique that can be used to provide structural information about a molecule in order to identify it, as well as providing information about the quantity.

One specific field in which MS plays an important role is that of chemical analysis of biological samples. In biology, a current trend is so called “omics”-approaches such as genomics, transcriptomics, proteomics, lipidomics and metabolomics. These techniques aim at preforming qualitative, and preferably also quantitative, detection of all respective biochemical compounds in a sample. By providing these chemical snapshots of a dynamic system, the hope is to provide insight into biological function of a given system. Currently, the genome is the only object that comes close to be fully characterized thanks to the possibility of amplifying nucleic acids with the polymerase chain reaction. For the proteome, lipidome and metabolome, no such amplification method exists. However, some strengths of MS is that it can be used to detect up to thousands of chemical species simultaneously, even at extremely low abundances. Therefore, MS has been popular in proteomic [1] as well as lipidomic,[2] and metabolomic studies.[3] And as constant developments in instrumentation keep pushing the limits of detection down, a larger portion of the chemical composition becomes available for investigation.

In addition to qualitative and quantitative analysis, sometimes the question “where?” needs to be answered. Also for this issue, MS can be a helpful tool through the use of mass spectrometry imaging (MSI) techniques. The question “where?” is very much at the center of this thesis, which revolves around a surface analysis technique called nanospray desorption electrospray ionization mass spectrometry imaging (nano-DESI MSI). Previous studies have shown that nano-DESI MSI can be used for spatially resolved analysis and imaging of metabolites and lipids in biological tissues, something that has been further evaluated in this work.
Mass Spectrometry

An ion is defined as an atomic or molecular particle having a net electric charge. [4] As such, its motion can be manipulated in an electromagnetic field; an ion can for example be accelerated, deflected or trapped. The motion of charged particles in an electromagnetic field is dependent on both the mass and charge of the particle. These basic concepts lay the foundation for mass spectrometry, an analytical technique in which ions in gas phase are separated based on their mass-to-charge ratio (m/z).

Three components are essential in a mass spectrometer: an ion source which transfers analytes into gas phase ions, a mass analyzer which separates ions based on their m/z, and a detector which detects the ions (Figure 1). [5] The detected signal of each m/z is then used to generate a mass spectrum, in which the signal intensity is plotted against the m/z.

Electrospray ionization

It can be noted that the ion source may or may not be in vacuum depending on the technique. However, the setup used in Papers I-IV was based on electrospray ionization (ESI), which is performed under atmospheric pressure. ESI provides an efficient way of generating gas phase ions from analytes in a liquid phase. [6] This property makes ESI a suitable interface between liquid chromatography (LC) and mass spectrometry. The useful LC-ESI interface in combination with the capacity to generate gas phase ions even from large biomolecules such as proteins [7], lead ESI to quickly become incredibly popular.

ESI is a soft ionization technique, which generally produces adduct ions without any fragmentation. In Papers I-IV, typical adduct ions were proto-
nated molecules ([M+H]⁺) or molecules cationized on alkali metals ([M+Na]⁺ and [M+K]⁺). In negative ion mode, deprotonated molecules ([M-H]⁻) or chloride adducts ([M+Cl]⁻) are examples of common ions. Large biomolecules that contain several protonation sites, such as proteins, typically form multiply charged species during the ESI process.

The key to ESI is the application of a high voltage, of about 3-4 kV, between the sample capillary and the mass spectrometry inlet. Due to the electric field, a charge will accumulate at the tip of the capillary and electrostatic forces will start to fight the surface tension of the solvent. The result is the formation of a Taylor cone from which a spray of charged droplets is emitted (Figure 2).[8] As solvent evaporates from the generated droplets and they start to shrink, the charge density of the droplets will increase. At a certain point in this desolvation process, the surface tension is overcome by electric repulsion and coulomb fission will generate even smaller droplets. However, when the charged droplets become small enough, analyte molecules in the droplets start to form single gas phase ions through one of several possible mechanisms. [9] Two of the most common theories will be described below.

The dominant ionization mechanism for large biomolecules, such as proteins, is thought to be the charge residue model. [9, 10] This model suggests that the final small droplet contains one single analyte molecule and a number of charges (e.g. protons in positive mode). Complete desolvation leaves the analyte in gas phase and forces the molecule to pick up the residual charges that were in the droplet. As a random number of charges might be present the final droplet, this model would explain why proteins are multiply charged with a distribution over many charge states.

The other suggested ionization mechanism, which is thought to be the dominant mechanism for small molecular weight species, is the ion evaporation model. The hypothesis in this model is that when the droplets reach a radius of ~10 nm, the surface charge will generate an electric field so strong that Coulomb repulsion causes ionized analytes to eject from the surface. In contrast to the charge residue model, many charged analyte molecules can be generated from the same final droplet.
Ionization efficiency

Ionization efficiency in ESI, which is a measure of how well analytes in solution are transferred to gas phase ions, is to a large extent dependent on the desolvation process. In both the ion evaporation model and the charge residue model, generation of gas phase ions is dependent on the formation of small solvent droplets with a size of ≤10 nm [9]. If the solvent droplets never reach this size, analytes remaining in the droplet will never be transferred to gas phase ions that can be detected by the mass spectrometer. Incomplete desolvation therefore increases the limit of detection for the analytes of interest. Parameters that will affect the formation of small sized droplets include flow rate of the solvent, the applied voltage, capillary temperature, conductivity and liquid surface tension of the solvent.[11] To provide a good ionization efficiency in Papers I-IV, the applied voltage was manually optimized for the specific setup used in each project and the flow rate was kept at sub-µl/min flow rates (normally 300-500 nL/min). Conductivity was ensured by the use of protic solvents (typically 9:1 methanol:water) with or without addition of formic acid. However, the high surface tension of water has prohibited the use of pure water in nano-DESI analysis, and the highest previously reported water content was the use of a solution with 1:1 methanol:water.[12]

Pneumatically assisted ESI

To overcome the surface tension of highly aqueous solvents in the ESI process, and/or allow for an increase of the flow rate, it is possible to add a co-
axial sheath gas to the outside of the capillary (Figure 3). [13] The gas, typically nitrogen, aids in the nebulization process and does to a certain extent decouple aerosol formation from the charging event.[14] Pneumatic nebulizers have been extensively used in many ESI applications, and the development of a pneumatically assisted nano-DESI source is described in Paper IV.

![Figure 3 - Pneumatic nebulization using nitrogen gas.](image)

### Quadrupole-Orbitrap mass analyzer

The instrument used in Papers I-IV was a Q-Exactive™ Plus (ThermoScientific), a hybrid mass spectrometer consisting of a quadrupole and an Orbitrap mass analyzer. The quadrupole provides a high mass selection speed, whereas the Orbitrap provides high mass resolving power and mass accuracy.

#### Quadrupole

In a quadrupole mass analyzer, an oscillating electric field is generated between four electrode rods (Figure 4). One pair of two opposite electrodes have an applied potential of +(U+Vcos(ωt)), while the other pair has an applied potential of -(U+Vcos(ωt)). The value of U represents a constant DC voltage and the Vcos(ωt) component represents an oscillating AC voltage. The oscillating field will make incoming ions move in spiral as they travel between the rods, and differences in the m/z ratio of the ions will lead to different ion trajectories. Some of the trajectories will be stable, so that the ion can travel through the analyzer and reach the detector, whereas other ions will spiral out of control and not be detected. By changing the parameters U and Vcos(ωt) it is possible to select m/z of interest, and a quadrupole can be used to either scan entire mass ranges in a stepwise fashion or statically let specific ions through.
The Orbitrap mass analyzer is a relatively new invention which became commercially available in 2005. In essence, it is composed of a barrel shaped outer electrode and a spindle shaped inner electrode (Figure 5). When an ion package enters the analyzer, the ions are trapped and start to orbit around the inner electrode. In addition, the ions also start to oscillate along the axis of the inner electrode.

If the ion package contain species with different \( m/z \), these will be separated from each other along the axis as the frequency (\( \omega \)) of their harmonic ion oscillation will differ. The relationship between \( \omega \) and \( m/z \) is described in Equation 1, where \( k \) stands for field curvature.

\[
\omega = \sqrt{\left(\frac{m}{z}\right)^{-1} \cdot k}
\]  

(1)
The Orbitrap mass analyzer is capable of very high resolving power, and is only surpassed by the more expensive Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers. Resolving power is in essence the ability to separate two ions with similar \( m/z \). It is defined by Equation 2, where \( M \) is the mass of the peak and \( \Delta M \) is the width of the peak at a defined peak height.

\[
\text{Resolving power} = \frac{M}{\Delta M} \quad (2)
\]

The Q-Exactive™ Plus mass spectrometer used in Papers I-IV has a maximum resolving power of 280 000 at \( m/z \) 200, where \( \Delta M \) is defined at the Full Width of the peak at Half its Maximum height (Figure 6). At \( M=200 \) amu, \( \Delta M \) thus has a value of 7.0x10^{-4} amu. Manual inspection of mass spectra from Paper III shows that two compounds with a difference of about 2x10^{-3} amu will be fully resolved at \( m/z \) 200, with a spectral acquisition time of about 1.2 seconds. However, the resolving power of the Orbitrap is dependent on the \( m/z \)-value, as it is inversely proportional to the square root of \( m/z \). [5] Thus, the resolving power is roughly 140 000 at \( m/z \) 800 when the resolving power is set to 280 000 at \( m/z \) 200.

For comparison, it can be noted that the best FT-ICR instruments are capable of resolving powers up to 2 000 000 at \( m/z \) 400.[1] However, this resolving power requires detection times of about 12 seconds, and if a spectral acquisition rate of 1Hz is preferred the resolving power will be around 300 000 at \( m/z \) 400.

\[\text{Figure 6 - Image showing } \Delta M \text{ at the Full Width of the peak at Half its Maximum height (FWHM).}\]

In addition to resolution, mass accuracy is another important characteristic of a mass analyzer. It is a measure of how far the experimentally determined mass (\( m_e \)) deviates from the true mass (\( m_t \)) of an ion, as defined by Equation 3.
Detection

The Orbitrap mass analyzer utilizes image current detection, which takes place in the outer electrodes.[16] When ions are introduced into the trap, their charge will influence the metal of the electrode and lead to an induced charge on the surface. As ions with discrete \( m/z \) values oscillate with a certain frequency in the trap, the induced signal voltage will be a sum generated from the frequencies of all ions with individual \( m/z \) values.[20] This detected signal which is measured as a function of time is called a time domain transient, and in order to convert it to a mass spectrum the time domain signal is processed with Fourier transform mathematical operations. In short, the Fourier transform extracts all present frequencies from a combined signal (like a trained musician can extract individual notes, with specific frequencies, from a played chord), which in turn can be converted to \( m/z \) values (Equation 1). In essence, the high mass accuracy and resolution obtained by the Orbitrap and FT-ICR MS instruments can be achieved because frequencies can be very accurately measured. The longer the transient is measured, the more accurately the frequencies can be extracted. This is the reason that ultra-high resolution in FT-ICR instruments requires transients of over 10 seconds to be acquired. [21] However, as speed of analysis is important in many applications, and measurements above a second might be much too long, there is a trade-off between resolution and speed.

Tandem mass spectrometry in a Q-Exactive™ Plus

Despite the advantages of high resolving power and high mass accuracy, molecular identification of a specific \( m/z \) becomes complicated when a sample contains isomers. Isomers have the same exact mass and therefore they cannot be separated in a mass spectrometer. However, more information about the specific \( m/z \) can be obtained by tandem mass spectrometry (MS/MS), a technique in which a precursor ion is fragmented and the masses
of the fragment ions are measured. Different molecular structures give rise to specific fragmentation patterns, and comparison to databases or standards might enable identification of isomeric compounds.

In a Q-Exactive™ Plus, the precursor ion is isolated by the quadrupole and transferred to a collision cell where the molecule is fragmented. In Papers I-IV, fragmentation was performed with higher-energy collisional dissociation. [22] In short, the precursor ion is transferred to a collision cell which contains a neutral gas such as nitrogen. When the precursor ion(s) enter the cell it will collide with the gas, and if kinetic energy in the analyte is transformed to internal energy, dissociation of covalent bonds might occur.[23] The fragments formed upon dissociation are subsequently transferred to the Orbitrap mass analyzer, and their m/z-values are measured with high resolving power.
Mass spectrometry imaging

Although mass spectrometric analysis often is performed on analytes in solution, there are also methods to sample and ionize analytes directly from a surface. If mass spectra are collected at defined coordinates over a surface, it is possible to map the spatial distribution of detected analytes and generate ion images of specific ions (Figure 7). This technique is called mass spectrometry imaging (MSI), and can be used to study surface localization of organic as well as inorganic analytes. Different techniques for surface sampling have different strengths and weaknesses regarding what kind of samples that can be analyzed, what kind of chemical species that can be detected and at what lateral resolution they can be detected at.

Techniques for surface sampling and ionization in mass spectrometry

The first MSI experiments were performed in the 1960’s using a surface sampling and ionization technique called secondary ion mass spectrometry (SIMS).[24, 25] In SIMS, the surface is bombarded with a primary ion beam which causes neutral species and secondary ions to sputter from the surface. The ions can subsequently be transferred to a mass analyzer for detection (Figure 8).[25] As the secondary ions absorb a large amount of energy in the sputtering process, a large degree of fragmentation will occur, and SIMS has
therefore traditionally been used for elemental analysis of inorganic materials such as such as polymers, metals and glass.[26, 27] However, the development of cluster primary ion beams has led to SIMS methods that are capable of softer ionization, which has popularized the method for analysis of biological samples.[28, 29] The lateral resolution of SIMS is the highest to date, and generation of images with sub-micrometer pixel sizes are routine.[30]

Another method for surface sampling and ionization is matrix-assisted laser desorption ionization (MALDI), where a laser is used to ablate material from a surface. [31] A matrix capable of absorbing the energy of the incoming laser beam, typically a small organic molecule, is added to the sample surface to aid the ionization process (Figure 8). The matrix needs to be tailored to the specific application, as it is important that the analyte of interest and the matrix co-crystallize well.[32] In addition, it is important to choose a matrix which does not generate background peaks with the same mass as the analyte(s) of interest, which typically might happen at \( m/z <500 \). Like ESI, MALDI is a soft ionization technique which typically generates adduct ions or deprotonated ions without fragmentation, with the difference that ions generated in MALDI are almost exclusively singly charged, even proteins.[33] Proteins, peptides and lipids [34] have been the main compound classes analyzed with MALDI, although targeted studies have also looked at small metabolites using special matrices.[35, 36] Imaging with MALDI can be routinely performed at a spatial resolution of >20 µm, but can reach up to low µm-scale lateral resolution with optimized instrumentation. [37, 38]

![SIMS and MALDI Diagram](image)

**Figure 8 - Schematic overview of the sampling and ionization process in secondary ion mass spectrometry (SIMS) and matrix assisted laser desorption ionization (MALDI).**

Mass spectrometry imaging using SIMS and MALDI is performed in a rastering fashion much like the illustration in Figure 7. Stepwise sampling is performed either by moving the sample under the laser beam in MALDI, or
by directing the ion beam to new areas in SIMS. Therefore, MALDI and SIMS typically generate ion images with square pixels.

Although instrumentation exists for atmospheric pressure MALDI, [39] the sample is typically placed in vacuum during sampling and ionization in both SIMS and conventional MALDI. In certain applications this may be a problem, such as single-cell analysis where it might be of interest to keep the cells in a native state for as long as possible.

**Ambient surface sampling**

To ease sample preparation and avoid transfer of sample into vacuum, a wide range of ambient mass spectrometry techniques have been developed. [40] The definition of ambient mass spectrometry varies somewhat depending on who you ask, but the following requirements are often mentioned: (1) it has an ion source that is not enclosed and therefore can hold samples of various shapes and sizes, (2) it can be interfaced to different mass spectrometers with atmospheric pressure interfaces, (3) it provides soft ionization, and (4) it requires no or little sample preparation. [41] As for the last statement, I wish to quote Javanshad and Venter: [40]

> “While it is often said that ambient ionization methods do not require sample preparation, our view is that it is more accurate to say these methods frequently require no sample preparation, *other than the sample processing that takes place during the analysis* [emphasis added].”

Thus, in ambient ionization, a sample surface with any size or geometry (such as a flower petal, a tomato or a finger [42]) can be probed without further treatment right in front of the mass spectrometer, but analytes still have to be transferred in real-time from the surface using methods such as liquid extraction, laser ablation or thermal desorption. [40]

The most commonly used ambient MS technique today is desorption electrospray ionization (DESI), which uses liquid extraction for sampling. [42]. In DESI, a stream of charged droplets is sprayed onto a sample surface and the liquid desorbs analytes prior to “splashing” off the surface towards the mass spectrometry inlet. The analytes subsequently undergo an electrospray ionization process from these secondary droplets (Figure 9).[43]

Unlike SIMS and MALDI, mass spectrometry imaging using DESI is not performed in a rastering fashion. Instead, the sample is moved with continuous speed in the x-direction while mass spectra are continuously collected. When a line scan in the x-direction is finished, the sample is moved one step in the y-direction and a new line scan is initiated. The lateral resolution in the x-direction is thus dependent on the scanning speed of the instrument and the speed with which the sample is moved, whereas the resolution in the y-direction is set by the chosen step size.
Nano-DESI was developed in 2010 by Patrick Roach, in the lab of Julia Laskin, and was originally a modification of a commercial DESI setup.[44] In this technique, a liquid bridge is generated between a primary capillary, which continuously delivers solvent, and a self-aspirating secondary capillary, which transports the solvent to the MS inlet (Figure 10). [44] When the liquid bridge is placed in contact with a surface, analytes can be extracted and transferred through the secondary capillary. When a high voltage is then applied between the solvent and the inlet of the mass spectrometer, ionization of the analytes occurs at the tip of the secondary capillary.

Figure 9 - Schematic overview of the desorption electrospray ionization process

Figure 10 - Schematic representation of the nano-DESI setup in positive mode. Note that the figure is not to scale; the capillaries are in reality 90 or 150 µm wide.
Self-aspiration through the secondary capillary is aided by placing it in close proximity to the MS inlet, as the vacuum in the MS helps to pull liquid through the capillary. However, a short distance between the capillary tip and the inlet can result in a poor desolvation process which hampers the ionization efficiency. This issue was addressed in Paper IV.

As in DESI MSI, imaging with nano-DESI is performed by placing the sample on a motorized xyz-stage that can move the sample in three dimensions under the probe.[45] The sample is placed in contact with the liquid bridge of the nano-DESI probe, and sampling is performed through line scans in the x-direction. The sample is then moved in a stepwise fashion in the y-direction, commonly with a step size of 150-200 µm, and a new line scan is performed.[46] This is repeated for a desired number of lines, until the whole area of interest has been analyzed. This process has been automated through the use of a software in which the movements of the xyz-stage are programmed.[46] As a constant distance, in the range of ±2 µm, between the probe and the sample surface is of utmost importance for the quality of the data the software also accounts for tilted sample surfaces. This is achieved by first defining the coordinates (x, y, z) of three points on the surface, which in turn are used to define the plane tilt.[46] By programming the xyz-stage to move along this plane in the z-direction, the sample will be held at a constant height in relation to the nano-DESI probe.

Tissue imaging with nano-DESI MSI

Although nano-DESI has also been used to analyze samples such as organic aerosols,[47, 48] petroleum [12] and bacterial colonies [49, 50], one major application has been MSI of biological tissues.[45, 46, 51-55] A tissue imaging experiment can generally be divided into three steps: preparation of tissue, nano-DESI MSI analysis and data analysis (Figure 11).

Tissue preparation

As previously mentioned, ambient MS methods are often said to require no or little sample preparation. For nano-DESI MSI of biological tissues, three sample preparation steps are typically needed: 1) euthanization of laboratory animal, 2) dissection of organ and 3) cryosectioning. Euthanization and dissection procedures may influence quality of MSI data, as the levels of certain metabolites may be rapidly altered post-mortem. The brain used in Paper I was quickly collected from a decapitated rat, and the kidneys in Papers II and IV were surgically removed from anesthetized rats. The procedures were performed by a trained lab technician and the organs were instantly snap-frozen in liquid nitrogen to preserve the chemical composition. To obtain thin tissue sections from the organ, cryosectioning with a thickness of 12 µm
was performed followed by thaw-mounting onto a glass slide. For nano-DESI, regular glass slides can be used as no conductive surface is needed to prevent charge buildup, unlike in MALDI.[56]

Setting up nano-DESI MSI
One of the important considerations for nano-DESI MSI analysis is the choice of solvent. Certain questions need to be answered: Is it a targeted analysis so that the solvent can be tailored to extract and ionize that particular compound? Is it untargeted, so that as many analytes as possible should be extracted and ionized? Should any internal standards or reagents be added to the solvent? In Papers I-IV, we had the big advantage of having spare tissue samples that could be used to evaluate if the solvent was suitable for the specific analysis, by determining if the analyte(s) of interest could be detected from the sample at desired signal intensities.

To optimize a setup for nano-DESI analysis, it is necessary to find the optimal position for the secondary capillary in front of the MS inlet to obtain
the best possible pull of nano-DESI solvent through the capillary, as well as the best possible ionization. In a second step, the positioning of the primary and secondary capillary towards each other needs to be optimized in order to generate a contained liquid bridge, which only touches the surface without leaking over the tissue. This can be obtained by adjusting the position of the secondary capillary in front of the inlet and/or changing the angle and distance between the capillaries. It can be noted that many of these optimization steps were found to be easier to perform with a pneumatically assisted secondary capillary (Paper IV).

Another factor that will affect the collected data in nano-DESI MSI is the selection of lateral resolution. Two questions are important here: how small are the regions we would like to analyze in the tissue, and how fast does the analysis have to be? If oversampling is to be avoided, a step size of 200 µm in the y-direction has typically been used for capillaries with an outer diameter of 150 µm. However, a finer lateral resolution can be obtained in the x-direction and is determined in part by the speed with which the stage is moved. In part, it is also determined by the settings of the mass spectrometer. As the Orbitrap is an FT instrument, the longer the measured transient is, the higher the mass resolving power. As an example, in Paper I, a typical pixel was 24 µm in the x-direction when using a scan speed of 40 µm/s and a mass resolving power of 140 000. If a mass resolving power of 280 000 would have been chosen instead, the pixel would have been wider than 24 µm. Another important parameter in the MS settings is the polarity of the analysis. It can be noted that the studies in Papers I-IV have utilized positive ionization mode, and it is important to know that this will favor analysis of certain compounds but hinder the analysis of others.

Data analysis

Imaging data can be processed in a few different ways. Naturally, one of the data analysis steps is generation of ion images. In short, an ion image visualizes the signal intensity of a certain m/z-value over a surface, where the source of one pixel is a single mass spectrum. If a large mass range is scanned and hundreds of compounds are detected, a single MSI experiment can therefore generate hundreds of ion images, one for each m/z-value. In Papers I-IV, the program MSI QuickView [46] was used to extract intensity data of a certain m/z from each mass spectrum, and then generating a pixel with a color ranging from dark (setting the lowest detected intensity in the image at 0%) to bright (setting the highest intensity at 100%). If an internal standard was used in the MSI experiment, its intensity could was used to normalize the intensity of the analyte in each mass spectrum.

Sometimes the goal of the analysis is to evaluate the mass spectrometry data in different anatomical regions of the tissue, and two in-house scripts were developed specifically to meet these needs in Paper I-II. The first
script, called Massive, utilized the open software Decon2LS [57] to extract peak information from the acquired spectra. It then generated a data matrix with information on the location of each pixel and the peaks detected within it. A region of interest (ROI) was manually defined by drawing it in blue on an ion image (Figure 11). The second script, called ROIextractor, used the ROI image and the data matrix from Massive as input, and then extracted pixels with a location corresponding to the blue area. In the targeted analysis of Paper I, the script was used to perform spectrum-to-spectrum based quantification of the targeted analytes by use of their corresponding internal standards. The output of the analysis was an average concentration and standard deviation for each analyte within the ROI. In the untargeted analysis of Paper II, ROIextractor was used to extract all pixels in an ROI, while further data processing was performed using custom made in-house scripts. In short, all data in an ROI pixel was normalized to the total ion current (TIC), and an average intensity and standard deviation of each $m/z$ in the ROI was calculated. Welch’s t-test was then used to compare the normalized intensities of each $m/z$ between diabetic and control kidney.

Matrix effects

One major issue in MSI analysis is the presence of matrix effects, which might affect the reliability of generated ion images. It can be noted that I am no longer talking about a MALDI matrix, but instead the chemical background in which an analyte of interest is present. A “matrix” in this section is thus defined as all compounds in a mixture which are not the analyte of interest,[4] and if the matrix affects the detection of the analyte this is termed a matrix effect. For example, if the analyte is detected with a lower signal in the presence of the matrix, this is called ion suppression, whereas an increase in analyte signal in the presence of the matrix is called ion enhancement.

It can be noted that in nano-DESI, as well as DESI, MALDI and SIMS, a complex mixture of compounds will be sampled and ionized simultaneously, which means that there is always a potential risk of matrix effects. If the matrix is similar all over the sample surface, this might not be a problem for ion image generation. However, difficulties will arise if different regions of the surface have highly different chemical compositions. If this is the case, it might appear as if a compound which in reality is present in equal amounts all over the surface, has specific localizations.[55, 58]

One example of matrix effects is unequal distributions of alkali cations over a surface, which is of a huge importance in ionization techniques that generate adduct ions such as MALDI, DESI and nano-DESI. At set concentrations of sodium and potassium, the relative signal intensities of the $[M+Na]^+$ and $[M+K]^+$ adducts are fairly constant. However, if the concentration of the alkali ions change, so will the relative signal intensities of the

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adduct ions.[55] This was an important aspect in Paper II, where we wanted to compare data between diabetic and control kidney tissue. One role of the kidney is to balance electrolyte levels, and therefore it was not known if the sodium and potassium abundances could vary between samples. In order to see if any \(m/z\)-values changed between diabetes and control, we therefore counted a molecule to be significantly altered only if it showed a significant change in both the \([M+Na]^+\) peak and \([M+K]^+\) peak. The adduct formation was also used to our advantage in Paper III, where the formation of \([M+K]^+\) peaks was used as a diagnostic tool to verify the extraction of material from single cells. The reason for the increase of \([M+K]^+\) upon cell contact is that the intracellular concentration of potassium is higher than the extracellular concentration, while the opposite is true for sodium.[59]

Other molecules than alkali cations can give rise to matrix effects. It can be noted that matrix effects in ESI are thought to originate from a competition for charge between matrix molecules and analytes, which relates to the mechanisms of gas phase ion generation.[60] In the ion evaporation model of ESI, it is assumed that the charges reside at the droplet surface, while the inside of the droplet will contain neutral molecules and salt dissolved in solvent.[61] It has been suggested that the molecules are in a constant equilibrium between the charged state at the droplet surface and the neutral state inside the droplet, where only molecules at the surface can be transferred to gas phase.[61] This would explain why amphiphilic compounds such as membrane lipids are readily detected in electrospray ionization, since they are expected to have a high surface concentration. This was of high importance in Paper I, where differences in membrane lipid composition between grey and white matter in rat brain gave rise to substantial matrix effects that needed to be taken into consideration.

Compensation for matrix effects in nano-DESI MSI

One main advantage of nano-DESI MSI is the ease with which internal standards can be added to the nano-DESI solvent.[51, 52, 55] If matrix effects are absent, the signal intensity of the standard should remain constant over a surface, as standard is continuously supplied with a constant concentration. If, however, the signal intensity of the standard is not constant, it can be concluded that matrix effects are present. Internal standards that are closely related to the analyte of interest, preferably a deuterated form of the compound, should theoretically be affected by the matrix effect in the same way as the endogenous analyte. Normalization of the analyte signal intensity to the standard signal intensity will therefore compensate for matrix effects in the analyte ion image.
Quantification in MSI

Another major challenge in MSI, which at some point needs to be resolved if MSI is to keep thriving as a scientific field, is that of quantification.[62] If only localization is the burning issue, MSI is a relatively mature technique that can be used to provide reliable qualitative data if the issue of matrix effects is carefully considered and evaluated. But many scientific questions also require a quantitative answer – what is the surface concentration or amount? This can be very important in fields such as pharmacokinetics, where it is crucial to know if a drug reaches its site of action in its therapeutic dose. [63, 64]

Quantification is in essence the art of converting an instrument signal into the concentration of an analyte in a sample, usually through the use of calibration curves.[5] However, even using conventional LC-MS or LC-MS/MS methods, where the analyte of interest is baseline separated from other compounds in the sample, MS-based quantification is not entirely straightforward.[65] Many factors in addition to analyte concentration will influence the detected signal intensity in ESI-MS, such as matrix effects and drift in the instrument due to build-up of contaminant species in both the MS inlet and in the ion optics inside the machine. Problems connected to instrumental drift and matrix effects are typically tackled through the use of internal standards, often structural analogues or deuterated forms of the analyte.[65]

Biological quantitative MSI faces additional problems. One of them is the lack of separation, which means that each detected m/z value might be consisting of several compounds with the same mass. Of course, this needs to be evaluated for both imaging and quantification purposes to ensure that the correct analyte is analyzed. But perhaps the biggest problem of all is the, oftentimes, rather uncontrolled sampling process. In MALDI, signal intensity may vary depending on height differences in the sample, the topography of the target plate and variations in analyte extraction and co-crystallization with the matrix over the sample surface. [66, 67] In nano-DESI and DESI, variation in signal intensity arises from variations in ionization efficiency between setups and variations in the extraction process, which for example is dependent on the distance between the surface and the probe in nano-DESI. In addition, tissue imaging is further complicated by the presence of distinct anatomical regions in the samples. These regions possess characteristic chemical compositions and cell densities, so it is reasonable to believe that analyte extraction efficiency into either the MALDI matrix or the nano-DESI solvent differ between anatomical structures. In Paper I, this was evaluated through localized extraction of the targeted analytes in the two most different tissue types in brain, namely white and grey matter, which resulted in similar extraction profiles for the analytes of interest.

An additional problem in quantitative MSI is the lack of reference surfaces for quality control, and the generation of calibration curves could be a
whole scientific subject in its own right. Calibration is often performed by mimicking standard addition methods, such as spotting of standard solutions on the sample,[68] or addition of standards to tissue homogenate which subsequently is frozen, sectioned and sampled.[69] In MALDI, it has also been shown that quantification can be performed by spotting calibration solutions and an internal standard on the target and then placing the tissue section on top.[70] However, all of these approaches remove the site-specific matrix effects that can occur in specific regions. For example, internal standards spotted on grey matter may not ionize in the same way as an internal standard spotted on white matter in the brain. And if a homogenate is used, all of the substructures will be mixed together, and will not represent any of the substructures. To date, however, these calibration methods are the best available options.

Quantitative analysis is the holy grail of MSI, and it is definitely something that the MSI community should continue to strive for. However, at present most MSI methods struggle with repeatabilities ~20% which in combination with the above-mentioned difficulties gives rise to low accuracies.[71] A user needs to be aware of these limitations and know that quantitative MSI typically provides an estimate of a quantity rather than a precise answer.

Shotgun quantification using nano-DESI MSI

In the field of lipidomics, ESI-based quantification of lipids in complex mixtures has been an important tool over the last couple of decades.[72] Often referred to as “shotgun quantification”, the typical workflow is to homogenize a biological sample, add internal standards, perform several lipid extractions of the sample and finally analyze the extracts using direct infusion ESI-MS.[73] Extraction procedures are chosen to separate lipid classes that are best analyzed in either positive or negative mode. Quantification is performed based on the relationship between the signal intensity of the endogenous compound ($I_{\text{end}}$) and the internal standard ($I_{\text{std}}$), as described by Equation 4.[74]

$$\frac{I_{\text{end}}}{I_{\text{std}}} = \left( \frac{a_{\text{end}}}{a_{\text{std}}} \right) \times \left( \frac{c_{\text{end}}}{c_{\text{std}}} \right) \quad (4)$$

If the concentration of standard ($c_{\text{std}}$) and the response factors of both standard ($a_{\text{std}}$) and endogenous compound ($a_{\text{end}}$) is known, it is thus possible to calculate the concentration of endogenous compound ($c_{\text{end}}$). It can be noted that if the internal standard has the same response factor as the endogenous compound, the equation can be simplified (Equation 5).

$$c_{\text{end}} = c_{\text{std}} \times \left( \frac{I_{\text{end}}}{I_{\text{std}}} \right) \quad (5)$$
Typically, stable isotopically labeled versions of the analyte are considered to be the best internal standards, as they more often than not exhibit the same physicochemical properties as the analyte.\[65\] The standard and the analyte will therefore have the same response factors, and quantification can be performed with Equation 5. However, many lipidomic analyses aim at quantifying hundreds of analytes in a single study,\[75\] and it is not feasible to add a stable isotopically labeled standard for each analyte. Much effort has therefore been put into developing quantitative lipidomic approaches with a restricted number of internal standards.\[72\]

One important group of lipids, that was of special interest in Paper III, is polar lipids such as phosphatidylcholine (PC). A number of factors have been shown to affect the response of polar lipids in ESI analysis, including; lipid concentration, solvent composition, the structure of the polar head-group, acyl chain length and acyl chain unsaturation.\[76\] The polar head group, which defines a lipid class, has such a large impact on the signal response that a minimal requirement for the internal standard is that it belongs to the same lipid class as the analyte of interest.\[76\] Commonly used internal standards either have odd-carbon number acyl chains or unnaturally short acyl side chains, to differ in structure from endogenous lipids. As mentioned, also the acyl chain length of the lipid affects signal intensity, but this dependence has been shown to be linear at low lipid concentrations.\[77\] Therefore, experimentally determined carbon factors can be used to compensate for differences in chain length between an analyte and an internal standard within a lipid class.\[72\] It has also been reported that at low lipid concentrations, approximately below 10 µM total polar lipid, there is a linear correlation between ion intensity and lipid concentration.\[72\] All in all, if all factors are carefully evaluated, it is possible to perform shotgun quantification of hundreds of compounds using one internal standard per lipid class.\[76\]

It was early recognized that nano-DESI MSI provides the possibility to perform shotgun-like quantification directly from tissue, and the technique has been used to quantify phospholipids as well as nicotine (using a deuterated nicotine standard) in brain tissue sections.\[51, 52\] In this thesis, shotgun quantification was used to determine the relative abundances of three neurotransmitters in different regions of rat brain tissue, through the use of deuterated internal standards (Paper I).

Nano-DESI MSI quantification using Equation 5 will give the concentration of extracted analyte in the solvent, but will not provide direct evaluation of the absolute amount of analyte in the tissue. However, absolute quantification can be performed by careful determination of the extraction efficiency of the analyte from the tissue.\[51\] As a complete extraction of lipids could be seen from single cells in Paper III, an estimation of absolute PC amounts in single cheek cells could be performed.
Challenging samples for MSI and MS analysis

Generally, it can be said that the more heterogeneous a sample is, the higher the risk of a variable matrix effect over the surface in an MSI experiment. One such example is brain tissue. As an illustration, it can be noted that in vertebrates, the brain can be anatomically divided into hindbrain, midbrain and forebrain, all of which contain unique substructures.[78] In addition to the general risk of matrix effects in brain sections, further complicating factors cause small-molecule neurotransmitters to be particularly difficult to analyze, which will be discussed in greater detail below.

Another major challenge in the field of mass spectrometry is that of single cell analysis. Naturally, this is due to the restricted sample volume of a cell, which is typically around a few pL for normal-sized mammalian cells.[79] It can be estimated that in a cell of this size, high abundant proteins can be found at low attomole levels, common membrane lipids at high attomole levels and common metabolites at the mid-femtomole range.[80, 81] As MS is capable of detecting proteins and small molecules even down to zeptomole levels or below,[82, 83] it has therefore become an interesting technique for untargeted single-cell analysis. However, single-cell MS is still a difficult field, as analyte extraction, ionization and MS analysis all need to work optimally in order to detect a reasonable number of analytes.

Brain tissue

The two main classes of cells in the brain and the nervous system are neurons and glial cells. Neurons convey information from the surrounding world to the organism, and process this information in order to generate a response. They also convey information from our inner world, as they register information from the inner environment in order to maintain homeostasis. The neuron consists of a neuronal cell body, from which dendrites and one axon are projected (Figure 12). Dendrites receive information from other neurons while axons pass on the information through electrical impulses. While neurons are responsible for transfer of information, glial cells are non-neuronal cells with a wide range of functions in the nervous system.[84] One type of glial cells is the oligodendrocytes which create insulating myelin sheaths around the axons. The myelin sheath is a fatty substance, which facilitates
the transmission of electrical impulses from the neuronal cell body to the axon terminal.[85]

The brain consists of two distinct tissue types; white matter which mainly consists of myelinated axons, and grey matter which mainly consists of neuronal cell bodies.[86] These two tissue types have highly characteristic lipid compositions as grey matter has a higher molar percentage of phosphatidyl-ethanolamine and PC, while myelin and white matter has a higher molar percentage of cholesterol and cerebroside.[87] In MS analysis, membrane lipids and phospholipids in particular are known to cause ion suppression, [88] and different degrees of ion suppression will therefore occur in white and grey matter when performing MSI on a brain tissue section as was seen in Paper I.

![Figure 12 - Schematic illustration of a neuron. Image taken from: https://openclipart.org/detail/245373/neuron](https://openclipart.org/detail/245373/neuron)

**MSI of neurotransmitters**

The transfer of information between neurons occurs via release of neurotransmitters from the axon terminal upon arrival of the electrical impulse. The neurotransmitters then diffuse a short distance over the synaptic cleft, and bind to receptors on a dendrite of an adjacent neuronal cell body. Depending on the type of neurotransmitter, the neuron is either stimulated (excitatory neurotransmitters) or prevented (inhibitory neurotransmitters) from sending an electrical impulse through the axon. The most common excitatory neurotransmitter in the central nervous system is glutamate (Glu), while the most common inhibitory neurotransmitter is γ-aminobutyric acid (GABA). In several disease states, the systems of these neurotransmitters have been found to be dysregulated, and the levels of Glu and GABA have for example been found to be decreased in brains of patients with major depressive disorder.[89, 90] Another neurotransmitter that has been shown to be involved in
several disease states is acetylcholine (ACh), which has been implicated in the pathology in e.g. Alzheimer’s disease [91] and Parkinson’s disease.[92]

There are a number of examples in which MSI has been used to study neurological diseases in a variety of mouse models, such as Parkinson’s disease, Alzheimer’s disease, and migraine. [93-98] However, the majority of these studies have analyzed lipids, peptides and proteins.[99] It can be noted that despite the importance of neurotransmitters for the function of the nervous system, not many MSI studies have targeted small molecule neurotransmitters such as ACh, Glu and GABA.[100] The reason for this is that small molecule neurotransmitter analysis faces several technical challenges: 1) many small molecule neurotransmitters, where ACh is a notorious example,[101] are subjected to fast post-mortem degradation, 2) many small molecule neurotransmitters are present at very low in vivo concentrations and 3) matrix signals in conventional MALDI analysis often mask the signal of small molecule neurotransmitters.[100] This problem is more pronounced when the MALDI ion source is used with a time-of-flight mass analyzer, which currently has a lower mass resolving power than FT based instruments such as the Orbitrap. Methods to overcome the challenge of post-mortem degradation include very careful sample preparation [102, 103] whereas the challenge with overlapping MALDI matrix peaks has been tackled by using MALDI with high resolution mass analyzers, tailored MALDI matrices, and on-tissue derivatization.[68, 104-107]

In one study, the ambient MS technique laser ablation electrospray ionization was used to determine the spatial distribution of both lipids and small metabolites, such as the neurotransmitter GABA, in rat brain.[108] The technique utilizes a mid-infrared laser for sampling of the water-containing surfaces, and removes the need for any sample preparation such as matrix application. However, as the technique is sensitive to changes in water content due to the ablation process, specific measures such as a sample cooling were needed to keep the native water content constant in the tissue throughout the experiment. Sampling with nano-DESI is not dependent on water content and we therefore imagined that small-neurotransmitter imaging with nano-DESI would provide the benefits of ambient mass spectrometry without the need for a specific sample setup (Paper I).

Single cells

Single cell analysis has brought insight to a range of research fields over the last decades, from cancer [109-111] and stem cell biology[112, 113] to the study of drug resistance.[114, 115] The importance of studying single cells comes from the fact that even the most homogenous of cell populations harbor a wide plethora of unique phenotypes.[116] It is thus necessary to study individual cells to accurately understand the biology of a given multicellular
system. Classical single-cell analysis methods are patch-clamp for the study of cell electrophysiology,[117] the use of fluorescent tags to study intracellular interactions by fluorescence imaging[118] and polymerase chain reaction for single cell genomics and transcriptomics.[119, 120] High-throughput methods such as fluorescence activated cell sorting and other flow cytometry based techniques are also available for single cell analysis.[121] In these methods, antibodies are often used as reporter molecules to detect the presence of analytes of interest.[121] Antibodies are also utilized in mass cytometry, a technique in which the presence of antigens on single cells is measured with MS using heavy-metal isotopes as reporters.[122] All of these methods have been invaluable for the field of single-cell analysis, but none of them provide an untargeted analysis of metabolites. Over the last two decades, there has therefore been a growing interest in MS based single-cell analysis, which enables untargeted analysis of proteins, lipids and metabolites.[123]

Traditionally, MALDI and SIMS are the two main ionization techniques that have been used for untargeted single-cell MS analysis.[123] The unsurpassed lateral resolution of SIMS makes it ideal for cellular imaging, and it can even be used to generate 3D images of single cells.[80] However, SIMS is generally not suitable for the analysis of large biomolecules due to extensive fragmentation in the ionization process. For analysis of peptides and intact biomolecules, MALDI has thus been the typical method of choice, often using chemical fingerprinting (i.e. sampling of the whole cell without spatial resolution) rather than imaging.[123] However, substantial sample preparation of cells and sampling in vacuum makes MALDI and SIMS analyses of living cells impossible. As the metabolome might be altered under these conditions, there has been a recent boom in the development of ambient MS techniques for single-cell analysis as these techniques theoretically could be used to sample live cells in their native state.[124]

Sampling in ambient single-cell MS can be performed in several ways. One is through capillary microsampling, in which a sharp glass capillary is used to suck the contents out of a cell. The tip is then moved in front of the mass spectrometer for subsequent ESI, sometimes after adding ESI solvent to improve ionization.[125-127] In one capillary microsampling study, 22 metabolites and 54 lipid species from single human hepatocytes were identified through the use of ion mobility separation prior to MS detection.[127] This is to my knowledge the highest number of identified compounds reported in a cell as small as ~25µm [79]. Sampling in ambient single-cell MS can also be performed through liquid extraction techniques such as DESI,[128] and the single-probe.[129] The single-probe has a setup that is similar to nano-DESI, but in this technique the primary and secondary capillary are placed inside a dual-bore quartz needle which is used to pierce the cell and extract analytes from the cytoplasm. Much like in nano-DESI, reagents can be added to the extraction solvent, and addition of dicationic ion-pairing
agents was shown to improve detection of negatively charged species from single cells through the formation of positively charged adducts.[130] The aim of Paper II was to push the limits of nano-DESI and evaluate if this technique too can be used for single-cell analysis.
Studying histopathology with MSI

Histology can be defined as the study of microscopic structures within cells and tissues, and its influence on the field of anatomical pathology cannot be overstated.[131] Many diseases alter the morphology of tissues and organs in characteristic ways, and thus biopsies of affected organs can be used for histopathological diagnosis of diseases like cirrhosis,[132] infectious diseases [133], kidney diseases,[134] and, of course, cancer.[135] In addition to the commonly used Hematoxylin and Eosin (H&E) stain, which stain nucleic acids and cytoplasmic proteins respectively, visualization of some other chemical compound classes can be performed with stains such as Oil Red O for neutral lipids and Periodic Acid Schiff for carbohydrates.

With the advent of immunohistochemistry it also became possible to follow pathological changes with higher specificity on the molecular level, as antibodies can be used to stain for antigens involved in disease progression.[136] An immunohistochemistry analysis involves extensive sample pre-treatment of the tissue section, followed by binding of antibody to the antigen (often a protein) with subsequent detection of antigen-bound antibody over the surface.[137] The information obtained from such an experiment is thus protein localization and possibly quantitative data on protein abundance.[138] However, much like MSI, quantitative immunohistochemistry typically provides estimates rather than precise answers. [139] Although immunohistochemistry has proven to be an invaluable tool for histopathologists, the technique is targeted in its nature as specific antibodies are needed for specific antigens. In addition, antibodies typically bind larger chemical structures such as proteins or polysaccharides, and small molecules are generally only recognized as antigens when bound to specific carrier proteins.[140] Tissue analysis with MSI can therefore complement immunohistochemistry, as in addition to protein analysis it can be used for untargeted analysis as well as analysis of small molecules.[141]

To date, MSI has been used to perform untargeted metabolite and lipid analysis in a wide range of cancer tissue biopsies, such as lung cancer,[142] breast cancer,[143, 144], brain tumors,[145] bladder carcinoma,[146] and renal cell carcinoma,[147] to name a few. Thus, as altered lipid metabolism in cancer has become a more recognized phenomenon, MSI has become an increasingly important technique.[148] The use of MSI has not been restricted to cancer, however; it has also been used to study the mechanisms behind preterm birth,[54] rheumatism,[149] metabolic disorders,[150] and kidney
However, among the MSI studies of kidney disease so far, I have only found two looking at diabetes. This is despite the fact that diabetes is now the number one cause of end-stage renal disease in the western world.

**Diabetic nephropathy**

The kidneys have several important functions, such as removing waste products from the blood and adjusting the water- and electrolyte levels in the extracellular fluid. This is performed in the functional units of the kidney, which are the nephrons (figure 13). Blood is filtered in the glomeruli, and the filtrate is then concentrated and processed into urine in the tubule. It has long been recognized that in diabetic patients, it is common for kidney function to slowly decline over time. This condition is called diabetic nephropathy (DN), and affects about 30% of diabetic patients.

![Diagram of kidney anatomy](http://unckidneycenter.org/kidneyhealthlibrary/glomerular-disease)

DN has traditionally been said to progress in 5 distinctive steps with the first symptom being hyperfiltration, where an excessive amount of glomerular filtrate is formed followed by a second stage of “silent nephropathy”. In this second stage, few symptoms are exhibited, but biopsies often reveal morphological changes in the kidney tissue, mainly in the glomeruli. Patients may stay in this phase the rest of their lives, but if they do
not, stage three to four indicate progressively higher levels of persistent albuminuria. The presence of albumin in the urine shows that the kidneys are no longer able to filter the blood in a proper way, and that the filtration system in the glomeruli has now started to leak and reabsorption in the tubule cannot keep up. In stage four, the patient also exhibits an increased blood pressure and a lowered glomerular filtration rate.[156, 158] The fifth and final step is end-stage renal disease, which is a fatal condition where dialysis is required and renal transplantation is the best therapeutic option.[159]

In addition, a nonalbuminuric phenotype with progressive loss of kidney function has become more recognized over the last years, meaning that albuminuria cannot be used as a predictor for end-stage renal disease in all patients.[156] This illustrates that the pathogenesis in DN is highly complex and not completely understood despite decades of intense research, and accordingly, a large number of molecular mechanisms have been suggested to be involved.[160, 161] Many studies have looked at how protein levels are altered in the urine of patients with DN, which has given some important clues to the pathogenesis,[162] but fewer metabolomic studies have been performed.[163] In addition, very few studies have performed metabolomic studies directly in diabetic kidney tissue. The aim of Paper II was therefore to perform an untargeted metabolomic analysis of diabetic rat kidney at an early time point after diabetic onset to evaluate early molecular changes in response to hypoinsulinemia and hyperglycemia. Nano-DESI MSI was chosen as the method for analysis, as it was also of interest to obtain information about the localization of endogenous compounds within the kidney.
Results and discussion

Nano-DESI MSI of small molecule neurotransmitters

In Paper I, the aim was to evaluate if nano-DESI could be added to the toolbox for small-molecule neurotransmitter MSI. For a first proof-of-principle, the neurotransmitters ACh, GABA and Glu were chosen for analysis (Figure 14). When the experiment was planned, we knew that the risk of variable matrix effects over the tissue was extremely high, as described above. We also knew that different substances might react entirely different to regional matrices, and therefore decided to use three deuterated internal standards, ACh-D₉, GABA-D₂ and Glu-D₃ to correctly image each neurotransmitter (Figure 14). The internal standards were all added to the nano-DESI solvent and were delivered at a constant concentration throughout the experiments. Three replicate imaging experiments were performed on brain tissue sections from one female Sprague-Dawley rat.

The first question to answer was if the neurotransmitters could be detected at all from rat brain tissue using nano-DESI. The results showed that m/z values corresponding to all targeted neurotransmitters were indeed readily detected, and the identities of the compounds were further confirmed with MS/MS. In addition, it was shown that the peaks of the deuterated standards did not overlap with endogenous compounds.

Figure 14 - Chemical structures of the endogenous small-molecule neurotransmitters acetylcholine, γ-aminobutyric acid, glutamate and their respective deuterated standards used in Paper I.
Correction of matrix effects

The second question was if the detection of the neurotransmitters would be affected by the various matrices in the tissue. This was evaluated through inspection of the ion images generated of the deuterated standards. As a reminder, if there would be no matrix effects, the signal intensity would be equal all over the image as they were delivered at a constant concentration. However, it is clear from Figure 15a-c that for all neurotransmitters, and especially for GABA and Glu, the signal intensity drops dramatically over the brain as compared to the glass outside the tissue. This shows that there is ion suppression at play in this analysis. In addition, for all neurotransmitters it is clear that the ion suppression is stronger in grey matter as compared to white matter, where the signal intensity is regionally increased (white matter is highlighted in yellow in Figure 15k).

Figures 14d-f show what the ion images of the neurotransmitters look like without any normalization to internal standard. Out of these, it is especially interesting to note the appearance of Glu which before normalization seems to be localized to the white matter (Figure 15f). This would be a highly curious result as white matter only consists of axons, and it would be expected that a neurotransmitter would be found in grey matter where synapses are localized. However, after normalization to the deuterated standard a whole other picture emerges (Figures 15g-i). Identical ion images to those in Figures 15g-i were obtained for all detected cation species, when normalizing to the internal standard ionized on the same cation. The normalized ion images now show all neurotransmitters are detected with higher signal intensity in grey matter than in white matter, as expected. This illustrates the great importance of matrix effect evaluation before drawing any conclusion about the biology from an ion image. In this case it was clear that localization of Glu to white matter seemed odd, but in other cases where there is no knowledge about the biological role of the analyte one might just accept non-normalized ion images without questioning their validity.

The most striking feature after normalization is the clear localization of GABA to the medial septum-diagonal band complex (highlighted in orange in Figure 15k). This is in accordance with previous observations which have also shown that GABA is found with higher abundance in this region.[68, 108] The same region also shows a lower abundance of Glu.
Shotgun quantification and post-analysis dissection

One major benefit of MSI is the possibility of performing in silico dissection of the tissue post analysis. The brain sections used in Paper I contained more than a handful of anatomical substructures, (Figure 16a) and by selecting these as specific regions of interest (ROI:s) it is possible to extract the pixels (i.e. mass spectra) and look at regional differences in the data.

Figure 15 - Ion images of ACh, GABA and Glu in a rat brain tissue section. (a) Non-normalized ion image of [ACh-D9]+, (b) non-normalized ion image of [GABA-D2 + H]⁺, (c) non-normalized ion image of [Glu-D₃ + Na]⁺, (d) non-normalized ion image of endogenous [ACh]+, (e) non-normalized ion image of endogenous [GABA + H]⁺, (f) non-normalized ion image of endogenous [Glu + Na]⁺, (g) normalized ion image of [ACh]+, (h) normalized ion image of [GABA + H]⁺, (i) normalized ion image of [Glu + Na]⁺, (j) optical image of the analyzed brain tissue section, (k) optical image of analyzed brain tissue section with white matter regions highlighted in yellow and the medial septum-diagonal band complex highlighted in orange. Scale bar: 5 mm. The signal intensity of the ion images scale from dark to bright. Reprinted from Paper I with permission from The Royal Society of Chemistry.
Figure 16 - Quantification of neurotransmitters in selected regions of interest (ROI). Data points were pooled from the defined ROIs in 3 replicate nano-DESI MSI experiments. (a) Defined ROI:s. The optical image shows one of the brains from the three replicate nano-DESI MSI experiments. COR = Cortex (the number of acquired mass spectra (n) used for quantification within this region was 10 261), CG = Cingulate cortex (n = 4280), WM = White matter (n = 4488), CPu = Caudate Putamen (striatum) (n = 14 749), LS = Lateral septal nucleus (n = 4227), NA = Nucleus accumbens (n = 5675), MSDB = Medial septum-diagonal band complex (n = 4159) and Whole = entire brain tissue section (n = 76 649) (b) graph showing ACh concentrations in nM per pixel in eight ROIs (c) graph showing GABA concentrations in μM per pixel in eight ROIs (d) graph showing Glu concentrations in μM per pixel in eight ROIs. Error bars represent ±1 standard deviation. Reprinted from Paper I with permission from The Royal Society of Chemistry.

To determine the relative abundances of the three neurotransmitters in different regions, quantitative data was extracted from eight defined ROIs (Figure 16). The result in Figure 16 is given in μM/pixel, or nM/pixel for ACh, representing the concentration in the nano-DESI solvent at the time of ionization. However, comparisons of analyte abundance between different regions can only be performed if the extraction efficiency is similar across the tissue. To evaluate this, we performed localized extraction experiments in grey and white matter (shown in Figure S3 in supplemental information of Paper I). These showed an overall similar behavior in the two tissue types, indicating that the differences seen in Figure 16 should not be due to varying extraction. In addition, we showed that quantification data of GABA, which was detected as [M+H]+, [M+Na]+ as well as [M+K]+, gave the same results
independent of cation species as long as quantification was performed against the same cation species of the internal standard. According to the results, GABA was about twice as abundant in the medial septum-diagonal band complex as compared to cortex while glutamate showed the opposite relationship. We tried to find literature values to compare our results with, but were only able to find literature data for cortex vs caudate putamen (striatum). The lack of literature values for other anatomical structures might reflect that these are difficult to isolate by dissection, and highlights the possible use for MSI to provide data on chemical localization. In any case, literature values showed a relative abundance of about 1:1 for Glu in cortex vs striatum,[164] which is the same as the result in Paper I. For GABA, literature values of the cortex-to-striatum ratio are found between 1:2 [165] and 1:1 [164], which corroborates our result of 1:1. ACh was present at relatively equal abundances over the surface, which is in disagreement with data obtained from brains fixated with microwave irradiation.[101] These studies show that the levels of ACh in striatum should be about four times higher than in cortex.[101] This illustrates one of the analytical challenges in small-molecule neurotransmitter imaging, namely post-mortem degradation of the analytes of interest. Microwave irradiation instantly causes denaturation of the enzyme acetylcholine esterase which is responsible for the diminished ACh-levels post-mortem, but this method of euthanization requires highly specialized personnel and obtaining ethical approval is more difficult than for conventional methods. I have not found any regulations regarding this in Sweden, but I imagine it might not be possible to perform such experiments in this country. However, this proof-of-principle study shows that nano-DESI MSI can be used to detect ACh even at diminished concentrations, and could therefore be used to image ACh in specifically prepared samples in the future.

Nano-DESI MSI of diabetic and control kidney tissue

While Paper I represented a targeted analysis, Paper II represents an untargeted nano-DESI MSI analysis. The aim was to compare diabetic kidney tissue to control kidney tissue, to see if any differences could be detected in their chemical composition. Diabetes was induced in male Sprague-Dawley rats by injection of the drug streptozotocin (STZ) which is highly toxic to the insulin producing β-cells of the pancreas.[166] The model thus simulates type I diabetes in the rat as hyperglycemia is as a result of insulin deficiency, and it has been extensively used to study DN.[167] The rats were euthanized two weeks after STZ treatment, when histopathologists are yet unable to tic
Detected compounds in control kidney

To set the baseline for the data obtained from diabetic tissue, we first evaluated data from the control tissue sections. Overall, roughly 250 unique chemical formulas (that is, not counting several cationic species of the same molecule) corresponding to endogenous metabolites were found in the kidney. Several of these were detected with specific localization to anatomical substructures within the tissue (Figure 17a). Specifically, about half of the detected chemical species were found with localization to the outer stripe of the outer medulla (OS, Figure 17b), the cortex (Figure 17c) or both (Figure 17d). Roughly 50 compounds were found with an even distribution over the whole tissue and most of the remaining compounds were localized to the inner strip of the outer medulla (IS, Figure 17e). Only a few compounds were found specifically in the inner medulla (IM, Figure 17f).

Figure 17 - Analysis of kidney using nano-DESI, showing TIC normalized ion images. a) Optical image of kidney section. OS = outer strip of outer medulla, IS = inner strip of outer medulla and IM = inner medulla. b) Ion image of m/z 170.0923, identified as [Methylhistidine+H]^+. c) Ion image of m/z 218.1386, identified as [Propionylcarnitine+H]^+. d) Ion image of m/z 741.5307, tentatively assigned as [Sphingomyelin 34:1+K]^+. e) Ion image of m/z 140.0681, identified as a combination of [Betaine+Na]^+ and [Valine+Na]^+. f) Ion image of m/z 205.0681, tentatively assigned as [Sorbitol+Na]^+. Scale bar = 1 mm. The relative signal intensity of the ion images scale from dark to bright.
Matrix effects in kidney

The attentive reader will note that the ion images in Figure 16 are made with normalization to the TIC, although I spent most of the last section telling you about the importance of compensating for matrix effects using standards. Well, here is the problem with untargeted MSI analysis: when you do not know what you are looking for, you do not know which standards to add for normalization. However, a few things can be said about nano-DESI analysis of kidney tissue. Notably, marked differences were seen in the spectra collected over the cortex/OS (Figure 18a) and spectra collected over the IS/IM (Figure 18b). First of all, the signal intensity of betaine increases dramatically and completely dominates the spectrum in the IS/IM, as can be seen when comparing Figure 18a and 18b. Secondly, a clear shift in cationization is observed towards favored formation of [M+Na]^+ in this region. These changes correspond well to the biological function of the loop of Henle, which is located in the medulla. In this region, the concentration of urine (i.e. osmolarity) is regulated so that the kidney can either excrete or conserve water in order to keep a constant body fluid osmolarity. To achieve this function, an osmotic gradient is present in the extracellular fluids of the kidney, going from 300 mosmol/L in the cortex up to 1200 mosmol/L in the inner medulla.[155] The high osmolarity is in part due to reabsorption of sodium and chloride, leading to very high sodium concentrations. The cells in this region are thus subjected to incredibly high osmotic stress, and to protect the cells from shriveling, they contain high concentrations of osmoprotective compounds such as betaine and sorbitol (Figures 17e-f).[168]

This lead us to the conclusion that the major differences in matrix effects are found between the cortex/OS and IS/IM. We commonly noted that a molecule cationized on sodium exhibited a high relative abundance in the IS/IM (Figure 18c), whereas the same molecule cationized on potassium (Figure 18d) showed a low relative abundance in the IS/IM. During analysis, a small amount of lysophosphatidylcholine 19:0 was present in the solvent in order to monitor the stability of the ESI signal. Although it was not meant to be added as an internal standard, it could be used to generate the normalized ion images of other PC species. This is illustrated by Figures 18e and 18f, which show that if an appropriate standard is added to the nano-DESI solvent, the matrix effects caused by differences in cation abundances can be effectively compensated for. This is in accordance with previous studies.[55] In addition, the figures show that normalization has a smaller impact on the appearance of the cortex/OS.
Figure 18 - Analysis of control kidney tissue with nano-DESI. a) Typical mass spectrum collected over the cortex and outer stripe of outer medulla, b) Typical mass spectrum collected over the inner stripe of outer medulla and inner medulla, c) TIC normalized ion image of PC 36:4 cationized on sodium, d) TIC normalized ion image of PC 36:4 cationized on potassium, e) Ion image of PC 36:4 cationized on sodium, normalized to internal standard lysophosphatidylcholine 19:0 cationized on sodium, f) Ion image of PC 36:4 cationized on potassium, normalized to internal standard lysophosphatidylcholine 19:0 cationized on potassium.
Differences between diabetic and control kidney

In this study, three kidney sections from each of three control rats and three diabetic rats were analyzed with nano-DESI MSI. Some of the diabetic kidney tissue sections showed signs of hypertrophy, and had been damaged in the IM during sectioning (Figure 19). This in combination with the clear matrix effects displayed in the IS/IM, caused us to focus the data analysis on the cortex/OS. To achieve this, kidney tissue sections were H&E-stained post analysis and the cortex/OS was chosen as the ROI (Figure 19). As many of the functional changes seen in diabetic nephropathy are connected to the function of the glomeruli, which are located in the cortex, the omission of the IS/IM was seen as acceptable. Mass spectra from the ROI were extracted and used to compare the signal intensities of detected \( m/z \)-values in control and diabetes.

Figure 19 – Optical image of control and diabetic kidney, showing a typical damage seen in diabetic samples after sectioning. After analysis, the sections were H&E-stained and the cortex/out strip of the outer medulla was chosen as the region of interest, shown in dark grey. Scale bar = 2mm.

In total, 24 compounds identified with MS/MS were found to be detected with significantly increased signal intensity in diabetic kidney tissue, while 14 compounds identified with MS/MS were found to be detected with significantly decreased signal intensity (Table 1). Among the compounds detected with increased intensity was a molecular formula corresponding to glucose, which was seen as a proof of the validity of this approach. Many of the altered chemical species were fatty acids, acylglycerols and long-chain acyl-carnitines, which points to a dysregulated fatty acid oxidation. An increase of several short-chain acylcarnitines, which are products of branched chain amino acid catabolism, was also detected. In addition, a decrease of several amino acids species and their metabolites was seen, suggesting a dysregulated amino acid metabolism.
Table 1 - Molecules detected with significantly altered signal intensity in rat kidney sections from STZ-treated rats (2 weeks post treatment) compared to control using nano-DESI MSI analysis. FA = fatty acid, MG = monoacylglycerol, DG = diacylglycerol.

<table>
<thead>
<tr>
<th>Increased signal in diabetes</th>
<th>Compound</th>
<th>Decreased signal in diabetes</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical formula</td>
<td></td>
<td>Chemical formula</td>
<td></td>
</tr>
<tr>
<td>C4H6O3</td>
<td>E.g. 2-ketobutyric acid</td>
<td>C3H7NO3</td>
<td>Serine</td>
</tr>
<tr>
<td>C6H8O4</td>
<td>E.g. 3-Hexenedioic acid</td>
<td>C2H7NO2S</td>
<td>Hypotaurine</td>
</tr>
<tr>
<td>C6H10O5</td>
<td>E.g. 3-hydroxy-methylglutaric acid</td>
<td>C3H7N3O2</td>
<td>Guanidinoacetic acid</td>
</tr>
<tr>
<td>C6H12O6</td>
<td>E.g. Glucose</td>
<td>C5H9NO3</td>
<td>E.g. Hydroxyproline</td>
</tr>
<tr>
<td>C9H17NO4</td>
<td>C2-carnitine</td>
<td>C7H7NO2</td>
<td>E.g. Anthranilic acid</td>
</tr>
<tr>
<td>C10H19NO4</td>
<td>C3-carnitine</td>
<td>C7H13NO2</td>
<td>Proline betaine</td>
</tr>
<tr>
<td>C11H21NO4</td>
<td>C4-carnitine</td>
<td>C5H11N3O2</td>
<td>4-Guanidinobutanoic acid</td>
</tr>
<tr>
<td>C12H23NO4</td>
<td>C5-carnitine</td>
<td>C7H15NO2</td>
<td>E.g. Dehydro-carnitine</td>
</tr>
<tr>
<td>C11H21NO5</td>
<td>C4-OH-carnitine</td>
<td>C5H9NO4</td>
<td>Glutamate</td>
</tr>
<tr>
<td>C18H30O2</td>
<td>FA 18:3</td>
<td>C6H9N3O2</td>
<td>Histidine</td>
</tr>
<tr>
<td>C18H32O2</td>
<td>FA 18:2</td>
<td>C7H11N3O2</td>
<td>Methylhistidine</td>
</tr>
<tr>
<td>C18H34O2</td>
<td>FA 18:1</td>
<td>C9H11NO3</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>C19H34O2</td>
<td>Methyl linoleate</td>
<td>C11H12N2O2</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>C10H14N5O7P</td>
<td>AMP</td>
<td>C38H76NO8P</td>
<td>Phosphatidylcholine 30:0</td>
</tr>
<tr>
<td>C21H36O4</td>
<td>MG 18:3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C21H38O4</td>
<td>MG 18:2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C21H40O4</td>
<td>MG 18:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C23H45NO4</td>
<td>Palmitoylcarnitine (C16:0)</td>
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<td></td>
</tr>
<tr>
<td>C25H45NO4</td>
<td>Linoleylcarnitine (C18:2)</td>
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<tr>
<td>C25H49NO4</td>
<td>Stearoylcarnitine (C18:0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C35H68O5</td>
<td>DG 32:0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C37H68O5</td>
<td>DG 34:2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C37H70O5</td>
<td>DG 34:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C39H68O5</td>
<td>DG 36:4</td>
<td></td>
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</table>

We became very intrigued with the number of increased lipid species, and that almost all compounds contained an unsaturated fatty acyl side chain.
with 18 carbons. Due to the similar side-chain composition between the compounds, a concern was raised that the free fatty acid (FFA) and monoacylglycerol (MG) species could have been a result of in-source fragmentation of increased diacylglycerol (DG) species. To verify that the FFA and MG species were truly increased, a standard of DG 13:0/13:0 was bought and infused at the same settings used in Paper II. The results showed that virtually no in-source fragmentation of \([DG 26:0+Na]^+\) or \([DG 26:0+K]^+\) occurred. Curiously, a neutral loss of water from \([DG 26:0+H]^+\) was clearly observed, but loss of the side chain 13:0 to form MG 13:0 was not detected. We therefore draw the conclusion that MG 18:1, MG 18:2, MG 18:3 and the respective FFAs truly are present with a higher abundance in diabetic kidney tissue. Several studies have previously shown that the renal lipid content increases in response to diabetes,[169] but we became painfully aware that there is a lack of studies looking at the acyl group compositions of this lipid content. Paper II is therefore among the first studies to provide comprehensive data showing a preferential accumulation of unsaturated FFA, MG and DG species.

Interestingly, we found that an increasing number of studies suggest that mitochondrial dysfunction may be one of the driving forces behind DN.[170] As both fatty acid oxidation and amino acid metabolism is tightly linked to the mitochondria,[171, 172] the results reported in Paper II support this theory, as the changes were observed prior to any histopathological changes. However, the results cannot detail if the lipid accumulation and amino acid depletion is a cause or effect of mitochondrial dysfunction, and we specifically would like to see more studies relating to the mechanisms of possible toxic effects of lipid accumulation in order to paint a clearer picture.

**Single-cell analysis using nano-DESI MS**

As the interest in mass spectrometry based single-analysis has increased over the years, we wondered if nano-DESI MS would be sensitive enough to detect such small sample volumes. The aim of Paper III was to investigate this question. The single cells of choice were buccal epithelial cells that were sampled with cotton swabs and spread on glass slides.

**Adapting nano-DESI for single cell analysis**

The typical nano-DESI probe consists of fused silica capillaries with an I.D. of 50 µm and an O.D. of 150 µm, where the liquid bridge generally has the same size as the O.D. of the capillary. As the cheek cells had a typical size of 60-90 µm, we wished to downsize the probe in order to minimize sample dilution. Initially, I tried to pull the capillaries to an O.D. of ~30 µm. But as it was difficult to get repeatable sizes of the tips, and they became extremely
prone to damage when they were so thin with the glass exposed, we soon realized that this was not a viable approach. Instead, fused silica capillaries with an I.D. of 20 µm and an O.D. of 90 µm were used.

In previous nano-DESI MSI studies, the solvent has generally consisted of 9:1 methanol:water without any additives such as formic acid, and ionization has been accomplished by adduct formation with sodium and potassium that has been extracted from analyzed samples. However, for single-cell analysis, 0.3% formic acid was added to the nano-DESI solvent to improve ionization.

With the abovementioned modifications, it was possible to detect a number of endogenous metabolites and lipids from single cells. To ensure that only single cells were sampled, optical images were taken of the glass slide to find areas with a suitable single-cell density (Figure 20a).

Contact between a single-cell and the nano-DESI probe was characterized by a temporary shift in cationization of background peaks, from [M+Na]+ to [M+K]+, due to the high intracellular concentration of potassium (Figures 20b-c).[59] During this shift, a number of endogenous compounds could be detected (Figures 20d-e). Identification was performed using MS/MS and resulted in a list of 14 detected amino acids, six other small metabolite species, and 26 membrane lipids within several lipid classes. Among them were 10 plasmalogen species, which to the best of our knowledge have not been previously reported using ambient-, SIMS- or MALDI-based single-cell MS analysis. It should also be mentioned that a much larger number of compounds were detected during single cell extraction, but have not yet been identified with MS/MS, leaving room for improvement for nano-DESI in single-cell analysis. However, this number of identified species is among the highest obtained in any ambient single cell method without any form of separation.[124]

The aim of a single-cell analysis is often to compare cells based on their chemical composition. To test if single-cell nano-DESI could be used to compare cells, cheek cells from three human donors were sampled and analyzed. It was noted already during analysis that there were obvious differences in cells between people, as would be expected due to individual differences in diet and bacterial flora of the oral cavity. These differences could be visualized using non-metric multidimensional scaling, which showed that the cells from the same donor had highest similarity.

As it was noted that analyte extraction followed a recognizable pattern in all cells (as seen in figure 20d-e), with a quick increase in signal followed by a few seconds of continuous depletion, we performed a lipid quantification using PC 46:0 as internal standard. Calculations were performed according to Equation 6.

\[
n_{end,tot} = \sum \left( \frac{n_{end}}{l_{std}} \times c_{std} \times t \times \frac{CF_{end}}{C_{F, std}} \times F \right)
\]  

(6)
In short, the concentration of the specific lipid in the nano-DESI solvent was calculated by \( \frac{I_{\text{end}}}{I_{\text{std}}} \times c_{\text{std}} \). The amount of lipid could then be calculated from the concentration as the scan time \( (t) \) and flow rate of the solvent \( (F) \) were known. As PC 46:0 had longer fatty acyl chains than all endogenous PC species that were detected, compensation for differences in ionization efficiency were taken into account by using the carbon factor for PC 46:0.

Figure 20 - Static mode nano-DESI MS of endogenous molecules in a single cell. Contact between the single cell and the nano-DESI probe was achieved at 0.12 min. (a) Optical image of an area with six single cells. One typical cell has been enlarged for clarity. (b) Extracted ion chronogram of an unidentified background peak (BG) cationized on sodium, m/z 353.2658. (c) Extracted ion chronogram of an unidentified background peak cationized on potassium, m/z 369.2398. (d) Extracted ion chronogram of protonated creatine at m/z 132.0767. (e) Extracted ion chronogram of protonated PC 36:2 at m/z 786.5995. Adapted from Paper III with permission from The Royal Society of Chemistry.
(CF$_{\text{std}}$) and the endogenous PC (CF$_{\text{end}}$). The amounts were then summed over the cell extraction ($n_{\text{end, tot}}$).

The result showed that PC 34:1 and PC 36:2 were the most common lipids, in agreement with previously reported data on fatty acid composition in cheek cell phospholipids.[173] In addition, the total amount of PC in single cheek cells was estimated to be 7 amol $\mu$m$^{-3}$, assuming a spherical shape and a diameter of 70 $\mu$m. This is comparable to the amounts found in other kinds of cells, for example 10 amol $\mu$m$^{-3}$ for Chinese hamster ovary cells.[174, 175] Although a suitable reference material would be necessary to fully evaluate the capabilities of single-cell quantification with nano-DESI, this proof-of-principle study displays that the technique has the sensitivity needed to characterize membrane lipid composition.

One desirable quality of a single-cell analysis method is high-throughput. Nano-DESI single-cell analysis as described above requires that a technician manually finds single cells and places them in contact with the probe, which is a method that might enable analysis of up to 100 cells per day if a method for systematic placement of single cells over a surface was developed. However, automated high-throughput single-cell analysis will need to be developed for single-cell MS to be able to compete with targeted methods such as fluorescence activated cell sorting. We therefore spread cells over a surface and performed a nano-DESI MSI experiment, in which we scanned the surface to see if any cells could be detected. Resulting ion images showed that a number of endogenous compounds were detected with specific localization to the cells. The result opens up for the possibility of developing an automated nano-DESI based single-cell MS analysis with higher throughput.

Pneumatically assisted nano-DESI MS

The benefit of using ambient mass spectrometry for single-cell analysis is the possibility of keeping cells alive until the moment of analysis. To achieve this, the cells need to be kept in their native state for as long as possible and one way is to sample them using a physiological buffer. However, these types of buffers are water based, and as described previously it is difficult to overcome the high surface tension of purely aqueous solutions in nano-DESI MS. In order to increase the solvent versatility, a pneumatically assisted nano-DESI setup was developed as described in Paper IV (Figure 21).

Effect on metabolite analysis

When the properties of nano-DESI MS analysis using the pneumatically assisted device were evaluated, it was soon realized that it had a big impact on the detection of metabolites from biological samples. Specifically, it was
shown that the signal-to-noise of small molecular species was improved up to a thousand times as compared to conventional nano-DESI. However, for larger species such as lipids with m/z 700-900, the signal-to-noise dramatically decreased when the nebulizing gas was added. This was somewhat surprising, as we assumed that a more complete desolvation would benefit the detection of all species. One likely explanation for the decrease in signal intensity of heavier species is the longer distance between the probe and MS inlet that is used with the pneumatically assisted device.[176]

\[ \text{Figure 21} - \text{Schematic representing the experimental setup for pneumatically assisted nano-DESI device. Part (a) representation of the nano-DESI nebulizer device drawn to scale, and (b) schematic of the secondary capillary detailing solvent and gas flows. Reprinted from Paper IV with permission from The Royal Society of Chemistry.} \]

**Decreased height dependence on signal intensity**

Another important difference between conventional nano-DESI and pneumatically assisted nano-DESI was a decreased dependence in the height between the sample and the probe on the signal intensity of the analyte. Experiences from conventional nano-DESI MSI show that if the height is altered
by 5-10 µm during a run, this will have a big impact on the signal intensity of extracted analytes, and therefore the appearance of their ion images. However, the same effect was not noted when using the pneumatically assisted device. The reason for the decreased height dependence is not clear as it is impossible to tell if the effect originates from altered fluid dynamics in the extraction process or an altered ionization process. In any case, this phenomenon should be evaluated in further detail due to the potential benefits that can be gained from it.

Increasing solvent versatility

As mentioned, the initial aim of developing the pneumatically assisted nano-DESI source was to facilitate the use of aqueous solutions as the nano-DESI solvent. Comparison of mass spectra acquired from a biological sample using either 9:1 methanol:water or pure water as solvent showed that the spectra were highly similar in the low mass region between m/z 100-300. However, about 300 peaks were detected exclusively or detected with higher signal intensity using pure water as solvent. Not surprisingly, detection of membrane lipids was not achievable with water. However, this opens up for new possibilities in metabolite analysis as ion suppression effects should be minimized in the absence of surface active lipids.

An imaging experiment was performed to compare the MSI characteristics of conventional nano-DESI and pneumatically assisted nano-DESI using 9:1 methanol:water, as well as pneumatically assisted nano-DESI using pure water (Figure 22). Although the images overall showed similar tissue locations, some differences between the methods could be noted. The sharpest contrast was found in images generated with pneumatically assisted nano-DESI using 9:1 methanol:water, due to the increased sensitivity obtained for low weight molecular species using this device. For the compound with m/z 235.1650, it can also be noted that detection in the cortex was achieved with pneumatically assisted but not conventional nano-DESI MSI (Figures 22d-e). Paper IV also presented the first ever ion images generated with pure water in a liquid extraction MSI technique. Although imaging using pure water was a bit more challenging than 9:1 methanol:water due to the higher surface tension of the liquid bridge, the features in the tissue could still be successfully imaged (Figure 22c and f). It was also possible to find m/z-values with specific localization in the tissue, that could not be detected using 9:1 methanol:water (Figure 22g-i)
Figure 22 - Ion images attained from rat kidney tissue sections. Ion images for m/z 146.1175 (a, b, and c), m/z 235.1650 (d, e, and f), and m/z 129.0397 (g, h, and i). Images a, d and g were obtained with conventional nano-DESI, while b, e and h were generated using pneumatically assisted nano-DESI. Water was used as an extraction solvent in conjunction with the nebulizer set up for c, f, and i. The color scale is relative for each individual image, where a brighter color depicts higher intensity. Reprinted from Paper IV with permission from The Royal Society of Chemistry.
Conclusions and future directions

The field of MSI is continuously developing, and a repertoire of methods is now available for imaging of a wide range of analytes in a wide range of samples. It has overcome a number of challenges, but a few issues still need to be resolved. During my time as PhD student, I have seen that one of the major challenges within this field is that of variable matrix effects over biological samples, and that it is something that the community as a whole needs to address with combined efforts. In this thesis, two new studies add to the existing body of work which shows that nano-DESI MSI is a powerful technique in this respect, as internal standards added to the continuously supplied solvent can be used to compensate for differences in chemical composition (Paper I) as well as cation abundance (Paper II).

A question relating to matrix effects is the challenge of quantification. Today, MSI can be used to estimate absolute quantities or to perform relative quantification of analytes over a surface, similar to immunohistochemistry-based techniques. Therefore, the challenge is to push MSI towards becoming a routine method for accurate absolute quantification. This would open up new possibilities for MSI in research areas such as pharmacology and biomarker discovery. One barrier in the development of quantitative MSI is currently the lack of good reference surfaces. How do you evaluate the technical variability of a method when you cannot analyze the same sample multiple times? How do you test variability when you don’t know exactly how the analyte abundance changes over the surface? When you do not have complete control over sample topography? The development of standardized reference surfaces with homogenous abundances would greatly improve the possibilities of optimizing and controlling factors that impact signal variability in quantitative MSI. One of the main factors impacting signal variability in nano-DESI today is height differences between the probe and sample. Therefore, the finding that pneumatically assisted nano-DESI is less sensitive to these differences opens up for the possibility of developing a more reliable quantitative nano-DESI MSI method (Paper IV). This is definitely something that should be studied in greater detail. In addition, a few recent studies by Nguyen et al. show that nano-DESI can be coupled to shear force microscopy to more precisely monitor the distance between the probe and the sample, something that has the possibility to benefit quantitative nano-DESI MSI.[177, 178]
Despite these challenges, nano-DESI MSI could be successfully used to detect differences in chemical composition between kidney sections from control and diabetic rats, in one of the few MSI studies performed on diabetic kidney tissue (Paper II). The study was performed at such an early time point (two weeks after induction of diabetes) that no histopathological changes correlating to DN could yet be seen in the tissue. The detected changes relate to the energy metabolism of the kidney, supporting one of the current theories that mitochondrial dysfunction may be one of the driving forces in the pathogenesis of DN. Imaging data shows that not one specific region of the kidney is affected first, but differentially detected compounds are found with localization to a number of anatomical substructures. In future work, appropriate internal standards should be used to verify the localizations of affected compounds and quantify the changes.

In this thesis, two new applications of nano-DESI MS for challenging samples have been presented, namely analysis of small-molecule neurotransmitters in brain tissue (Paper I) and single-cell analysis (Paper III). In the neurotransmitter study, three internal standards were simultaneously added to the nano-DESI solvent for multiplexed analysis, and it would be interesting to see how far this multiplexing can be taken and how the system is affected by an increased number of standards. All in all, we believe that nano-DESI MSI is well suited for the study of small-molecule neurotransmitters, and that it could be applied to the analysis of pathological changes in localization and abundance of neurological disease models. We additionally show that nano-DESI MS can be used to detect a large number of metabolites and lipids from single cells. As buccal epithelial cells are rather large (on average ~70 µm) compared to other cells in the human body (typically ~10-20 µm), future experiments need to be performed to evaluate if nano-DESI can be used for analysis of smaller cells. A first approach should be to analyze cells using the pneumatically assisted nano-DESI device, as its increased sensitivity for metabolites might counterbalance the decrease in sample volume. Future studies should further be aimed at improving throughput, to enable the study of large cell populations.

In summary, this work demonstrates that nano-DESI MS is a versatile tool for imaging as well as analysis of limited sample amounts. Although it is still used by relatively few groups, due to the lack of a commercial setup, continuous efforts keep bringing the technique forward and it will be interesting to see what the future brings.
Populärvetenskaplig sammanfattning

Analytisk kemi handlar i mångt och mycket om att få fram svar om ett prov där någon frågar ”vilka kemiska substanser innehåller det här provet, och i vilka halter?”. Beroende på vilka kemiska substanser och vilken typ av prov som är av intresse så behöver specifika analytiska metoder användas. Den här avhandlingens fokus har varit att använda och utveckla en relativt ny metod för att analysera prov som placerats på ytor.

Metoden, som heter nanospray desorption elektrospray jonisering med masspektrometri (nano-DESI MS), bygger på att två mycket tunna kapillärer placeras i en vinkel med varsin öppning mot varandra, mellan vilka det kontinuerligt pumpas en vätska. Då vätskan rör sig från den ena kapillären till den andra så bildas en mycket liten vätskebrygga mellan de två kapillärerna. Om denna vätskebrygga placeras i kontakt med ett prov som lagts på en yta, så kommer kemiska substanser från provet att tas upp i vätskan och föras vidare genom kapillären till en masspektrometer. Genom masspektrometrisk analys får man veta molekylvikternas för de kemiska ämnen som finns i provet, vilket ger en bra grund för att säkerställa de kemiska ämnenas identitet. Man kan även med hjälp av internstandarder få en god uppfattning av de enskilda ämnenas mängd.


Även om nano-DESI idag används av relativt få forskargrupper, då tekniken inte finns tillgänglig kommersiellt, så vill vi hävda att den är högst användbar inom ett stort antal vetenskapliga frågeställningar. Denna avhandling visar på att metoden kan appliceras för att studera ett antal olika typer av vävnader och prov, och att det finns potential att hitta ännu fler applikationer tack vare den nya utvecklingen som använder sig av kvävgas vid joniseringen-
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In my licentiate thesis, I wrote that I would save the really long acknowledgement section for next time. Here we are, and I have many to thank for the fact that this thesis came into existence.

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