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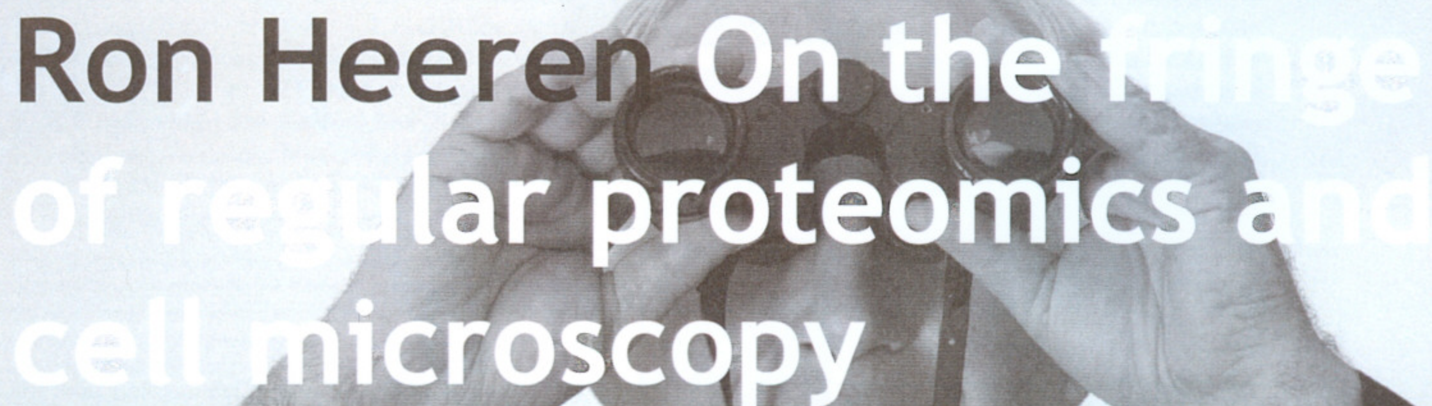
Ron Heeren On the fringe of regular proteomics and cell microscopy



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Imaging Mass Spectrometry can provide local chemical information on a wide variety of biological surfaces. This allows us to construct extensive molecular maps of cells and tissue surfaces using modern mass spectrometric techniques. In collaboration with various manufacturers and pharmaceutical industries and with the benefits of the NPC network we are able to bring IMS a step further in the direction of proteomics.

In terms of surface analysis, Imaging Mass Spectrometry (IMS) has already been known as a very valuable tool for many years, especially in the field of semi-conductors, with elemental composition analysis at a nanometer scale using secondary ion mass spectrometry (SIMS). However, its application to biomolecular surface imaging has been, and still is, a challenge. In particular the development of reproducible sample preparation, desorption and ionization technologies that allow the concise study of intact biomolecules at surfaces. This has extended the complexity of IMS well beyond atomic species.

This development in IMS required among others the adaptation of the SIMS technique to meet these new targets. Good examples are the development of polyatomic primary ion guns or the use of specific sample preparation techniques like matrix deposition and metal coating of the sample to enhance sensitivity and reduce fragmentation (respectively matrix enhanced ME- and metal assisted meta-SIMS) [1]. At the same time, matrix assisted laser desorption and ionisation (MALDI) also began to be used for imaging purposes. As MALDI-MS is a method mainly used for conventional proteomics it seems an ideal imaging tool because of its soft ionisation, capable of ionising and transferring intact bio-molecules to the gas phase. Recently an ion microscope (Physical Instruments) has been coupled to a MALDI source. This allowed direct tissue imaging with MALDI-specific sensitivity at so far unsurpassed sub-cellular spatial resolution [2].

Advantage of a consortium In order to identify and localize specific bio-molecules like cancer biomarkers, in the context of NPC, a tight collaboration with clinicians, pharmaceutical companies and instrument manufacturers such as Bruker-Daltonik has been established. We take advantage of this consortium, together with the NPC partners Erasmus Medical Centre and Groningen University, to develop and establish innovative analytical instruments and protocols for the analysis of tissue sections from biopsies or test animals by Imaging Mass Spectrometry. The addition of pathologists' morphological knowledge of the tissues with the chemical images that IMS provides for each component ideally allows the identifica

About creating value with proteomics:

Imaging Mass Spectrometry proves to be a versatile technology

"Proteomics is such an interdisciplinary field that it is impossible to be successful without collaboration with other disciplines," says Ron Heeren, a professor in Imaging Mass Spectrometry (IMS) at the FOM-institute AMOLF. Heeren's group works with various academic and industrial partners to apply IMS to the imaging of biomolecules on the surface of tissue. Collaborators include the pharmaceutical company Organon. They are interested in the fate of their medicine and its metabolites. IMS is ideally suited to follow these molecules in tissue. Another collaborator of a more academic nature is the Johns Hopkins Medical School in the US and involves the application of IMS techniques to breast cancer research. "In this case it is truly a bilateral collaboration, where IMS assists the discovery process. In the case of industrial partners such as instrument manufacturer Bruker Daltonik, we share our knowledge with them. In turn we benefit from access to the newest technologies they develop," Heeren says.

These collaborations are often initiated during conferences or NPC-meetings. "This is the consortium's strength," Heeren explains. "People from different disciplines in academia and industry are brought together and start collaborating. We are mass spectrometrists and FOM is an institute of physical science. We do not have patients or tissue samples. NPC offers us access to these samples and to partners in our project."
"For me it is important that there is a technology roll-out. We do not want to remain on our own little island with our new technologies," says Heeren when asked about valorisation of his research. This roll-out is already taking place. Detectors, developed by Heeren and co-workers specifically for IMS, are now produced for commercial use. Also, Bruker-Daltonik is implementing protocols for sample preparation developed by Heeren and his group.

Heeren works, within NPC, with many different hospitals in applying the newly developed imaging technologies to protocols for biomarker discovery. But technology valorisation is not the most important according to him. "For me, the transfer of the knowledge and skills our researchers have gained to other institutes, such as academic medical centres and analytical companies, is the ultimate 'human' valorisation."

Valorisation and Internationalisation

Figure 1 | Sagittal sections of a rat brain (collaboration with Organon, Scotland). Left (fig 1a): from left to right an optical image of a section covered with DHB and coated with 2 nm gold afterwards, followed by MS images of 2.5-dihydroxybenzoic acid (DHB) (M+H, 155m/z), Au+ (196m/z) and their overlay (scale bar 2 mm). The DHB image (in blue) exhibits very sharp contours corresponding exactly to the brain shape, whereas the gold image (in red) seems homogeneous on the whole measured area. The red fringe on the overlay of the two previous images highlights the sole presence of gold signal at the periphery of the tissue on the glass itself, whereas the matrix peak appears exclusively on the tissue.
Right (fig 1b) brain section covered with sinnapinic acid (SA). From left to right, MS images of protonated SA (M+H, 225m/z), sodiated SA (M+Na, 247m/z) and with potassium (M+K, 263m/z). The matrix peaks appear with a highest intensity from the glass, with hardly any signal coming from the tissue.

tion and localization of markers. This information can be used for a more precise diagnosis allowing potential differentiation between types and stages of development of a disease, and the subsequent choice of the most appropriate treatment. Within the framework of the NPC biomarker project we have developed new protocols for sample preparation relevant for the imaging of biomolecules in breast tumour sections. This allows us to link the histopathological observations to molecular distributions determined by MRI and imaging mass spectrometry in xenografted human breast tumours. This interdisciplinary study is performed together with Johns Hopkins Medical School in Baltimore, MD (USA).

In our case the identification of a marker is obtained through the correlation between the mass spectrometric image and an optical image of the tissue. This allows the association of a biomolecular distribution with a specific anatomical part of the tissue or organ identified by the pathologists. This has to be followed by an identification step, which does not necessarily need to be performed on tissue directly. Indeed, when compared with 'regular' proteomics experiments, IMS only analyses the material located on the surface layer of tissue instead of the bulk. This prohibited the use of the cleaning or separation steps that are often employed for tissue homogenates or laser capture microdissected samples. This restricted array of sample preparation techniques prior to tissue analysis induces a higher complexity in the resulting mass spectra. This can result in a variable sensitivity for a specific component with non-negligible ion suppression effect when analyzing tissue samples. Ion suppression is usually attributed to salts and irrelevant analytes also present on the tissue. These molecules ionize easier than the analyte of interest due to different gas-phase basicities, thus decreasing the analyte intensity on the mass spectrum. The same kind of tissue can display very distinct ion suppression effects along with the nature of the matrix (see Figure 1 and 2). The complexity of the phenomenon makes it difficult to anticipate its effect in tissue imaging and to draw very firm quantitative conclusions from the analysis of such images. However it often does allow for the differentiation between different tissue types based on molecular fingerprints.

The sensitivity of tissue imaging and spatial resolution are crucially determined by sample preparation diligence. It affects both the quality and quantity of chemical information contained in the mass spectra and can enhance the spatial resolution down to the cellular level [3]. There are many reasons why the sample preparation steps, among which matrix deposition on tissue is one of the most sensitive, are so important for IMS [4]. The first reason is that the actual spatial resolution in high resolution studies is no longer limited by the probe size but by the matrix crystal size. Thus the quality and nature of the sample preparation directly influences the spatial resolution (see Figure 3). Secondly, the matrix deposition protocol also has a tremendous influence on the sensitivity of the mass measurement towards large bio-molecules like peptides and proteins. The growth of the matrix crystals on tissue acts as a purification step; extracting peptides from tissue and segregating them from the salts for reduced ion suppression. It is widely accepted that the slower the crystal growth, the better the analytes' signal. It was found that on-tissue digestion performed before matrix deposition could significantly enhance the amount of signal coming out of the tissue section.

Figure 2 | Electron Microscope images of the matrix crystal layer applied on breast cancer tissue with the ImagePrep apparatus (Bruker Daltonik). Crystal sizes range from 2 to 20 microns. Right panel shows a hole drilled into the same matrix by laser pulses. Laser diameter is much bigger than the crystal size but, in ion-microscope mode imaging, the crystal size limits the spatial resolution.

Different matrix deposition systems At AMOLF we tested several commercial and lab-built matrix deposition systems. Apart from the classic dried droplet method requiring only a micropipette and a steady hand, we have tested a nano-droplet chemical inkjet printer (ChIP, Shimadzu), a manual pneumatic sprayer (Sigma), an environmentally controlled pneumatic sprayer (in collaboration with Novartis), a home built electrospray deposition system and the ImagePrep vibrational sprayer from Bruker. The results were compared from the point of view of homogeneity and crystal size.

The droplet techniques (dried droplet and ChIP) are good for tissue profiling but not amenable to high resolution imaging due to the droplet size (typically larger than 100 microns with the ChIP, even bigger with a micropipette), inhomogeneous coverage and variable crystal size. Pneumatic spray produces very good coverage, a homogeneous matrix layer and controlled crystal size, with better control, repeatability and optimisation of the deposition conditions (temperature, hygrometry) in the automated mode as compared with the manual mode.

However, these pneumatic systems consume large quantities of matrix material. The electrospray deposition (ESD) produces very small crystals in a homogeneous layer all over the tissue, but due to fast evaporation of the solvent, the tissue analytes have less time to migrate into the crystals, resulting in reduced sensitivity. ImagePrep from Bruker is also excellent in terms of homogeneity and repeatability, with good control of the saturation of the atmosphere over the tissue sample to prevent fast ce the sensitivity of the subsequent

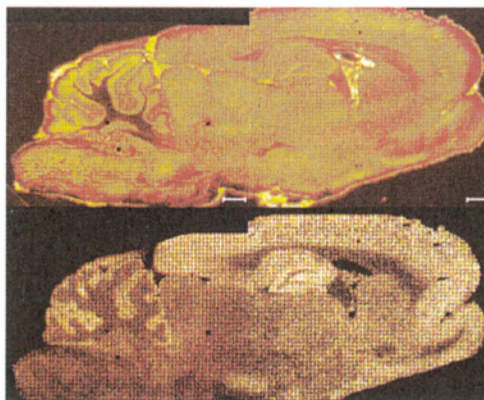

Figure 3 | Lipid distribution and tissue morphology in SIMS imaging (a collaboration with Organon, Scotland). Mass selected ion images of a rat brain: the potassium distribution (upper panel) shows well resolved anatomical details which can also be recognised on the phospholipid image (lower panel).

measurements, which turns out to be a key parameter for sensitivity enhancement. The use of fixed protocols helps in increased reproducibility of MS imaging results. The last two methods, ESD and ImagePrep, also display the ability to cover the tissue with very small quantities of solution, which finds an immediate application in deposition of digesting enzymes (100-200 µL at usual concentration of about 10 µg/µL of trypsin), with control of the incubation conditions, for on-tissue digestion followed by matrix deposition and IMS to readily identify tryptic peptides with specific localisation on tissue. This could not be reasonably achieved with the pneumatic sprays, considering the amount of solution consumed (50-100mL of matrix solution with concentration between 15-30mg/mL CHCA or SA).

Parallel to those preparation techniques, our lab recently acquired a novel MALDI-TOF-TOF instrument (UltraflexIII, Bruker Daltonik). This instrument was initially developed for regular proteomics analysis after a typical LC-separation step. Using this instrument for IMS, it becomes possible to fully benefit from the increase in sensitivity determining the choice of the matrix deposition method. Indeed, the mass range can be extended considerably with regard to our other instruments. Thus investigating intact proteins up to several hundreds of kDa becomes achievable directly on tissue. In addition, this instrument is designed to perform MS/MS measurements. Therefore pre-digesting of tissue sections becomes even more interesting as, after a first MS analysis of the tissue, it is then possible to re-analyse the sample and identify or confirm the attribution of the previous peptides sequences by tandem mass spectrometry. This brings IMS a step further in the direction of proteomics.

These exciting features in imaging MS will be applied to cancer biomarker targeted projects in the NPC programme and will benefit from the NPC network involving the complementary knowledge of histologists, clinicians, and proteomic research.

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Detectors, developed by Heeren and co-workers specifically for IMS, are now produced for commercial use. Also, Bruker-Daltonik is implementing protocols for sample preparation developed by Heeren and his group.

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Summary

Imaging mass spectrometry lies at the centre of a number of collaborations in the biomarker program of the NPC. The understanding of the spatial organization of biomolecules in tissue requires an intensive interdisciplinary collaboration with instrument manufacturers, academic hospitals and pharmaceutical industries. Within the framework of the NPC biomarker project we have developed new protocols for sample preparation relevant to the imaging of biomolecules in breast tumour sections. This allows us to link the histopathological observations to molecular distributions determined by MRI and imaging mass spectrometry in xenografted human breast tumours. This interdisciplinary study is performed together with Johns Hopkins Medical School in Baltimore, MD (USA). Developing and evaluating innovative matrix deposition methods such as the vibrational sprayer (ImagePrep) on tissue has been performed as part of this study, in collaboration with Bruker Daltonik GMBH. With homogenous matrix coatings, it was shown that ion suppression can seriously hamper the generation of representative images depending on the protocol used. Standard proteomics protocols are employed in the tissue imaging workflow allowing for the combination of on-tissue digestion with imaging MS. These protocols have been successfully tested in a collaborative study with Organon focused on the elucidation of the spatial distribution of various lipids in saggital sections of mouse brains.

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