ron heeren mass spectrometer identifies and localises biomarkers in cells

When Anthony van Leeuwenhoek first looked through his microscope, he could not have envisaged how his eye as detector would come to be replaced by fast, sensitive cameras and his illuminating candle by intense light sources and lasers. Now, microspectroscopic imaging tools are used to study the distribution and interactions of biomolecules within cells and tissue.

Chemical imaging mass spectrometry is revolutionising the field of biological surface analysis. It provides both the chemical information of a mass spectrometer and the spatial organisation of each component on a surface. High spatial resolution combined with high sensitivity for large molecules remains the goal of chemical imaging mass spectrometry. Both matrix-assisted laser desorption/ionisation (MALDI) and secondary ion mass spectrometry (SIMS) based approaches are being developed to achieve this target. These two techniques offer different starting positions for the ultimate goal of high mass, high-resolution imaging, namely high mass and relatively low spatial resolution (MALDI) and low mass and high spatial resolution (SIMS).

The research within the NPC aims at identifying and localising biomarkers in cells and tissues using MS imaging techniques. Knowledge of particular biomarkers may be used to diagnose illnesses or abnormalities such as tumours, it may be used to develop a therapy or the pathology of the illness may be studied.

Through the use of well-established multiple fluorescent labelling techniques (colouring of tissue), it is possible to study the spatiotemporal behaviour of selected biomolecules in parallel in a single experiment. Despite the valuable insights already realised, any labelling technique suffers from several innate weaknesses. Arguably the most important is the need to identify the molecules of interest prior to the experiment in order to apply the appropriate label. In addition, the small number of molecules that can be investigated simultaneously in a single experiment, the possible interference of the label with the protein's normal function, and the difficulties in distinguishing between specific posttranslational modifications of proteins limit the application of labelling techniques as a discovery tool.

This last aspect is especially important considering that several diseases and immune responses have been associated with altered posttranslational modifications. Clearly, the desire to study disease and the complexity of biological mechanisms demand a molecule-specific imaging technique that can be applied to tissues or cells directly. Mass spectrometry possesses the chemical specificity and high sensitivity required for biomolecular imaging experiments without the use of chemical modifications or labels.

Two main approaches were taken towards chemical imaging mass spectrometry at AMOLF. Firstly, matrix-enhanced SIMS

What this research is about:

Exploiting physical knowledge for biological purposes

"There is no single method that combines biomolecular sensitivity with high spatial resolution," says prof. dr. Ron Heeren. He aims at chemical imaging mass spectrometry, which he developed to look at specific biomarkers in cells and tissue. Heeren's group is specialised in the physics of mass spectrometry and has been involved in imaging since 1995. He defines his activities as 'exploiting physical knowledge for biological purposes'.

Imaging mass spectrometry provides both chemical information and the spatial distribution of each analyte detected. A challenge in this field is to combine high spatial resolution with high sensitivity for high-mass molecules. Heeren and his team used two methods to this effect. Matrix-enhanced SIMS imaging is used to gather chemico-spatial information that cannot be provided by established SIMS or MALDI imaging techniques. The addition of a matrix to a sample improves the sensitivity of SIMS for higher mass molecules. When combined with a primary ion column capable of delivering high-resolution images, such as a liquid metal ion gun or an ion-optical microscope, ME-SIMS allows images of intact molecular ions to be recorded with high spatial resolution.

Secondly, Heeren used a novel approach to MALDI molecular imaging that combines high spatial resolution with high-speed analysis while operated at the preferable lower laser fluences. The group has demonstrated the feasibility of MALDI microscope mode imaging. The test samples that were used delivered the recognisable structures needed to prove the concept of MALDI microscope mode imaging. In addition, they served as useful targets to estimate the experimental spatial resolution. The high spatial resolution, the increased speed and the greater versatility of potential ion sources offered by the mass microscope approach will prove important in the development of the mass spectral molecular imaging of biomolecules.

Research Theme NPC6: Biomarker discovery

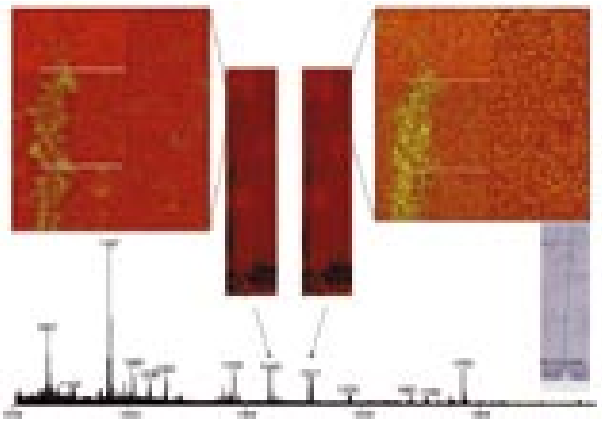

Figure 1 | **ME-SIMS images of rat brain tissue.** The biomarker at m/z = 1511 is present in all cells, the biomarker at m/z = 1443 is localised in specific cells.

(ME-SIMS) was applied to mollusc, insect and rat brain tissue. The high spatial resolution images, in the low micrometer range, of intact molecular ions demonstrate the ability of ME-SIMS imaging to provide chemico-spatial information that cannot be provided by established SIMS or MALDI imaging techniques.

Secondly, a novel MALDI microprobe instrument has recently been reported that is capable of imaging mass spectrometry with a micrometer-size laser probe. The drawback of this approach is the high laser fluence needed to reach the ionization threshold for MALDI from such a small spot, which leads to a high degree of fragmentation and a significant loss of sensitivity. Moreover, because each pixel is sequentially analysed, to increase the spatial resolution by a factor N requires an increase in analysis time of $N2$. We present a novel approach to MALDI molecular imaging that combines high spatial resolution with high-speed analysis while operated at the preferable lower laser fluences.

Brains in a matrix In ME-SIMS, a sample is prepared in a matrix which increases sensitivity for high-mass molecules with respect to conventional SIMS. Analysis of cockroach brain tissue shows that the ME-SIMS images clearly resolve features that are separated by less than 10 μm, and that the cholesterol, choline and diacylglycerol distributions differ significantly.

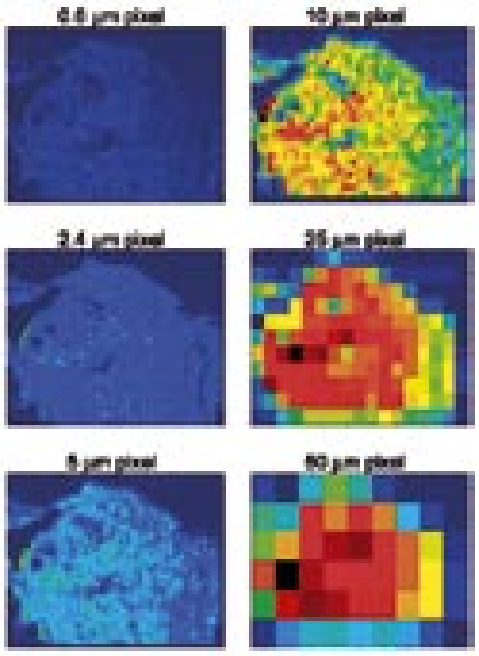

Figure 2 | The effect of resolution on images of cholesterol in tissue by ME-SIMS.

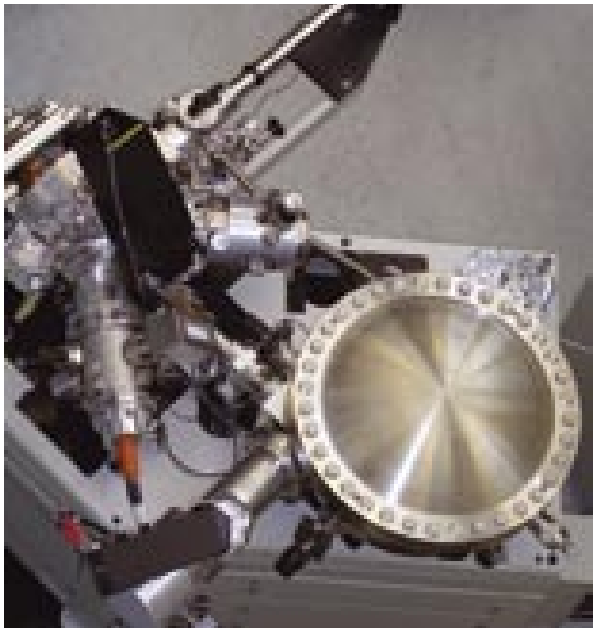

Figure 3 | Imaging SIMS set-up.

To obtain information from within an organ, thin tissue sections can be cut using a microtome. The enhanced information obtained from tissue sections was demonstrated with SIMS spectra obtained from a thin section of the parietal ganglion of Lymnaea stagnalis before and after matrix deposition.

The results demonstrate that ME-SIMS provides both the spatial resolution and the sensitivity for molecular ions necessary to reveal subcellular distributions of analytes, in addition to providing an in situ quality assessment of the images obtained (height map). Without matrix the low yields and lack of chemical specificity of the resulting low-mass fragments prompted a leading figure in the SIMS community to reflect that, 'There are few compounds that exist at sufficient concentrations and display sufficient secondary ion yields to permit any sensible spatial analysis' [1]. The addition of a matrix helps to alleviate these sensitivity/specificity problems. This facile improvement on the technique provides information not currently obtainable with regular SIMS or conventional ≥25 μm laser spot MALDI chemical imaging mass spectrometers.

Finally, rat brain tissue was prepared using gold deposition and analysed using ME-SIMS. Images were obtained using a microprobe with a 100 nm beam, giving images of 150x150 μm. It was clearly shown that one of the biomarkers with m/z 1443 was localised to specific cells, whereas another of m/z 1511 was present in all cells in the brain tissue. There is no other method which combines this biomolecular sensitivity with high spatial resolution.

Microscope One disadvantage of ME-SIMS combined with the mass microscope used at our laboratory is the limitation of molecule size up to ~2000 Dalton. Using MALDI on the same instrument, molecules up to 10,000 Dalton can be imaged. We have used an ion microscope in combination with MALDI-MS to record macromolecular images of the spatial distributions of intact peptide and protein ions [2]. Using a mass spectrometric microscope, the spatial resolution is independent of the spot size of the ionizing beam. This decoupling allows a much larger area to be examined without having to move the sample or the laser spot. The ions produced by a single laser shot pass through the time-of-flight mass spectrometer forming an ion-optical image on a position sensitive detector, much like in wide-field optical microscopy. In this manner, a mass-to-charge ratio (m/z) separated series of molecular images is generated showing spatial detail from within the laser spot.

The small features obtained in our MALDI microscope experiments would not have been discernible with the ≥25 μm spot sizes of a typical MALDI microprobe. In contrast, multiple squares identified in the images would have been sampled by a single laser position and thus be represented as a single pixel. The spatial resolution of around 4 μm obtained in this current work is comparable to that of the micrometer-size microprobe studies. Moreover, these high-resolution images were recorded without incurring the loss of sensitivity at increased laser threshold fluences associated with a highly focussed laser.

A distinct advantage of the microscope over the microprobe approach is its improved speed of analysis. Compared to a 2 μm microprobe (at least two pixels are necessary for 4 μm experimental resolution), the mass microscope can analyse the same area, delivering equivalent performance, using much fewer individual positions. In addition, the decoupling of the spatial resolution from the source conditions offers an advantageous extension of useful ionization methodologies for imaging purposes. In particular, the microscope approach allows the use of ion sources that cannot provide convenient small spot sizes for microprobe experiments, such as infrared laser sources in IR-MALDI.

Time for validation We can conclude that the addition of a matrix to a sample improves the sensitivity of SIMS for higher mass molecules. When combined with a primary ion column capable of delivering high-resolution images, such as a liquid

metal ion gun, or an ion-optical microscope, ME-SIMS allows images of intact molecular ions to be recorded with high spatial resolution. The mechanistic differences between ME-SIMS and MALDI suggest that there is significant scope for improving the sensitivity of ME-SIMS for macromolecules. This would improve the contrast of the images already attainable in addition to increasing the number of analytes amenable to high spatial resolution analysis.

Furthermore, we have demonstrated the feasibility of MALDI microscope mode imaging. The test samples we have used delivered us the recognisable structures needed to prove the concept of MALDI microscope mode imaging. In addition, they served as useful targets to estimate the experimental spatial resolution. The high spatial resolution, the increased speed, and the greater versatility of potential ion sources offered by the mass microscope approach will prove important in the development of the mass spectral molecular imaging of biomolecules. The quality of the results obtained justifies further studies in which microscope mode imaging will be applied to biological samples.

Using chemical imaging mass spectrometry we have taken a clear step towards localising biomarkers that are present in tissue only in very small amounts. Their identification will rely heavily on the NPC infrastructure provided in the biomarker discovery program. Building infrastructure, initiating cooperations and gathering expertise have been our main occupation. Now it is time to validate and optimise our methods for application in diagnosis and pathology.

References

1 McDonnell, L.A. et al. (2005) Subcellular imaging mass spectrometry of brain tissue. *J. Mass Spectrom.* 40, 160–168.

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summary

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Through the use of well-established multiple fluorescent labelling techniques (colouring of tissue), it is possible to study the spatiotemporal behaviour of biomolecules in parallel in a single experiment. The disadvantages of this technique include the need to identify the molecules of interest prior to the experiment, and the small number of molecules that can be investigated simultaneously. Clearly, the desire to study disease and the complexity of biological mechanisms demand a molecule-specific imaging technique that can be applied to tissues or cells directly. Mass spectrometry possesses the chemical specificity and high sensitivity required for biomolecular imaging experiments without the use of chemical modifications or labels.

Two main approaches were taken towards chemical imaging mass spectrometry. Firstly, matrix-enhanced SIMS (ME-SIMS) was applied to mollusc, insect and rat brain tissue. High spatial resolution images were obtained and it was demonstrated that ME-SIMS has the ability to provide chemico-spatial information that cannot be provided by established SIMS or MALDI imaging techniques. Secondly, a novel approach to MALDI molecular imaging is presented that combines high spatial resolution with high-speed analysis while operated at the preferable lower laser fluences. Using these techniques, we have taken a step towards finding biomarkers that are present in tissue only in very small amounts.