

Minireview

## Biological imaging using secondary ions

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### Abstract

Biological materials are morphologically and chemically complex. A quantitative imaging tool is now available that can produce chemical, and even metabolic, information from morphological features as small as a few nanometers.

Biological materials are immensely complex structures whose chemical composition can vary both temporally and on an extremely fine spatial scale. Biologists operate with mental models of the abundance, distribution, movement and metabolism of molecular and ionic species within these complex structures. A chemical imaging tool is now available that can illuminate such models by visualizing and quantifying these species with a spatial resolution of around 30 nm and the ability to obtain chemical information from features as small as a few nanometers. This tool is a novel mass spectrometer that generates chemical images and derived isotope ratio images, using ions ejected directly from the surface of a sample by a probing ion beam focused to a spot as small as around 30 nm. In this issue of *Journal of Biology*, Lechene and co-workers [1] report impressive results from such an instrument, utilized by a collaboration of biologists and instrument experts at the National Resource for Imaging Mass Spectrometry established by Lechene at Harvard Medical School and Brigham and Women's Hospital. They call their technique multi-isotope imaging mass spectrometry (MIMS).

The first imaging secondary-ion mass spectrometer was developed by Georges Slodzian in the late 1950s for his PhD thesis at the University of Paris [2]. This novel instrument utilized kiloelectron-volt ion beams ('primary' ions) impinging on a sample surface to eject, or 'sputter', ions characteristic of the

chemical composition of the surface ('secondary' ions). The secondary ions were accelerated into a mass spectrometer, where they were identified (via their mass/charge ratio) and quantified (via the ion intensity). By using the mass spectrometer itself as an ion-optical microscope (in Slodzian's original design), or, in later designs, by focusing the primary ion beam to a tiny spot and rastering it over the surface as in a scanning electron microscope, it is possible to produce element-specific (or even molecule-specific) ion images with exceptional sensitivity. The lateral resolution of the initial Slodzian design was around 500-1,000 nm, comparable to an optical microscope; the modern instrument used by Lechene *et al.* (also designed by Slodzian) can achieve a resolution of around 30 nm. After his graduation in 1962, Slodzian was invited to Stanford University by Joshua Lederberg, who was interested in exploring the utility of this novel technology for biological research. Lederberg was interested not just in terrestrial applications but also was looking ahead for analytical techniques to search for life in future missions to Mars - this only a year after the start of the United States lunar mission program and 14 years before the Viking missions to Mars (G. Slodzian, personal communication). It is a tribute to the difficulty of this challenge that only now, almost half a century later, is the full power of quantitative biological imaging with secondary ions starting to be realized, as reported here by Lechene *et al.* [1].

## Issues with biological samples

Application of this powerful imaging technique to biology initially moved quite slowly, for a number of reasons. Biological samples are inherently incompatible with the high vacuum needed for ion microanalyzer operation, and pioneering early work by researchers such as Bellhorn and Lewis [3] and Galle [4] was subject to artifacts arising from facile migration and loss of mobile ions such as Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> during sample fixation and dehydration; the final positions of those ions that remain in the sample often do not correspond to their original distributions. Furthermore, much of the information in biological samples is encoded in molecular form, and because secondary ions are produced by bombarding the sample surface with primary ions whose energies are orders of magnitude greater than chemical bond energies, molecules are rapidly degraded by the bombardment, and their information lost. Even images obtained using atomic ions are extremely difficult to quantify, owing to the extreme variability of ionization probabilities. Ionization probability is the fraction of ejected atoms or molecules that become ionized in the ejection process (there is no auxiliary means of ionization). This probability varies exponentially with the ionization potential (for positive ions) or electron affinity (for negative ions) of the ejected species, and also depends strongly upon the chemistry of the sample surface from which atoms or molecules are sputtered (so-called 'matrix effects').

Many structures of interest in biology are significantly smaller than the resolution limit of the optical microscope, so that sub-microscopic image resolution is desirable, achieved in the MIMS instrument by shrinking the size of the primary ion beam to some tens of nanometers. Of particular importance for sub-microscopic imaging is the fact that the imaging technique is destructive - the image is produced by ions ejected from the sample by the energetic primary ion impact - so that shrinking the information volume requires maximizing the efficiency with which the sputtered ions can be created and sampled, issues specifically addressed in the design of the instrument reported in [1].

## Information issues driving instrumental design

An ion image is constructed of an array of pixels, each of which contains some number of secondary ions sputtered from a region in the sample surface defined by the primary ion beam position and size. The information in an image is governed by Poisson statistics: ion emission is a random process so that a number of ions,  $N$ , arriving at the detector has a standard deviation of  $\pm\sqrt{N}$ . Thus, a minimum of 100 ions are required to achieve a standard deviation of  $\pm 10\%$  for the signal in a given pixel; to measure the ratio of two isotope signals to an accuracy of  $\pm 1\%$ , at least 10,000 counts

of the less abundant isotope are required. Consider a primary ion beam 50 nm in diameter generating a single image pixel by eroding a 10-nm deep volume containing approximately  $10^5$  atoms. If the species of interest is, say, a 1% component of this volume, the ionization probability is 1% and the mass spectrometer transmission (the fraction of secondary ions that reach the detector) is 10%, then on average each pixel will contain only a single ion count (and because such small numbers fluctuate considerably, roughly half the pixels will contain zero counts and others will contain two or more). Thus optimization of the ionization probability and of the mass spectrometer transmission are primary concerns in achieving good image resolution: it is not sufficient simply to minimize the primary beam size. In the MIMS instrument, both ionization probability and mass spectrometer transmission can approach 100%.

## Molecular ion imaging

Several groups have pursued the idea of obtaining molecular image information using sputtered molecular ions themselves to form images. Somewhat surprisingly, given the energetic nature of the sputtering event, biomolecules are occasionally sputtered as intact molecular ions, or as structurally identifiable fragments. The information issues in directly imaging with molecular ion signals are challenging. A significant fraction (perhaps around 90%) of molecules near the impact point of a primary ion are destroyed by the impact; even worse, the primary ions penetrate deep below the surface and thus also damage many of the subsurface molecules even before they can be exposed at the surface to be sputtered. For imaging, the surface layer of a sample rarely contains enough molecules to form an image. Even if the species in question constituted 100% of the surface layer, there would be only a few thousand surface molecules in a 50-nm diameter spot; if only 10% of these survive the sputtering event to become intact molecular ions (because the ionization probability may be 1% or lower) most pixels would contain only a single count, even with 100% mass spectrometer transmission. In most cases, then, to form a molecular ion image it is necessary to sample molecules over an extended depth below the surface, so that there is a need to minimize or avoid the subsurface molecular destruction resulting from the penetrating primary ions [5]. One approach is to minimize primary ion penetration by using large cluster species as the primary projectiles. Work on this challenging problem is proceeding in the laboratories of Gillen [6], Winograd [7] and Vickerman [8] and others.

A nice example of direct molecular ion imaging is seen in the work of Ostrowski *et al.* [9]. These authors used a pulsed 200-nm diameter Ga<sup>+</sup> ion beam coupled to a time-of-flight (TOF) mass spectrometer to image lipid composition in the

membranes of fusing cells of *Tetrahymena*. They were able to show that phosphatidylcholine, which is postulated to resist high-curvature structures, is indeed absent from the membranes in the fusion site. This study shows the importance of designing the experiment to mesh with the strengths and limitations of the imaging approach. The target lipids constitute a large fraction of the membrane, so that image information in this case can be gained from just a bilayer of material. The TOF mass spectrometer both has a high transmission and collects an entire mass spectrum for each ion pulse, so that the researchers could subsequently examine the images and were able to select fragment ion species that were sufficiently intense and revealed the desired characteristic information. Although the ion signals were fairly low for the images, the scientific question posed - whether phosphatidylcholine is present at the fusion site or not - has a simple yes/no answer, and line scans of signal intensity through the fusion site confirmed the statistically significant depletion of phosphatidylcholine in the site.

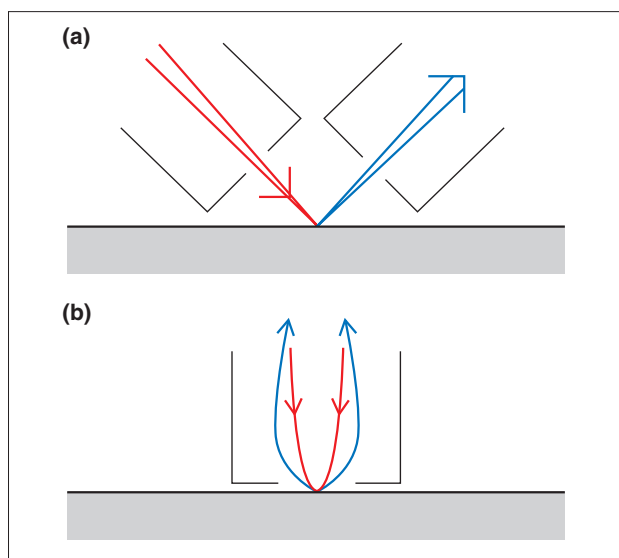
### Design of the MIMS instrument

For many samples, the molecules of interest may be present in low concentrations, or be too large to sputter efficiently (for example, proteins and nucleic acids), and if sub-micrometer lateral resolution is desired, ion statistical issues preclude the use of direct molecular ion imaging. Therefore, work with the MIMS instrument at Harvard has taken a very different approach. The target molecular species are labeled with stable isotopes such as  $^{13}\text{C}$  or  $^{15}\text{N}$  by feeding organisms with labeled precursors. Then isotope ratio images are obtained that quantitatively reveal the uptake of the precursor species and thus both localize the final molecular products (such as nucleic acids and protein) and provide information about metabolic uptake rates in the image features.

To obtain isotope ratios of the desired accuracy requires simultaneous optimization of ionization probability, primary beam size, ion collection efficiency and mass spectrometer transmission. About 25 years ago, working at the University of Paris, Slodzian began to address these instrumental issues to achieve the ultimate image resolution and sensitivity in the secondary ion microanalyzer. Optimum ionization probability is achieved at oxygenated surfaces (for positive ions) or cesiated surfaces (for negative ions). Operationally, the most convenient way to obtain such surfaces is to use oxygen or cesium primary ion beams. These primary ions are implanted into the target and accumulate to a steady-state concentration; for cesium impinging on organic material, a concentration of around 10-20% is typically achieved at the surface, which is roughly optimum for high negative ion yields. Under these conditions, species with electron affinities greater than about 2 eV are ionized

with close to 100% efficiency [10]; these include sulfur (S), the halogens and, importantly, cyanide (CN).

Obtaining the smallest possible primary ion beam diameter while simultaneously optimizing ion collection efficiency requires careful attention to ion optics, the rules of which are quite similar to the rules of light optics. The only way to achieve very small focused beams of oxygen or cesium ions is to use a final lens with an extremely short focal length. In addition, a high collection efficiency for the sputtered ions requires the highest possible acceleration field at the sample surface, which also means the smallest possible spacing between the sample, held at a high positive or negative potential, and a ground electrode. Combining these two requirements led Slodzian to use a single coaxial ion lens with very short working distance to serve the dual tasks of focusing the primary ions and efficiently collecting the secondary ions (Figure 1). The analogy is with the use of coaxial illumination through a short focal length condenser lens in the optical microscope. Finally, these ion-optical elements were combined with a specially designed secondary ion mass spectrometer that simultaneously focuses ions over a wide range of mass/charge ratios along a focal plane. In this design, several ion signals (up to five in the Harvard instrument) can



**Figure 1**

Lens design. (a) Ion lenses are irreducibly bulky and the use of separate lenses for primary and secondary ions forces long working distances and low demagnification for primary ions (red) and/or low collection efficiency for secondary ions (blue). (b) A coaxial single lens design allows the shortest possible working distance and optimum primary ion demagnification together with high secondary ion collection efficiency. Secondary ions of opposite polarity to the primary ions are separated downstream by electrostatic deflection.

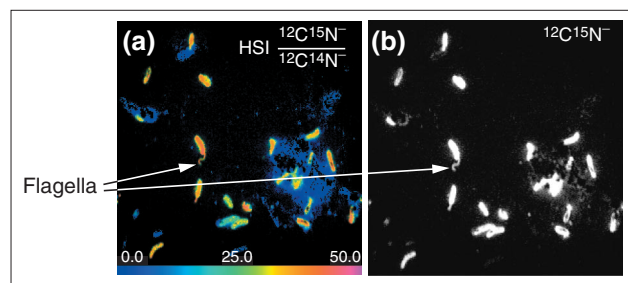
be detected simultaneously and ion ratios can be measured directly, avoiding the waste of scarce signal inherent in detecting only one species at a time, and ensuring that signal variations arising from primary ion beam fluctuations or sample changes do not affect ratio measurements. The mass spectrometer is designed to have high transmission (around 70-80% of sputtered ions reach the detector) together with high mass resolution, which allows separation of the targeted ion signal from interfering ion species at the same nominal mass/charge ( $m/z$ ) ratio. In particular, detection of  $^{15}\text{N}$  as the  $^{12}\text{C}^{15}\text{N}^-$  species at  $m/z$  27 requires separation from  $^{13}\text{C}^{14}\text{N}^-$ . These two ions differ slightly in mass by about 1 part in 4,000 due to the different nuclear binding energies of the constituent atoms, and this small difference is sufficient to allow them to be separated in Slodzian's mass spectrometer.

### Biological imaging

The first biological images from the prototype of this new device were reported by Slodzian and colleagues in 1992 [11]. The instrument was commercialized under the name NanoSIMS by Cameca (France), the company that had previously commercialized Slodzian's original ion micro-analyzer design and its successors; Slodzian's prototype instrument became the core of the National Resource for Imaging Mass Spectrometry at Harvard. As with the direct molecular imaging study of Ostrowski *et al.* [9], the work now emerging from the Harvard facility again shows the importance of careful experimental design to produce samples that are optimally matched to the capabilities of the imaging technique. The element migration problems that had blighted the early biomedical applications of the ion microanalyzer are largely avoided by an approach of imaging labeled molecules using either stable isotope or

halogen labeling. The best image resolution is obtained with a  $\text{Cs}^+$  primary ion beam, and fortunately biological material rich in C and N yields intense signals of  $\text{CN}^-$  ions, so that  $^{15}\text{N}$ -labeled nitrogen and  $^{13}\text{C}$ -labeled carbon (which may also be imaged directly as  $^{13}\text{C}$ ) can be imaged with high efficiency and quantified with good accuracy in the  $\text{CN}^-$  signal. The destructive effect of the primary ion impacts is here not a problem, as with molecular imaging, but rather a vital feature of the technique, allowing molecules to be degraded and the C and N atoms recombined into the exceptionally useful  $\text{CN}^-$  signal.

By feeding organisms with isotopically enriched material and developing isotope ratio images of selected features, Lechene *et al.* show that it is possible to identify sites of high metabolic activity, such as nucleic acid synthesis or protein turnover, and even to determine quantitatively the turnover rate of protein or nucleic acid in specific features of an organism. Owing to the exceptional sensitivity of the instrument, features even smaller than the primary beam size can be detected and their size can be estimated from the secondary ion signals. An example from [1] is an image of flagella, estimated to be about 10 nm wide, of *Teredinibacter turnerae*, a nitrogen-fixing bacterium found in the gill of a shipworm (Figure 2). On an even finer scale, images of stereocilia, the organelle of mechano-electrical transduction, show zones of high  $^{15}\text{N}$  incorporation that may correspond to tip links, fine filaments that are directly connected to mechano-electrical transduction channels and are estimated to be 5 nm in size. There is a range of possible labels, so that dual-labeling experiments are possible, as in the co-localization of DNA and RNA synthesis in rat embryo fibroblasts using  $^{15}\text{N}$ -labeled uridine and Br-labeled deoxyuridine. Even  $^{14}\text{C}$ -labeling can be used, as earlier noted by Hindie *et al.* [12]: direct detection of the  $^{14}\text{C}$  rather than waiting for its slow radioactive decay improves sensitivity roughly 1,000-fold and so minimizes radiation hazards while allowing lateral resolution much better than can be achieved with autoradiography. Isotope ratio images can also be obtained for samples directly labeled with  $^{13}\text{C}$  or  $^{15}\text{N}$ , as in the high-resolution images of lipid domains recently obtained by Kraft *et al.* [13] using a NanoSIMS instrument at Lawrence Livermore Laboratory. In the remarkable work reported by Lechene and co-workers, the performance of imaging secondary ion mass spectrometry is pushed close to its ultimate limits, opening new vistas in quantitative biological imaging.



**Figure 2**  
MIMS images of *Teredinibacter turnerae*, a nitrogen-fixing bacterium inhabiting a shipworm gill. The  $^{12}\text{C}^{15}\text{N}^-/^{12}\text{C}^{14}\text{N}^-$  image (left) allows the uptake of  $^{15}\text{N}$  by these organisms to be quantified. On the right, the image of  $^{12}\text{C}^{15}\text{N}^-$  ions shows that sensitivity is sufficient to image the flagellum of this bacterium (arrowed) even though the flagellum diameter is estimated from the ion signal to be only about 10 nm. From [1].

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