# MOLECULE SPECIFIC IMAGES FROM BIOLOGICAL SAMPLES

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## Introduction

A wide variety of techniques exist which can be applied to generating images of biological samples. Dynamic SIMS has been used by several laboratories to image different types of biological systems using a range of sample preparation techniques. For example, dynamic SIMS has been used to follow the distributions of Ca, K and Na ions [1] in tissue. But it is hindered by the fact that little true molecular information can be obtained since the ions obtained in the mass spectrum are only fragments of the material originally in the sample. Using static TOF-SIMS, direct molecular information about the surface can be obtained with submicron lateral resolution. Applying TOF-SIMS to biological samples presents many difficulties that must be overcome. The main difficulties being sample preparation and interpretation of the mass spectra obtained from the experiment.

During static TOF-SIMS, only a small number (~1%) of the surface atoms are perturbed during the analysis, which ensures that the ejected material is representative of the surface rather than the chemistry induced by the primary ion beam. Because of the very low dose of the primary ion beam the technique presents extreme surface sensitivity, therefore only the first layers of the sample are analyzed. Thus, any contamination during sample preparation will constitute the material detected by the analysis. One of the major challenges for the analysis of biological tissue is how it should be handled before and during the analysis so that the information present in the tissue is not lost.

Sample preparation of biological samples is difficult because the samples are inherently incompatible with *in vacuo* analysis. The first obstacle that must be overcome is how to stabilize the sample during the analysis. There are several options that are available and have been used to analyze biological material using other techniques. For most of the analyses we have performed to date, *in vacuo* freeze fracture and direct analysis of hydrated samples have yielded the best results. Rapid freezing of the sample preserves the integrity of the cell such that features smaller than 5 nm can be observed with an electron microscope. Since the lateral spatial resolution attainable in current TOF-SIMS instrumentation is at best 50 nm, artifacts below 50 nm in size will not affect the analysis. Different sample preparation procedures used to try to obtain molecular specific images of biological samples will be discussed.

## Experimental

Experiments were performed using a Kratos time of flight mass spectrometer equipped with an FEI gallium liquid metal ion gun (LMIG) which can be focused to a 200 nm spot size with a 500 pA current in dc mode or a 50 nm spot size when operated with 60 pA current. The mass spectrometer is capable of achieving a mass resolution of better than $10^4$ at a mass of 300 m/z. Charge compensation of the sample was performed by flooding the sample 50 μs after firing the LMIG with 30 eV electrons with a dc current density of 50 nA $cm^{-2}$ for 50 μs.

*In vacuo* freeze fracture was performed using a home built apparatus that was coupled to the Kratos TOF-SIMS apparatus and is described elsewhere [2]. Briefly, the freeze fracture chamber (base pressure of $3x10^{-8}$ torr) is equipped with a stainless steel knife which can be cooled to 90 K. Fracture of the sample can be performed using the knife directly or by prying off a cover which has been frozen to the sample. After the sample has been fractured, the sample can be transferred into the analysis chamber (base pressure $3x10^{-10}$ torr) without breaking vacuum. Most samples were frozen using liquid freon although some were frozen using liquid nitrogen. The paramecia were purchased from Carolina Biological and were concentrated by centrifugation at 150 g before the sample was frozen.

## Results and Discussion

Initial experiments were performed on sturgeon sperm and tobacco leaves embedded in a plastic resin, microtomed into 200 nm sections, and placed on a copper grid. The result of TOF-SIMS analysis on these samples yielded excellent spectra of polydimethylsiloxane, but nothing indicative of the sample of interest. Analysis of material that was freeze dried but never fractured also did not result in useful chemical information but it did provide morphological information.

The sample preparation method that has provided the best chemical information to date is freeze fracture followed by direct analysis of the hydrated sample. We have investigated two methods of freeze fracture: 1) preparing a sample sandwiched between two substrates followed by rapid freezing and fracturing *in vacuo* at the desired pressure and temperature, and 2) freezing the sample as a small droplet on a small copper holder, introducing the sample to the vacuum, and fracturing it open by bringing a knife up to the sample.

The temperature at which freeze fracture is performed is critical to obtaining chemically specific images. The amount of contamination from background gas condensing on the surface of sample increases if the sample is too cold when the freeze fracture is performed. For example, a sample fractured at a sample temperature of -160°C and a pressure of $1x10^{-7}$ torr produced a spectrum with prominent peaks for hydrogen, $H_2O$, $H_3O$ and clusters of $H(H_2O)_n$ (n=2-26) almost exclusively. Conversely, if the sample temperature is too high then a significant portion of the sample is lost to the vacuum chamber. This not only alters the surface, but ejects material into the vacuum chamber which may recondense on the surface, obscuring the analysis. The best chemical images were obtained when the freeze fracture was performed at a temperature between -100 and -120°C.

Samples were fractured using both the sandwich fracture and the knife fracture methods because both produce fractures that are useful for analysis. When examining paramecia using the sandwich freeze fracture method, the paramecia orient themselves preferentially with their long axis parallel to the plane of the sample holder (before the sample is frozen) and thus most of the fractures go along the membrane rather than through the cytoplasm. When the samples are frozen in droplets for the knife fracture, the paramecia are in a more random orientation so the paramecia fracture along different directions. In Figure 1 both types of fracture are depicted. Most of the fractures with the sandwich method result in fractures along the membrane as in Figure 1A. Using the knife results in fractures through the body (see Figure 1B) as well as along the membrane.

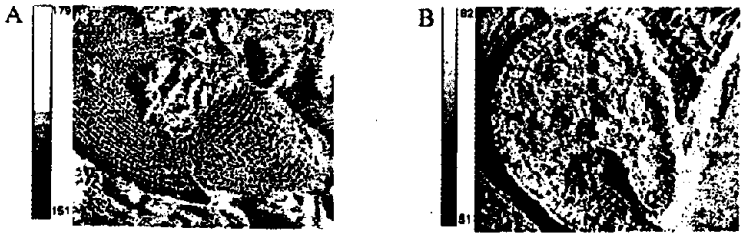


**Figure 1**: Freeze fracture along the membrane A) and through the body of the paramecium B). The image was collected using the dc primary ion beam and collecting all the ejected material.

To test the possibility of imaging complex biological samples with chemical specificity, paramecia were examined in their natural state and after being incubated with a dopant. Cocaine was used as a dopant because it is water soluble yet it interacts with the membranes of cells. The original impetus for incubating the cells in cocaine was to see if a midsize molecular species could be detected from a complex cellular matrix. Imaging cells without a dopant provides the baseline for the analysis. In Figure 2 a freeze fractured paramecium with no dopants is represented. The first aspect that is apparent is the chemical contrast between the localization of the water and other species. Also apparent are the kinetisomes that are the attachment sites for the cilia which are used by the paramecium to move through the medium. In the images of paramecia without dopants there is a marked difference between the ion concentration from the medium and the paramecium. Because it is difficult to differentiate between cells that are just below the surface of the ice optically, the ion gradient is a useful diagnostic since the SIMS signals for Na and K are large which allows exposed cells to be located quickly. Once the region of interest is identified then it can be imaged until the static limit is reached.

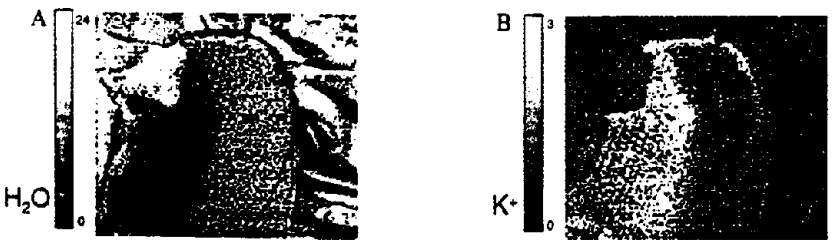


**Figure 2**: Static TOF-SIMS image of a freeze fractured paramecium. A) m/z 18-19 B) m/z 38.5-39.0. Note the complimentarity of the two images. The field of view is 67 μm.

After obtaining images of paramecia with chemical contrast the next step is to see whether a specific substance could be identified from a complex cellular matrix. Paramecia were incubated in different concentrations of a cocaine solution for 60 minutes and then rapidly frozen and freeze fractured in vacuum. At high cocaine concentrations (100 mM) the cells died and, when imaged, no chemical gradient was observed. The concentrations of $Na^+$, and $K^+$ were the same in the solutions surrounding the cell as well as in the cells. The loss of chemical contrast presumably resulted from the death of the cells. When the cells die, the ion concentration around and inside the cell appear to reach rapid equilibrium with the medium.

Reducing the concentration of the cocaine dopant to 10 μM or less resulted in viable cells that had an ion gradient similar to that of the undoped cells and cocaine was observed on the surface of the cell membrane. Two cocaine peaks were used to image for the presence of cocaine, the molecular ion plus hydrogen (M+H) as well as a major fragment at m/z of 105. The images collected are depicted in Figure 3. The signal obtained from the molecular ion and the major fragment are not strong enough to identify features on the paramecium but the signal is sufficiently intense that it can be identified as definitively associated with the cell membrane, as expected. The next step is to image material which is native to cells and is localized in specific parts of the cells.

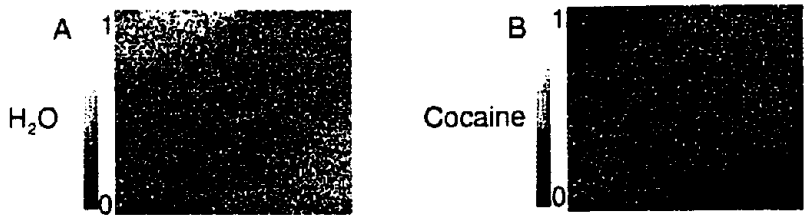


**Figure 3**: Static SIMS image of a paramecium doped with cocaine. A) m/z18-19 B) sum of 105 m/z and 304 m/z which correspond to a major fragment of cocaine and the M+H respectively.

## Conclusion

Sample preparation is critical for successful static SIMS analysis of biological samples because different sample preparations and treatments yield very different results. Also critical to obtaining better images from static SIMS is a better understanding of the ejection mechanism from a frozen aqueous medium, as well as studying the ionization process during ejection. Laser postionization of ejected neutral material may further enhance the signal obtainable from frozen hydrated samples.

## Acknowledgment

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## References

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