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Accelerated Articles

Atomic and Molecular Imaging at the Single-Cell Level with TOF-SIMS

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A complete cold chain freeze-fracture methodology has been developed to test the feasibility of using time-of-flight secondary ion mass spectrometry (TOF-SIMS) imaging for the molecular analysis of frozen hydrated biological samples. Because the technique only samples the first few monolayers of a sample, water on the surface of a sample can be a major source of interference. This problem can be minimized by placing a cold trap (fracture knife and housing at −196 °C) near the fractured sample that is held at a warmer temperature (−97 to −113 °C). This results in removal of surface water and prevents condensation on the surface. Although this approach is effective, it has been found that sample warming needs to be carefully controlled due to the volatility of other matrix molecules and the morphological effects imparted onto the cell surface during drying. By utilizing the above handling technique, it has been possible to demonstrate for the first time that TOF-SIMS imaging technology can be used to obtain images of molecular species across a cell surface with a submicrometer ion probe beam. Images of small hydrocarbons and the deliberately added dopants DMSO and cocaine have been obtained with TOF-SIMS of the single-cell organism *Paramecium*.

While conventional methods such as transmission and scanning electron microscopy are routinely used to create images of biological samples, such studies are generally limited to providing only morphological information. To complement these techniques, secondary ion mass spectrometry (SIMS) has been employed to spatially resolve important chemical information within biological tissue. SIMS utilizes a tightly focused primary ion beam to desorb chemical species from a solid matrix. Ejected ions are then collected and analyzed using a mass spectrometer. By rastering the ion beam across a sample, and collecting a mass spectrum for each point (pixel) from which ions are desorbed, it is possible to create a mass-resolved image depicting the spatial distribution of the chemical constituents of a sample.

Past SIMS analysis of single cells has fallen into the dynamic regime ($>1 \times 10^{13}$ primary ions/cm^2) and has been mainly limited to studying elemental ions.[1−3] Due to several key developments, SIMS can now be used to investigate the spatial distribution of important biological molecules within a single cell. These developments include the following: (i) the discovery that intact thermally labile molecules desorb directly into the gas phase using energetic ion beams,[4] (ii) liquid metal ion sources that can be focused to spot sizes of ≤50 nm,[5] and (iii) time-of-flight (TOF) detection schemes that allow the efficient transmission and simultaneous detection of all ions introduced into the mass spectrometer, with the sensitivity needed to analyze the small analytical volumes sputtered during static conditions ($\leq 1 \times 10^{13}$ primary ions/cm^2).[6]

In the last few years, there have been several applications of imaging TOF-SIMS to model systems demonstrating the potential of the technique for biological studies.[7,8] However, single-cell characterization has been hindered by the lack of reliable sample handling methodologies. Fortunately, sample preparation has been an area of much research in both the SEM and SIMS communities.[1,9] For example, soft tissue samples have been prepared for elemental SIMS imaging by using a variety of techniques including conventional electron microscopy fixatives such as gluteraldehyde.[3,10] To prevent the redistribution and loss of diffusible elemental ions resulting from chemical preparations, more reliable cryogenic techniques have also been used.[11] Once frozen, samples have been routinely analyzed after being freeze-dried[10,12] or freeze-substituted[3,13] and embedded in a plastic resin or after freeze-drying alone.[2,3]

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When choosing among established preparation methods, the molecular and surface sensitivity inherent to TOF-SIMS imaging must be considered. As a consequence of the sensitivity, freeze substitution and embedding techniques seem impractical, since either of these chemical components would contribute directly to unwanted background peaks in the mass spectrum and might disrupt the intrinsic distribution of target molecules. Frozen-hydrated samples have produced results that are inconsistent with freeze-dried preparations in dynamic SIMS studies in the past.[14,15] In contrast, freeze-drying seems impractical for static SIMS of molecules, as this process could result in both the redistribution and collapse of sample molecules onto the surface. For these reasons, we have been unsuccessful at using freeze-drying as a viable handling option for molecular imaging.

In this paper, we investigate the use of frozen-hydrated freeze-fractured biological samples as targets for direct molecular characterization by imaging TOF-SIMS. As a model system, concentrated *Paramecium* have been freeze-fractured and introduced into the TOF-SIMS instrument in a frozen and hydrated state. These single-cell animals are ideal for this preliminary study since their large size (~180−310 μm long[16]) allows unambiguous positioning of individual cells directly in the path of the primary ion beam. Visual placement is accomplished with the aid of an optical microscope attached to the TOF-SIMS instrument. In addition, *Paramecium* can be rinsed with distilled water without lysing, thereby minimizing any interfering background signals that might arise from the culture medium. In the frozen-hydrated state, sample and vacuum water became a major source of interference for freeze-fractured samples. Whereas other imaging studies have removed interfering surface ice by beam sputtering,[15] another more viable option utilized by the Cryo-SEM community is to warm the sample in vacuum to a temperature between −100 and −95 °C (this method of surface water removal is pressure dependent) to sublime or etch the surface ice.[17,18] This technique for water removal appears promising for TOF-SIMS experiments for several reasons. If properly controlled, freeze-etching can be used to remove the interfering surface ice, while simultaneously leaving less volatile components of the underlying biomatrix behind. This approach is also less invasive than beam etching considering that the molecular information of the sample is sensitive to the primary ion beam dosage. Finally, as will be shown here, by controlling the warming conditions, it is possible to create relatively artifact free biological surfaces that can be analyzed with imaging TOF-SIMS.

EXPERIMENTAL SECTION

Cell Preparation. *Paramecium multimicronucleatum* were sustained in 4-$^1/_2$ in. glass culture dishes with spring water (Carolina Biological) and inoculated wheat seed for nutrients.

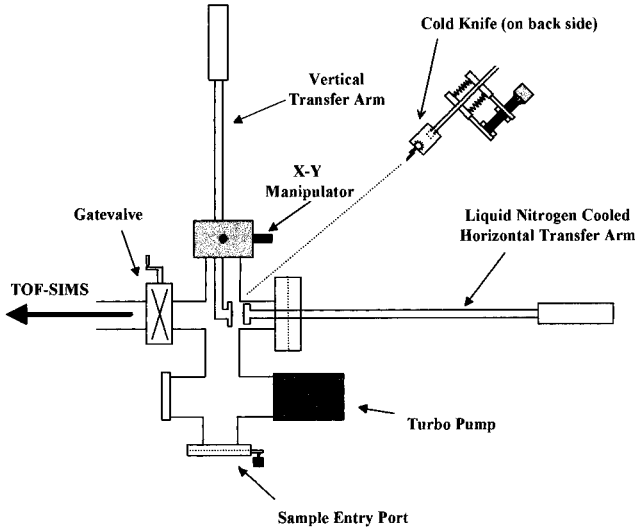

Figure 1. Schematic diagram of the freeze-fracture chamber attached to the TOF-SIMS instrument. Thermocouple attached to the bottom of the vertical transfer rod not shown.

Paramecia were generally subcultured and harvested for samples every three weeks. All culturing supplies and *Paramecia* were purchased from and cultured as suggested by Carolina Biological (Burlington, NC).

To minimize the amount of culturing debris present in the final preparation, *Paramecium* cultures were first strained through a 70 μm mesh nylon sifter (Carolina Biological) that allowed passage of the cells but removed larger particulate matter from the cell suspension. Similarly, a smaller 37 μm mesh nylon sifter, small enough to hold back the *Paramecia*, was then used to separate the cells from smaller debris in the culturing medium. After settling to the bottom, *Paramecia* were rinsed several times with distilled water and collected. This process produced 5−8 mL of a highly concentrated and relatively "clean" *Paramecium* suspension from four culture dishes (total volume ~160 mL). *Paramecia* were then gently centrifuged at 100*g* for 2 min to form a pellet directly after being isolated from culture, after being exposed to cocaine hydrochloride (Sigma, St. Louis, MO), or after being exposed to dimethyl sulfoxide (Fisher Scientific, Pittsburgh, PA).

Samples were prepared by loading the cell pellet onto one of two types of holders. Initially, *Paramecia* were placed directly onto a flat copper stub, and a sandwich was formed by placing a thin piece of copper on top of the cells. This technique was found to be useful, because the *Paramecia* consistently fractured along their long axis or through their outer membrane due to their confined orientation prior to freezing. Due to the large size of these holders, only a limited number of samples could be made from one pellet. As an alternative, small gold specimen carriers (Bal-Tec Products Inc., Middlebury, CT) were used. A *Paramecium* pellet was deposited as a mound (random orientation of the cells) directly on top of the gold carrier using a syringe. Once made, both types of samples were quickly plunged into either liquid nitrogen or, when available, liquefied Freon 22 (Falcon Safety Products, Inc., Branchbug, NJ). All samples were stored under liquid nitrogen until analysis.

Freeze Fracturing. The freeze-fracture chamber connected to the TOF-SIMS instrument is shown schematically in Figure 1. When not opened to the atmosphere, this chamber was evacuated to a base pressure of 5×10^{-7} to 2×10^{-6} Torr using 230 and 170 L/s turbomolecular pumps. Prior to being transferred into the

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fracturing chamber, samples were either taken from storage and placed directly into a dewar filled with liquid nitrogen beneath the entry port (sandwich type) or first loaded into a stainless steel housing under liquid nitrogen (small gold carriers). This stainless steel housing was adapted from a Balzers M360 freeze-fracture unit (Balzers, Liechtenstein) to fit onto the flat copper stubs that were directly compatible with the vertical transfer rod.

Prior to breaking vacuum, the gate valve to the imaging chamber was secured, and the vacuum pumps for the freeze-fracture chamber were turned off. To keep out atmospheric moisture and to shroud the sample during transfer, gaseous nitrogen was then introduced into the fracturing chamber. After the entry port was opened, the vertical transfer rod (not temperature controlled) was introduced into the liquid nitrogen dewar containing a sample and allowed to thermally equilibrate. Once samples were loaded onto the end of the transfer rod, they were transferred as quickly as possible (few seconds) into the fracturing chamber, where the vacuum was reestablished. To minimize the distance samples would travel through the open air, the dewar of liquid nitrogen was placed as close as possible to the bottom entry port.

While on the vertical transfer rod, the temperature of the sample was monitored by a thermocouple placed approximately 3−4 mm behind the surface of the copper sample holder. Due to technical constraints, the temperature of the sample was not monitored from any other position or during any other stage of handling. To determine the relationship between the temperature recorded during an experiment (i.e., from behind the holder) and the temperature at the surface of the sample, separate experiments were performed in which a second thermocouple was secured to the front of an empty sample holder. By comparing these two temperature readings, it was found that the experimental temperature varied by ∼±2 °C from the temperature near the surface of a fractured sample.

A different mode of fracturing was employed for each of the two different types of sample holders. Sandwich-type samples were fractured by first horizontally aligning them with the liquid nitrogen-cooled knife housing. Once in position, the knife housing was brought forward and secured behind the metal cover that extended over the edge of the sample. The top piece of metal was then fractured away by moving the vertical transfer arm away from the knife using the $x-y$ manipulator. Similarly, by attaching a precleaned injector blade (Electron Microscopy Sciences, Fort Washington, PA) to the knife housing, the mound of frozen *Paramecia* protruding from the top of the stainless steel specimen carrier could be chipped off. This was accomplished by raising the vertical rod to a height above the knife housing and then moving the cooled blade to a position directly beneath the mound of frozen cells. Rather than allowing the injector blade to slice through and drag across the surface of the sample, samples were fractured by slowly lowering them onto the blade so that a fracture plane could be initiated within the mound of cells. The fracturing process resulted in the exposure of a freshly cleaved surface.

All samples were fractured either immediately after being introduced into the freeze-fracture chamber (i.e., ∼−150 °C) or after warming on the vertical rod to a temperature of ∼−100 °C. Once the fracturing step was completed, the knife housing was retracted and the liquid nitrogen-cooled transfer rod was used to remove samples from the vertical rod. Samples were then transferred as quickly as possible (unless indicated otherwise) onto a liquid nitrogen-cooled stage inside the TOF-SIMS instrument (described in detail elsewhere[19]). The horizontal transfer rod was then retracted, and a base pressure of 5×10^{-10} to 2×10^{-9} Torr was reestablished within the imaging chamber. All fracturing and transferring steps were monitored through viewing ports on both the SIMS instrument and the freeze-fracturing chamber (not shown).

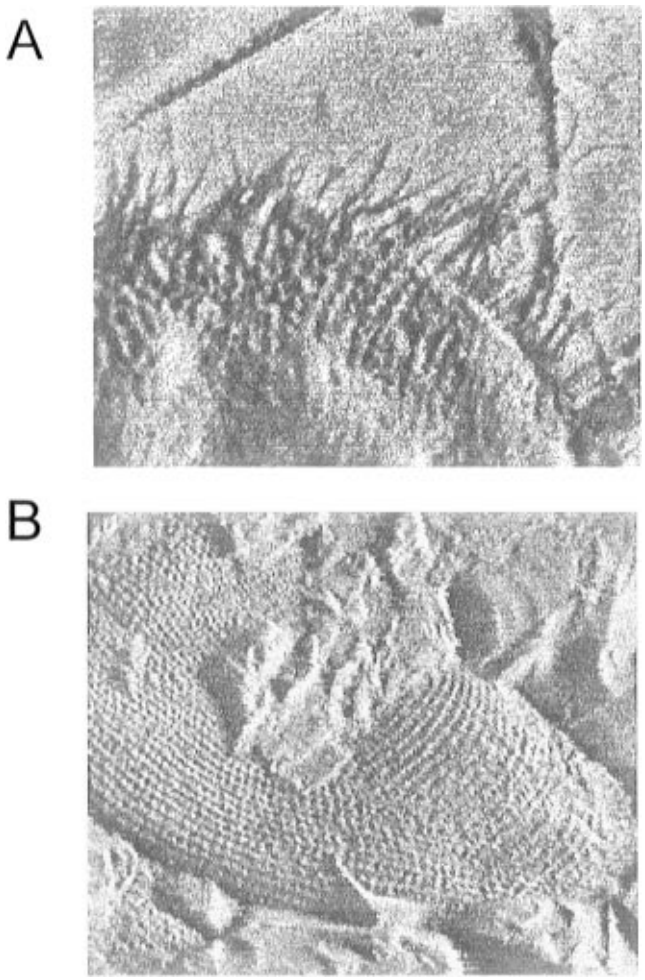

Figure 2. (A) SIM image of a *Paramecium* rapidly dried in the freeze-fracture chamber. The field of view is 33.3 μm. (B) SIM image of freeze-fractured *Paramecium* using the sandwich method. Note the longitudinal orientation of the cell as well as the regular depressions spaced ∼1 μm apart on the outer membrane. The field of view is 50 μm.

Analysis. Once inside the imaging chamber, a region of the sample containing exposed *Paramecium* was located using an optical microscope with 400× magnification and manipulated into the predetermined path of the liquid metal ion gun (LMIG). Imaging was performed using a Kratos time-of-flight mass spectrometer equipped with a gallium LMIG (FEI Co.), which can be focused to a 200 nm spot size with a 500 pA current or a 50 nm spot size when operated with a 60 pA current in dc mode (beam sizes as small as 100 nm have been obtained in the pulsed mode). The mass spectrometer is capable of achieving a mass resolution of better than 10^4 at a mass of m/z 300. Charge compensation of the sample was performed by

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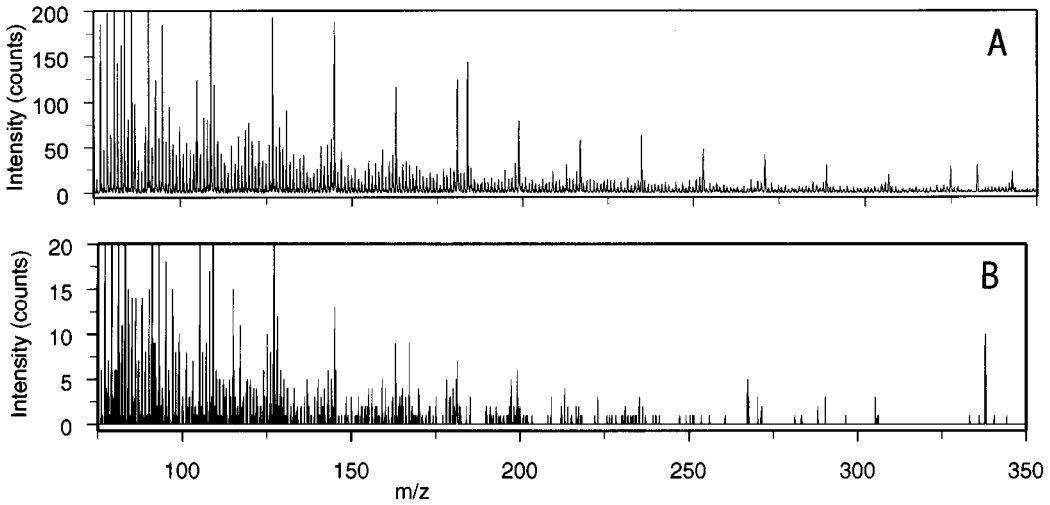

Figure 3. Mass spectra of a *Paramecium* sample (A) freeze-fractured at −147 °C (note the characteristic water peaks at intervals of *m/z* 18) and (B) at −114 °C.

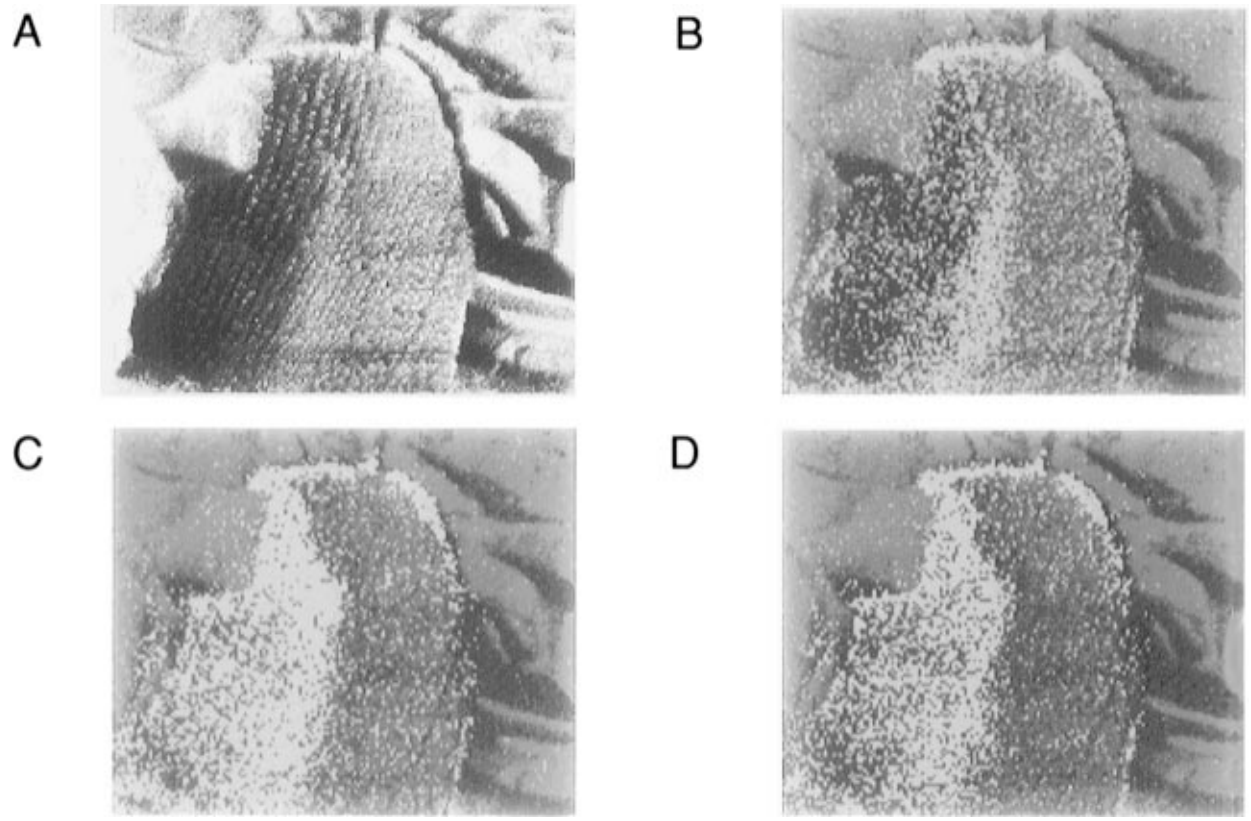

Figure 4. TOF-SIMS images of a *Paramecium* freeze-fractured using the sandwich-type holder. Images were produced either by mapping all the ions collected by the mass spectrometer (A) or by using a two-color overlay to show the relative distribution of two peaks selected from the mass spectrum (B−D). The color scales represent the intensity of the ions mapped; blue represents water and red represents hydrocarbons in (B), K+ in (C), and Na^+ in (D). The color intensities for each pixel within an image are normalized to the most intense pixel of the same color which corresponds to the maximum number of counts shown for that ion. The mass and count ranges used to map the distribution of water were *m/z* 17.86−19.32 and 0−24 counts. For the hydrocarbons, K^+, and Na^+ these were *m/z* 25.58−33.61 and 0−3 counts, *m/z* 38.74−39.35 and 0−1 counts, and *m/z* 22.62−23.40 and 0−1 counts, respectively. The field of view is 67 μm for all images. Interestingly, the pellicular depressions shown in the total ion image (A) resemble those shown in the SIM image shown in Figure 2B.

irradiating the sample with a pulsed beam of 30 eV electrons having a dc current density of 50 $\mu A/cm^2$. The electron beam was allowed to strike the sample for 50 μs after each LMIG ion pulse. During this time, the sample stage voltage was held at 0 V with 30 eV electrons having a dc current density of 50 nA/cm^2 for 50 μs.

Images were produced by either collecting and counting the secondary ions with the mass spectrometer (TOF-SIMS) or detecting virtually all the ejected material, including electrons, with a Channeltron detector placed next to the entrance of the TOF detector. This mode of data collection is referred to as scanning ion microscopy (SIM). By examining a line scan across a feature of interest, the resolution of an image was defined as the distance required to move from 20 to 80% of the total intensity change. All TOF-SIMS images were acquired using less than 10^{13} Ga^+ ions/cm^2.

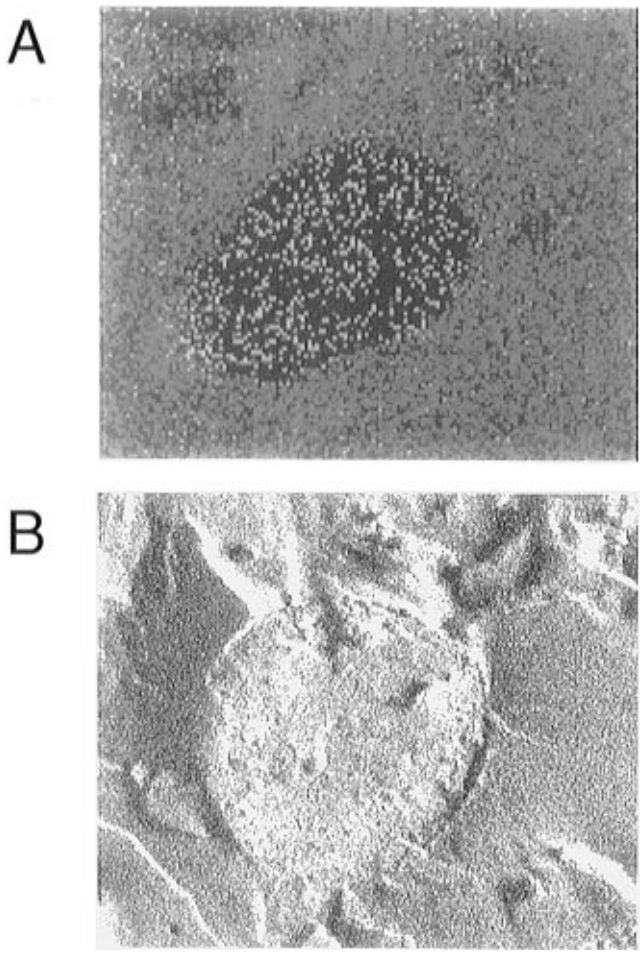

Figure 5. TOF-SIMS (A) and representative SIM (B) images of two different *Paramecia* from the same freeze-fractured sample. Note how cells are exposed in cross section using this gold-type specimen carrier. The color scaling scheme and mass ranges used here are the same as those used in Figure 4. Blue represents water and red represents K^+. (B) is a representative SIM taken from this sample demonstrating the relatively featureless sample surface (see text). The fields of view are 100 and 67 μm in (A) and (B), respectively.

RESULTS AND DISCUSSION

A SIM image of a *Paramecium* deposited directly from culture onto a sample holder without freezing and allowed to dry for several hours at room temperature in the vacuum of the freeze-fracture chamber is shown in Figure 2A. While large features such as the cilia can be easily recognized in this SIM image, the resultant TOF-SIMS images contained limited chemical information specific to the cell due to the simple preparation technique. These results are not unexpected, considering it is likely that many solvated components relocated during the drying process. To produce meaningful TOF-SIMS images, chemical species clearly need to be spatially fixed within the biological sample.

As shown by the SIM in Figure 2B, by freezing and then fracturing *Paramecium*, it is possible to preserve more of the fine structural details within the cell. Interestingly, this image resembles freeze-fractured *Paramecia* shown elsewhere.[20] While these results are an improvement over those shown in Figure 2A, the fracture temperature is the most important factor in determining the quality of the TOF-SIMS image and requires optimization. A representative SIMS spectrum of a *Paramecium* sample fractured at −147 °C is shown in Figure 3A. At this temperature, hydrogen, H_2O, H_3O^+, and clusters of $H(H_2O)^+_n$ ($n = 2-26$) dominate the spectrum. To remove surface water and prevent the condensation of water from the vacuum, samples have been freeze-fractured at warmer temperatures while keeping the fracture knife (−196 °C) near the sample to act as a cold trap. For example, the mass spectrum in Figure 3B, is of a *Paramecium* sample freeze-fractured at −114 °C. By fracturing at this warmer temperature, interference from water is minimized so that a TOF-SIMS spectral analysis can be carried out on the surface of the biological material. Several TOF-SIMS images have been obtained of a *Paramecium* freeze-fractured in the sandwich-type holders at −110 °C (Figure 4). Although K^+ (red in Figure 4C) and Na^+ (red in Figure 4D) appear to be colocalized in the cell and segregated from water (blue), the small hydrocarbons (red in Figure 4B) are more homogeneously distributed across the surface of the cell. Figure 5 shows the TOF-SIMS and SIM results obtained from two different *Paramecia* freeze-fractured in a gold-type specimen carrier at −110 °C. Due to the random orientation of cells within the gold holders, it is possible to analyze both cross-fractured and longitudinally (not shown) oriented *Paramecia* with the TOF-SIMS instrument.

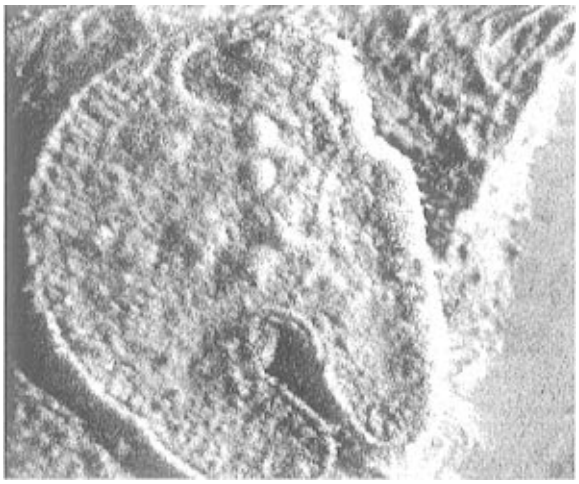

Figure 6. SIM image of a *Paramecium* held in the vacuum chamber for ~10 min prior to imaging. Note the prominent round food vacuoles distributed throughout the cell cytoplasm as well as the discontinuity in the outer cell membrane, the oral groove. The field of view in this image is 67 μm.

In an attempt to reveal cell features beneath the original ice layer exposed during freeze-fracture, some samples have been exposed to more severe etching conditions. For example, the SIM image shown in Figure 6, is from a *Paramecium* maintained on the horizontal transfer rod for ~10 min prior to being transferred into the TOF-SIMS instrument. Comparing these results with a sample that has been freeze-fractured at approximately the same temperature and transferred immediately into the analysis chamber (Figure 5B), it is clear that the sample in Figure 6 has lost a greater amount of ice, revealing more of the detailed structural morphology inside of the *Paramecium*. Although the temperature of the sample while on the horizontal transfer rod cannot be measured at present, this dramatic increase in surface morphology appears to result from prolonged sample warming which takes place because of the poor thermal contact between the sample holder and the liquid nitrogen-cooled horizontal transfer rod.

While deep-etching techniques such as these are useful in improving the morphological quality of the SIM images, they

(20) Janisch, R. *J. Protozool.* **1972**, *19* (3), 470−472.

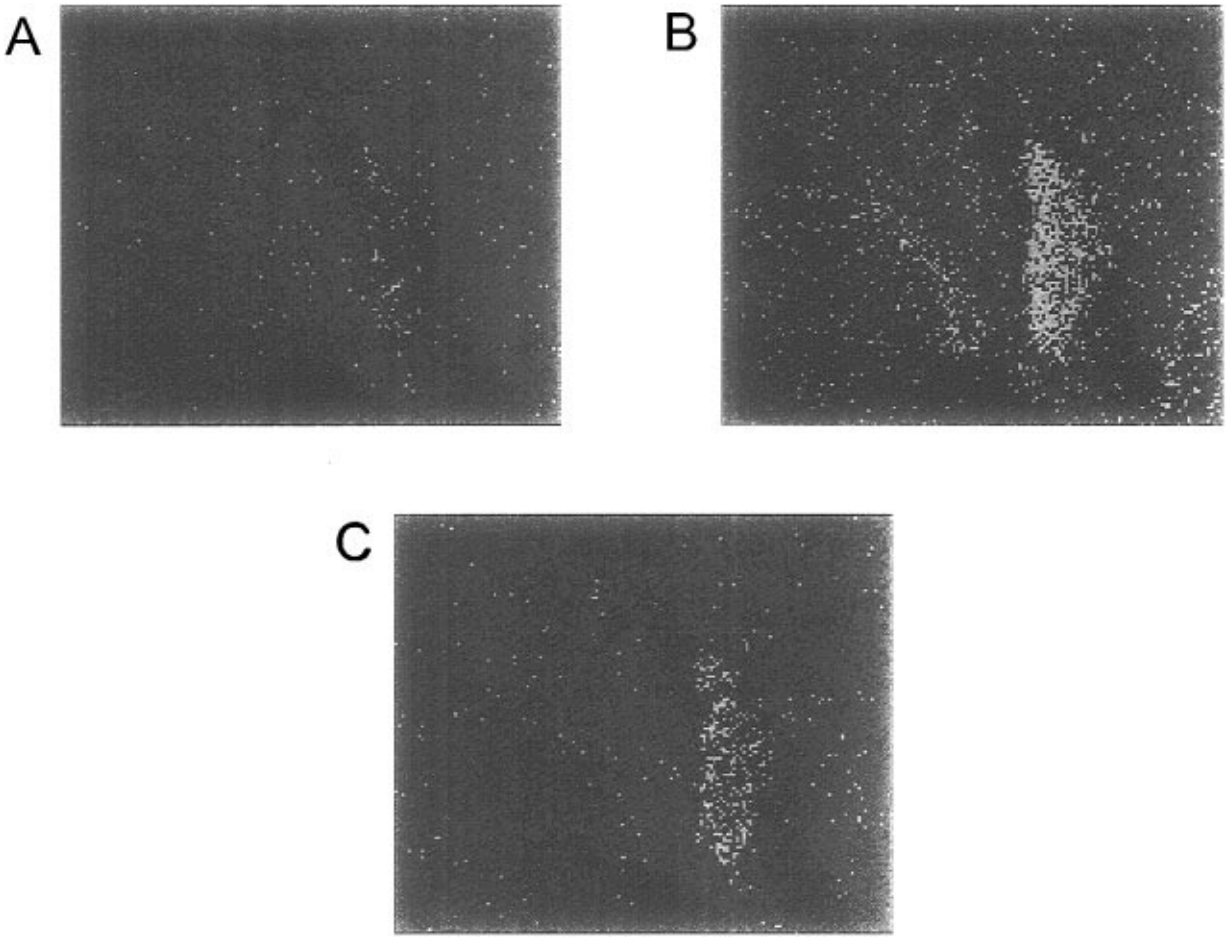

Figure 7. TOF-SIMS images of a *Paramecium* sample previously exposed to a 10 μM cocaine solution. The cocaine image in (A) was produced by creating a two-color overlay of the parent cocaine ion M + H with *m/z* 304 (the range of counts was 0−1) and a characteristic fragment *m/z* 105 (both in red) with water (blue). This image is compared to overlays of K^+ and Na^+ with water in (B) and (C), respectively. The color scaling scheme and mass windows used are the same as those described in Figure 4. The field of view is 100 μm.

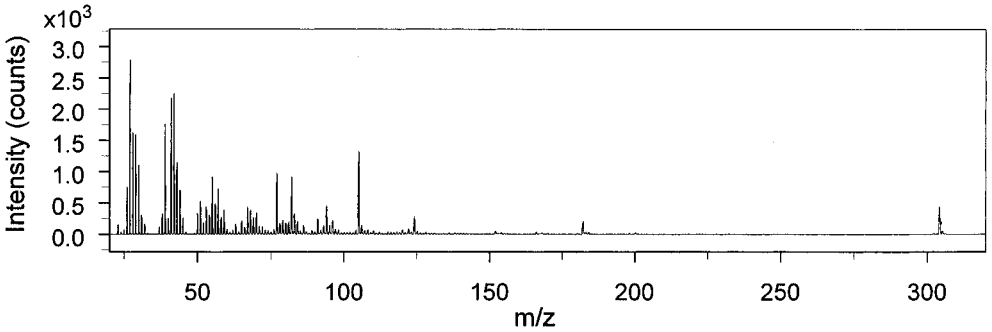

Figure 8. Mass spectrum of a *Paramecium* doped with cocaine similar to that shown in Figure 7.

unfortunately decrease the possibility of producing meaningful TOF-SIMS images. Generally, when samples are exposed to extreme etching conditions, the resultant TOF-SIMS images depict a relatively homogenous distribution of molecules across the surface of the sample as well as distorted cell features. Assuming that biological molecules other than water take on a significant vapor pressure during extreme warming conditions, then one of these artifacts can be explained. Specifically, if the vapor pressure of non-water molecules within the sample is not high enough to allow them to completely sublime away into the vacuum, then it is possible that they can recondense back onto the sample or collapse onto the surface as more volatile molecules escape. Both of these phenomena would result in a "masking" of the surface of the sample. The manner in which TOF-SIMS images are acquired can also be used to explain the difficulty of producing recognizable TOF-SIMS images for well-etched biological surfaces such as the one shown in Figure 6. In the TOF-SIMS instrument used here, the Ga^+ ion beam strikes the solid sample ~45° from the surface normal. Because of this geometry, any high surface variations in the *Z* direction are predisposed to angle-of-incidence artifacts, which have been shown to distort TOF-SIMS images.[8] To eliminate most of these "drying" artifacts, and to reproducibly create biological surfaces amenable for TOF-SIMS imaging, approximate conditions have been determined indicating that samples should be fractured in a temperature window between −115 and −100 °C and transferred onto the analysis chamber cold stage in less than 2 min.

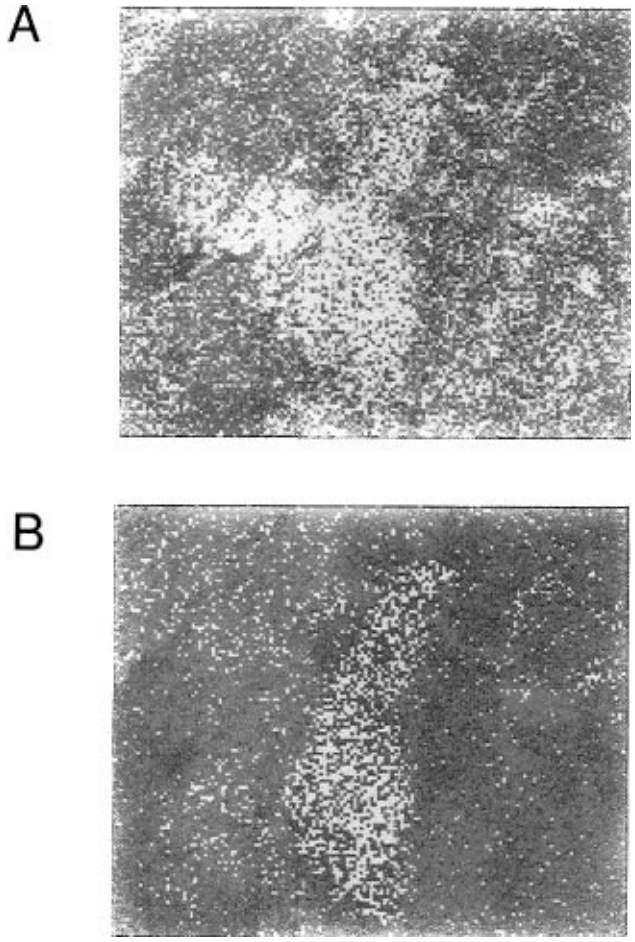

Figure 9. TOF-SIMS images of a *Paramecium* that was exposed to a 25 mM DMSO solution for 1 h. Panel A shows the distribution of DMSO (red) relative to water (blue) throughout the field of view. When compared to Ca^+ [red in (B)], these results indicate that the DMSO signal is localized to the cell. The mass and count ranges used to map DMSO's distribution were *m/z* 78.59−79.4 and 0−1, while those for Ca were *m/z* 39.43−40.49 and 0−1, and for water were *m/z* 17.72−19.41 and 0−13. The field of view is 143 μm.

With this handling protocol defined, identifiable molecular dopants have been added to the *Paramecium* to test the possibility of imaging the distribution of that molecule within a complex biological matrix. Because cocaine is lipophillic, and has been detected at physiologically significant concentrations in separate water/ice solutions (not shown), it was chosen as one of the first dopants. TOF-SIMS images of a freeze-fractured *Paramecium* sample, previously exposed to 10 μM cocaine for 1 h, are shown in Figure 7. The cocaine image shown in Figure 7A demonstrates the ability to spatially resolve the distribution of this molecule within a single cell using TOF-SIMS technology. Although this image suggests a relatively uniform distribution for cocaine throughout the field of view, by spatially comparing the signal for cocaine with the elements (K^+, Na^+) localized to the *Paramecium*, it is possible to confirm the presence of the dopant in the cell (i.e., compare panel A of Figure 7 to panels B and C). Figure 8 shows a mass spectrum obtained from a *Paramecium* doped with cocaine. The characteristic peaks for cocaine are observed at *m/z* 105 and 304.

Separate experiments support this conclusion and confirm the dopants' interaction with the *Paramecium*. For example, when *Paramecia* are exposed to a 0.1 M solution of cocaine, elements specific to undoped cells (such as K^+ and Na^+) show little or no localization (not shown). Presumably, this loss of elemental ion gradients resulted from cell death, since the *Paramecia* are observed to quickly die when exposed to the more concentrated cocaine solution. Complementary results are produced using a second dopant, dimethyl sulfoxide (DMSO). As DMSO is highly lipophilic, it is assumed to be concentrated in the membrane of the *Paramecium*. When *Paramecia* are exposed to a 25 mM solution of DMSO for 1 h, the resultant TOF-SIMS images indicate a much more localized cell specific signal for the dopant (Figure 9).

CONCLUSIONS

The results of this study confirm that freeze-fracture techniques can be used to prepare biological samples for TOF-SIMS imaging. Use of the freeze-fracture knife as a cold trap in combination with freeze-fracturing at relatively warm temperatures (∼−100 °C in vacuum) results in mild freeze-etching, decreasing water contamination and making it possible to acquire TOF-SIMS images of molecules from a single *Paramecium*. The relatively simple method of monitoring the temperature of the sample used here is adequate for defining some limitations of this method; however, events occurring at the sample surface during the drying process clearly need to be further investigated. These studies will be especially important in determining whether or not molecules such as the hydrocarbons shown in Figure 4B are specific to the cell or are a contaminant from the vacuum chamber. To do this, efforts are currently being focused on developing an accurate means of monitoring and controlling the temperature of the sample.

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