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Identification of Cellular Sections with Imaging Mass Spectrometry Following Freeze Fracture

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Freeze-fracture techniques have been used to maintain chemical heterogeneity of frozen-hydrated mammalian cells for static TOF-SIMS imaging. The effects the fracture plane has on scanning electron microscopy and dynamic SIMS images of cells have been studied, but the implications this preparation method has on static SIMS have not been addressed to date. Interestingly, the chemical specificity and surface sensitivity of TOF-SIMS have allowed the identification of unique sections of rat pheochromocytoma cells exposed to the sample surface during freeze fracture. Using the extensive chemical information of the fractured surface, cellular sections have been determined using TOF-SIMS images of water, sodium, potassium, hydrocarbons, phosphocholine, and DiI, a fluorescent dye that remains in the outer leaflet of the cell membrane. Higher amounts of potassium have been imaged inside a cell versus the surrounding matrix in a cross-fractured cell. In other fractures exposing the cell membrane, phosphocholine and DiI have been imaged on the outer leaflet of the cell membrane, while phosphocholine alone has been imaged on the inner leaflet. In this paper, we discuss how imaging mass spectrometry is used to uniquely distinguish three possible sections of cells obtained during freeze fracture. The identification of these sections is important in choosing cells with a region of interest, like the cell membrane, exposed to the surface for a more thorough investigation with imaging static TOF-SIMS.

Freeze-fracture techniques allow molecule-specific imaging of cells with static secondary ion mass spectrometry (SIMS).[1−3] SIMS analysis has been used to identify both elements and compounds at single-cell and subcellular levels in freeze-dried samples. Dynamic SIMS, which probes elements at various depths from the sample surface, has imaged elements and isotopically labeled molecules at the single-cell level in biological imaging studies.[4−9] In addition to being less destructive than dynamic SIMS, static SIMS, a softer ionization technique, analyzes the first few layers of a sample surface and detects molecules in their native state. Freeze-dried biological systems have been studied with static SIMS.[10] In addition to freeze-dried samples, frozen-hydrated samples have been analyzed by time-of-flight (TOF)-SIMS.[3,11,12] In these studies, cells are temporally preserved in their aqueous environment by quickly freezing samples and keeping them at low temperatures throughout preparation and TOF-SIMS analysis. New challenges in exposing the appropriate section of frozen-hydrated cells to the surface and in preserving the heterogeneity of molecules on the surface arise because static SIMS is surface specific.[11] Thus, freeze-fracture techniques have been adapted from electron microscopy (EM) for static TOF-SIMS analysis.

A sandwich fracture technique used in EM has been modified for TOF-SIMS and ion microscopy.[13−16] In this technique, cells in aqueous media and spacer beads of cellular dimensions are frozen between two silicon wafers. The spacer beads prevent crushing of the cells and influence the propagation of cleavage planes through the ice. In addition, cellular morphology, ice crystallization, and cellular-surface adhesion forces influence which section of the cell is exposed during freeze fracture. In scanning electron microscopy (SEM), a highly detailed morphological view of a metal-coated fractured sample is obtained. An interpretation of the cellular components at the surface is used to determine which part of the cell is exposed. For example, when a membrane is exposed to the surface, the identity of the membrane leaflet is determined by correlating the density and shape of the intermem-

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brane proteins.[17−19] Direct transfer of these methods to TOF-SIMS is inappropriate because the surface is coated with metal in SEM and individual membrane proteins are not detected by the technique. Furthermore, fracturing criteria have been shown to be more stringent when concerned with chemical versus morphological preservation. For example, condensation of ambient water onto the surface masks cells in static TOF-SIMS imaging. Unfortunately, EM methods in which the sample is heated to remove this water[17] cannot be used due to movement of diffusible species at the surface.[11]

New sample preparation and fracture techniques have been developed to preserve chemical information in frozen-hydrated membranes for TOF-SIMS imaging.[11] Freeze-fracture methods have been developed for paramecia (∼70 μm wide) to acquire molecule-specific images with sufficient sensitivity and spatial resolution to image a single cell.[1] In these studies, the cell is either fractured in cross section or along the membrane surface. To identify cross sections, subcellular features such as food vacuoles have been resolved by imaging intense ions such as Na^+. Kinetisomes (∼1 μm in diameter) have been located in Na^+ images when the fracture has occurred along the membrane surface. In addition, small lipophilic dopants such as dimethyl sulfoxide and cocaine have been imaged without resolution of cellular features. Improvements in the freeze-fracture technology have allowed static SIMS imaging of phospholipids in red blood cells and frozen-hydrated liposomes.[3,11] In addition, in situ bright-field, fluorescence, and scanning ion (analogous to scanning electron) imaging have been used with TOF-SIMS, to image membranes of rat pheochromocytoma (PC12) cells in the ice of frozen-hydrated samples.[20,21] The localization of several molecular ions and fragment ions in single PC12 cell membranes has been demonstrated. A key factor in TOF-SIMS analysis is that the cell section of interest must be exposed at the surface during freeze fracture.

In this paper, we discuss a novel approach to freeze-fracturing cells that is conducive to static TOF-SIMS imaging. Using this approach, we use molecule-specific images to identify regions of cells exposed during the fractures. The chemical environments inside and outside the cell and in the membrane are exploited to identify cellular sections exclusively with TOF-SIMS imaging. Molecule-specific images are shown for each of these sections. These chemical signatures identified can be used to locate appropriately sectioned cells and investigate their molecular components using static TOF-SIMS. Most importantly, we can determine which cells have been fractured to expose the cell membrane to the surface for static TOF-SIMS imaging. Imaging the molecular composition of these cellular membranes on a subcellular scale provides the potential to study phospholipid domains in the membranes of neurochemical cell models during membrane functions such as exocytosis.

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EXPERIMENTAL SECTION

Time-of-Flight Secondary Ion Mass Spectrometry. All analyses were performed in a Kratos (Manchester, U.K.) Prism TOF-SIMS spectrometer using a FEI (Beaverton, OR) gallium liquid metal ion beam (25 kV, 500-pA, 200-nm diameter). The ion beam had a 50-ns pulse width and was oriented at 45° to the sample. A liquid nitrogen-cooled stage (Kore Tech. Ltd., Cambridge, U.K.) was biased at + 2.5 kV with an extraction lens biased −4.7 kV. Secondary ions followed a 4.5-m time-of-flight path, including a reflectron, before being detected at a microchannel plate assembly (Galileo Co., Sturbridge, MA). A mass spectrum of a freeze-fractured sample was collected across the area to be imaged. Intensities of individual m/z ranges selected from the resulting spectrum were plotted in submicrometer pixels to generate an image. The instrument was also equipped with a channeltron detector (Burle, Lancaster, PA) positioned ∼0.5 cm from the sample to obtain morphological images similar to scanning electron micrographs. The channeltron collected charged secondary ions and electrons emitted from the sample, resulting in a morphological image of the surface.

Microscopy. Ex situ fluorescence images were obtained on a Zeiss (Thornwood, NY) epifluorescence microscope using a Hamamatsu (Bridgewater, NJ) monochrome digital camera for image capture. The essential components of a vertical illumination microscope (Olympus, Melville, NY) were mounted on the TOF-SIMS instrument for performing reflected bright-field and epifluorescent microscopy on the cold analysis stage. Olympus vertical illumination using a 100-W Hg light source was used. For fluorescence studies of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR)-labeled cells, an Omega (Battleboro, VT) XF101filter set was used for selection of excitation (549 nm) and emission (565 nm) wavelengths. Images were enhanced by altering sharpness, contrast, and brightness using standard graphics software.

Rat Pheochromocytoma (PC12) Cell Preparation. PC12 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured onto 5 mm × 5 mm Si wafers (Ted Pella, Redding, CA) coated with type I mouse collagen (Sigma, St. Louis, MO).[20] When desired, differentiation was induced by adding 100 ng/mL nerve growth factor (NGF; Sigma) to the media for 2 days. Cells were stained for 2 h with a solution of 10 μg DiI/mL media prior to freezing. Media and excess dye were removed by several rinses with phosphate-buffered saline (D-8662, Sigma) containing 5-μm-diameter polystyrene spacer beads (Duke Scientific, Palo Alto, CA). Next, a silicon shard was placed onto the wafer, and the entire assembly was frozen in liquid propane.

Sample introduction and freeze fracture were performed in ultrahigh vacuum as previously described.[11] Briefly, the sample stub was anchored onto a cold stage at −196 °C and slowly warmed to −105 °C. Next, the silicon shard was removed, exposing a fresh surface of the sample for analysis. The sample was cooled to −196 °C and moved onto the cold sample analysis stage where it was analyzed using fluorescence, scanning ion, and TOF-SIMS imaging.

RESULTS AND DISCUSSION

Cellular Imaging. PC12 cells have been employed as a single-cell model for neuronal membrane processes because they release catecholamines by exocytosis and differentiate into neuron-like

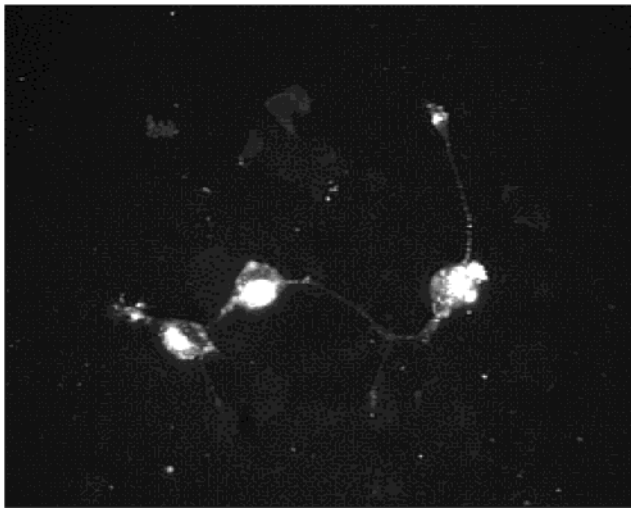

Figure 1. Fluorescence image of DiI-labeled, differentiated, PC12 cells that were cultured onto a Si substrate for subsequent fast-freezing, freeze-fracturing, and TOF-SIMS analysis. An approximate 180-μm-wide field of view (fov).

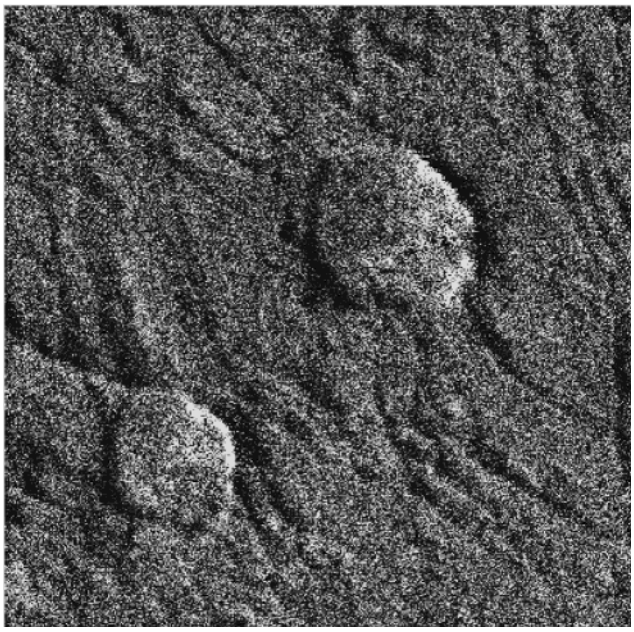

Figure 2. Representative scanning ion micrograph of freeze-fractured, frozen-hydrated PC12 cells exposed at the surface of the fracture ice layer. An approximate 50-μm-wide fov.

cells upon treatment with nerve growth factor. It is possible to culture these cells on collagen-coated SIMS targets while still maintaining their ability for cellular differentiation (Figure 1). The location of cells on the substrate is determined by staining cells with DiI and by imaging the sample with an epifluorescence microscope. In addition, scanning ion micrographs offer a morphological view of cellular structures in the ice and allow examination of the heterogeneity associated with the cellular surfaces. The scanning ion micrograph in Figure 2 shows two PC12 cells exposed at the frozen-hydrated surface. These images are typically acquired after static SIMS analysis to prevent surface damage from the ion beam before the static SIMS imaging experiments.

Since static TOF-SIMS is only sensitive to the first few layers of material at the surface, understanding the chemical makeup

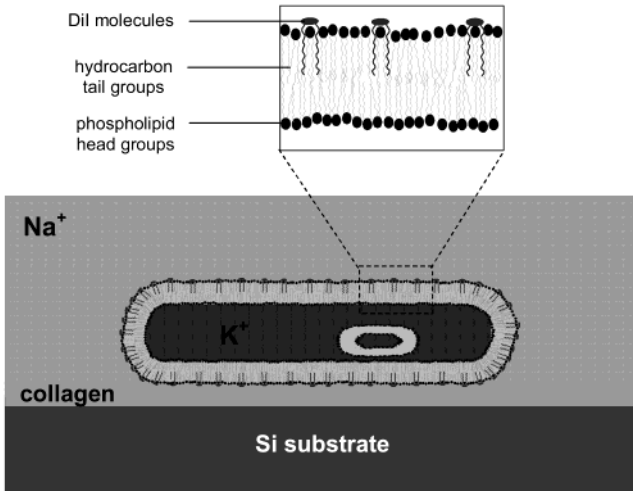

Figure 3. Illustration of chemical composition of frozen-hydrated single cell sample. The inset illustrates the location of DiI and phospholipids in the cell membrane.

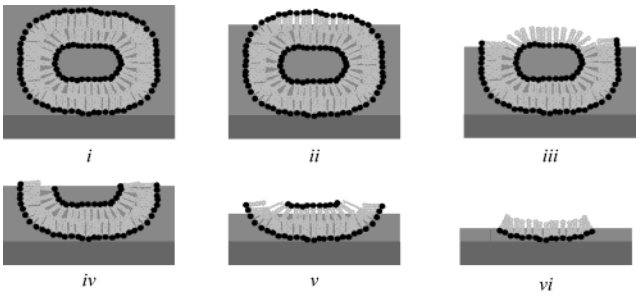

Figure 4. Schematic of possible surfaces exposed from a single cell for TOF-SIMS analysis: (i) covered with buffer, (ii) convex ES, (iii) convex PF, (iv) cross fracture, (v) concave PS, AND (vi) concave EF.

of the cell is crucial in determining which region is exposed to the surface. A schematic representing the simplified chemical composition of a PC12 cell is shown in Figure 3. Since the cells were living when they were fast-frozen, the concentration of sodium is greater in the surrounding buffer than inside the cells. Conversely, the concentration of potassium is greater inside the cell when compared to the outside. Both sodium and potassium are easily observed with TOF-SIMS. The molecular ion signal of DiI can be obtained with TOF-SIMS in the membranes of cells in frozen-hydrated samples.[21] The DiI used in these experiments contains long hydrocarbon tail groups (C18). This type of DiI inserts its tail groups into the lipophilic interior of membranes and remains in the outer leaflet of the cellular membrane as shown in the inset of Figure 3.[22,23] Other species present in the cell schematic that are routinely observed in TOF-SIMS images include hydrocarbons from the cell and collagen coating, water from the extracellular media, phosphocholine headgroup from the membrane,[3,11,22−25] and even silicon when the substrate is uncovered.[11]

Several surfaces are exposed during freeze fracture for TOF-SIMS analysis of cells. The different surfaces exposed are

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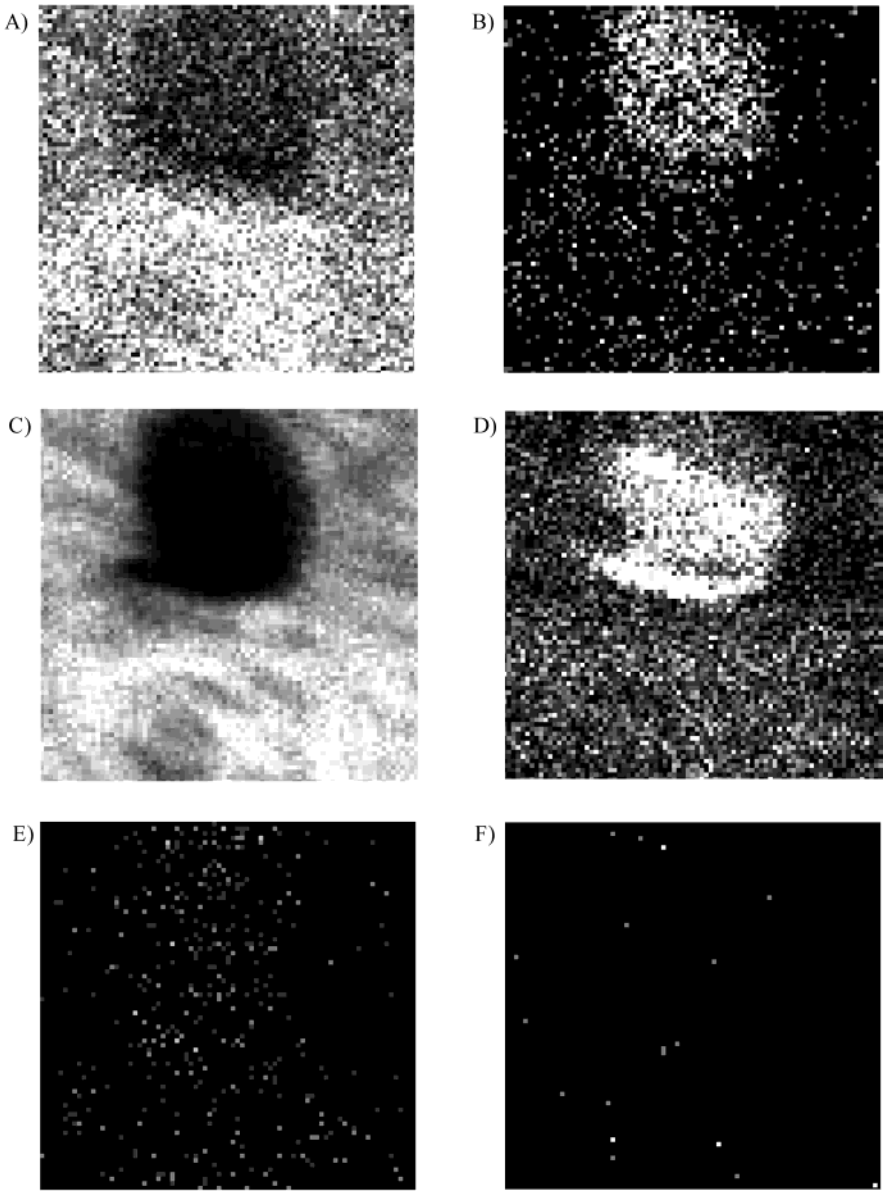

Figure 5. Positive ion molecule-specific images of a cross-fractured PC12 cell. (A) 18 and 19 *m*/*z* (H_2O^+, H_3O^+) on a scale of 0−12 counts, (B) 69 *m*/*z* ($C_5H_9^+$) on a scale of 0−3 counts, (C) 23 *m*/*z* (Na^+) on a scale of 0−150 counts, (D) 39 *m*/*z* (K^+) on a scale of 0−10 counts, (E) 184 *m*/*z* (phosphocholine headgroup, $C_5H_{15}NPO_4^+$) on a scale of 0−3 counts, AND (F) 833 *m*/*z* (DiI molecular ion) on a scale of 0−2 counts. An approximate 65-μm-wide field of view.

demonstrated schematically in Figure 4. The first surface occurs when the cell is covered by ice (i). This has not been formally described because it does not exhibit morphological features that can be detected by EM alone. However, when fluorescence microscopy is used, covered cells are observed. There are five additional surfaces described for EM[17−19] where different cell regions are exposed during freeze fracture. Specifically, they are (ii) a convex fracture exposing the extracellular surface (ES) of the membrane, (iii) a convex fracture exposing the protoplasmic face (PS) of the membrane, (iv) a cross fracture, (v) a concave fracture exposing the protoplasmic surface (PF) of the membrane, and (vi) a concave fracture exposing the extracellular face (EF) of the membrane.

Images of Cross-Fractured Cells. Cross-fractured cells (Figure 4iv) expose the inner cytoplasm containing elevated potassium levels. Hence, following location of cells with fluorescence imaging, a strong TOF-SIMS signal for K^+ indicates a cross fracture. In contrast, cells that are covered with buffer exhibit fluorescence but exhibit only species such as water and sodium in the TOF-SIMS images. Molecule-specific images of a cross-fractured PC12 cell are shown in Figure 5. The H_2O^+ signal (A) is depressed in the top of the image, corresponding to a fluorescent area seen in the microscope. Since cells are mostly composed of water, the depression of water in the cellular region is at first unexpected. However, the reduction in signal is likely due to the expected differences in ionization of water between the salty aqueous medium and the cell cytoplasm, which contains not only a variety of small ions but also a very complex mixture of hydrated macromolecules. Another possibility includes a slight etching of the surface water during fracture that is not detectable in any of the microscope or scanning ion images. This etching would concentrate the cellular species, thereby decreasing the

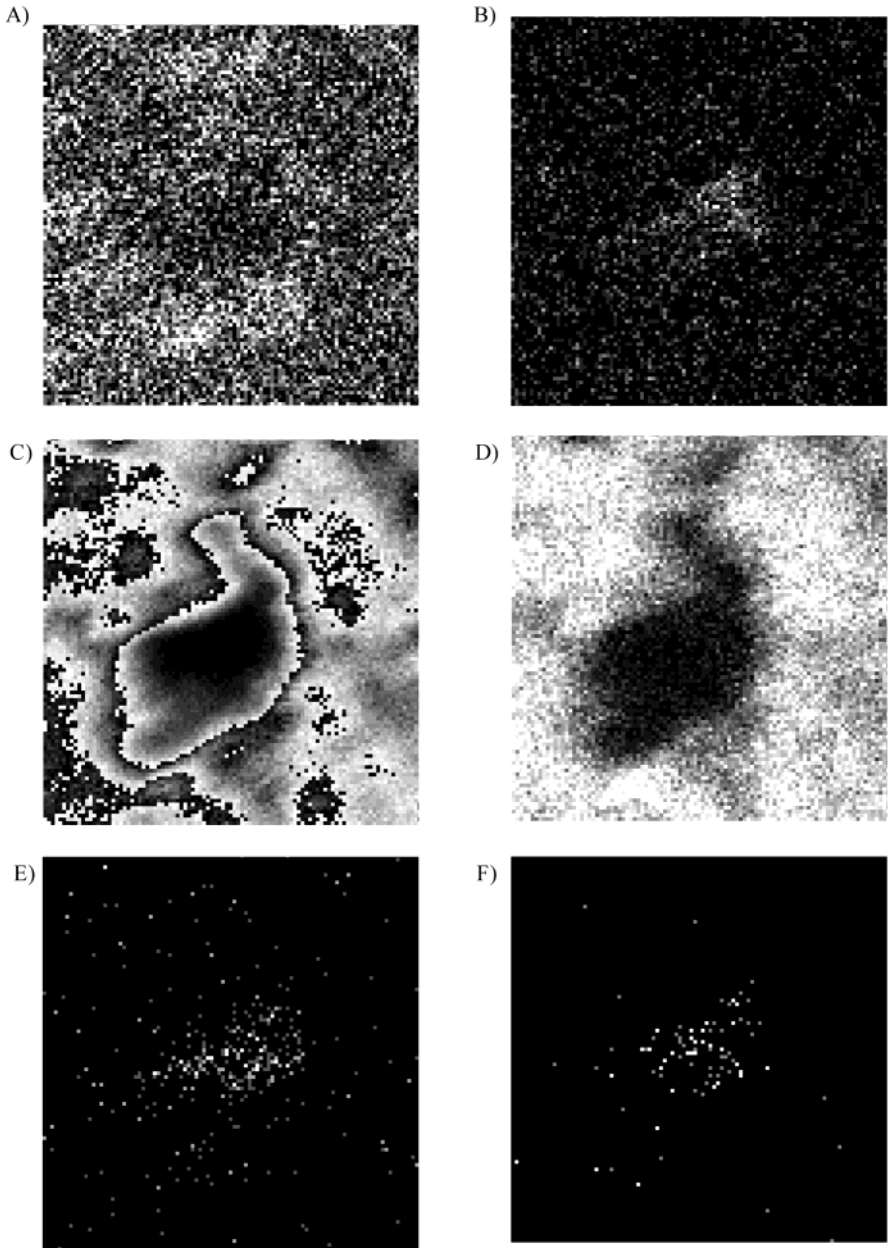

Figure 6. Positive ion molecule-specific images of freeze-fractured NGF-treated PC12 cells, representing the outer leaflet exposed cases: (A) 18 and 19 *m*/*z* (H_2O^+, H_3O^+) on a scale of 0−5 counts, (B) 69 *m*/*z* ($C_5H_9^+$) on a scale of 0−6 counts, (C) 23 *m*/*z* (Na^+) on a scale of 0−30 counts, (D) 39 *m*/*z* (K^+) on a scale of 0−30 counts, (E) 184 *m*/*z* (phosphocholine headgroup, $C_5H_{15}NPO_4^+$) on a scale of 0−3 counts, and (F) 833 *m*/*z* (DiI molecular ion) on a scale of 0−2 counts. An approximate 80-μm-wide field of view.

apparent concentration of the water in the cellular region. The $C_5H_9^+$ ion (B) is used as a chemical marker of hydrocarbon-containing structures that are localized within the cell. The Na^+ image (C) clearly contrasts the cell from the surrounding matrix. The reverse location of K^+ in the cell (D) with respect to Na^+ in these images is characteristic of cross-fracturing a cell to reveal the cytoplasm at the sample surface. Only the edge of the cell membrane is exposed in this fracture and the width (~100 Å) is too small to image. Thus, the phosphocholine headgroup (E) and DiI (F) ions are not localized in the images of this cross-sectioned cell.

Images of the Membrane Outer Leaflet. In Figure 6, TOF-SIMS images of differentiated PC12 cells demonstrate the fracture of the outer leaflet of a cell. The reduction of H_2O^+ signal (A) occurs where the cell is exposed from the surrounding ice. As expected, there is still water present in the cellular region, since the cell and the cell membrane are hydrated. The hydrocarbon image (B) shows inverse localization to H_2O^+ for additional confirmation that a cell is present. The Na^+ signal (C), although off scale in the surrounding area, shows an absence of signal in this region as well. A depression in the K^+ image (D) is also evident, indicating that the cell is not cross-fractured to expose the high concentration of potassium in the cytoplasm. Phosphocholine headgroup (E) and DiI (F) signal are clearly localized in the TOF-SIMS images of the cell.

Exposure of any membrane surface results in a strong signal for phosphocholine in the TOF-SIMS images. DiI images allow discrimination between signals from the outer leaflet and the inner

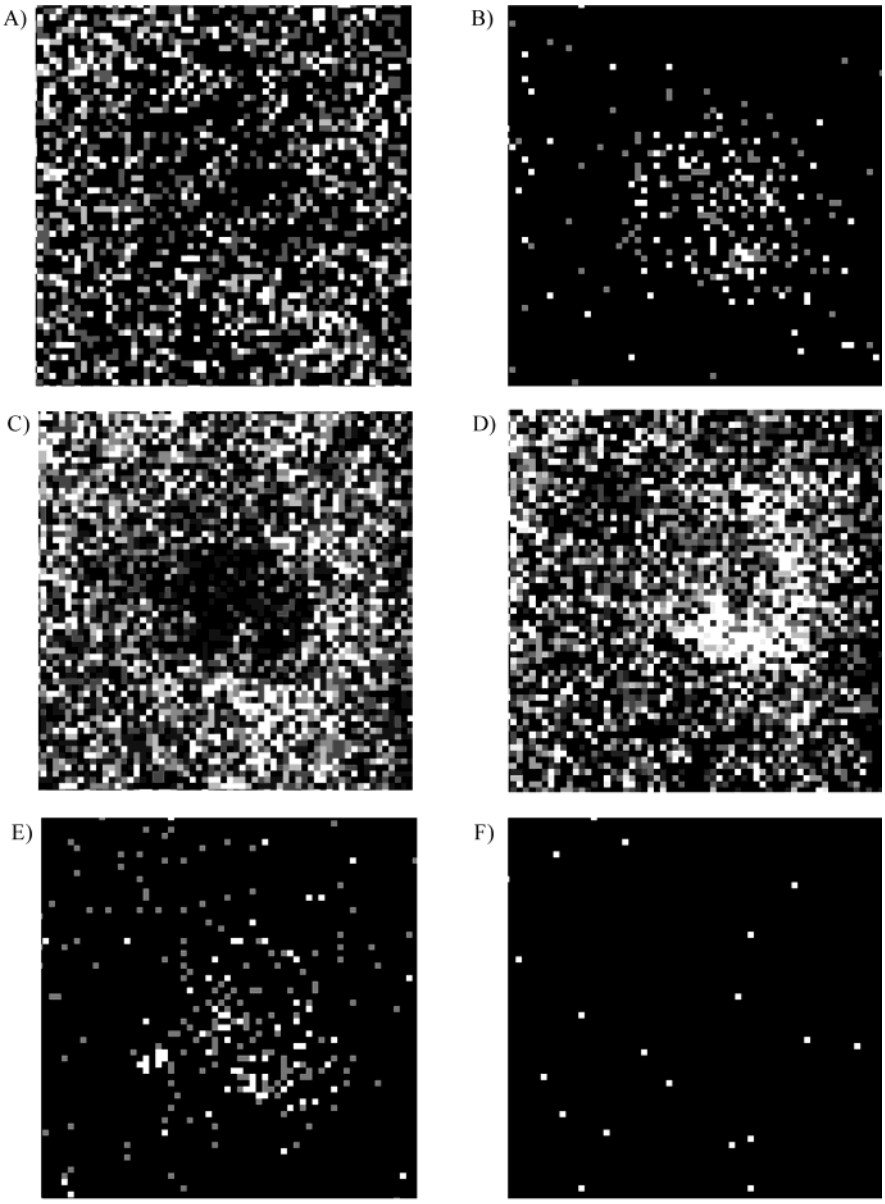

Figure 7. Positive ion molecule-specific images of a freeze-fractured NGF-treated PC12 cell, representing the inner leaflet exposed cases: (A) 18 and 19 *m*/*z* (H_2O^+, H_3O^+) on a scale of 0−4 counts, (B) 69 *m*/*z* ($C_5H_9^+$) on a scale of 0−2 counts, (C) 23 *m*/*z* (Na^+) on a scale of 0−6 counts, (D) 39 *m*/*z* (K^+) on a scale of 0−20 counts, (E) 184 *m*/*z* (phosphocholine headgroup, $C_5H_{15}NPO_4^+$) on a scale of 0−2 counts, and (F) 833 *m*/*z* (DiI molecular ion) on a scale of 0−1 counts. An approximate 50-μm-wide field of view.

leaflet of the cell membrane. Incubating cells in DiI solution labels the entire outer leaflet of unfixed cells, including the leaflet embedded in the collagen-coated substrate. Therefore, the colocalization of phosphocholine and DiI in the cell image signifies fractures in which the outer membrane is exposed. The most common fracture described here results in uncovering the outer leaflet of the cell membrane as shown in Figure 4ii but could also result in the surface shown in Figure 4vi.

Images of the Membrane Inner Leaflet. An occasional surface exposed during freeze fracture of cells is the inner leaflet of the membrane, as shown in Figure 7. The H_2O^+ image (A) shows a lower intensity, whereas the hydrocarbon image (B) is localized in the region where the cell resides. Likewise, the Na^+ (C) signal is decreased and K^+ (D) signal is increased in the region of the cell. Interestingly, the K^+ signal does not appear to be uniform across the cell. The phosphocholine headgroup (E) signal is localized, while DiI (F) is not localized in these TOF-SIMS images.

A lack of DiI signal in the images suggests that the inner membrane leaflet is exposed, as shown in Figure 4iii and 4v. In the first case, the fracture plane passes through the top bilayer, removing the outer leaflet and revealing the top inner leaflet as shown in Figure 4iii. In the second case, the fracture plane passes just above the bottom bilayer, exposing the bottom inner leaflet as shown in Figure 4v. The inner leaflet is exposed in either case, revealing phospholipids for imaging but not DiI. In addition, a slightly higher K^+ signal is expected since the headgroup originates from the inner leaflet where the phospholipid headgroups interact with K^+ in the cytoplasm. Intensity variations occur possibly due to surface uniformity effects or due to a fracture exposing inner cell regions containing membrane-bound organelles to the surface.

SUMMARY

Static TOF-SIMS imaging requires new sample preparation protocols for cellular analysis because SEM methods used to preserve morphology do not accommodate the molecular specificity of mass spectrometry imaging. Chemical signatures have been established with TOF-SIMS to identify which part of the PC12 cell is exposed at the surface during freeze fracture. Selected molecule-specific images are used to determine whether (1) the cell is cross-fractured exposing the cytoplasm, (2) the outer leaflet of the cell membrane is exposed, or (3) the inner leaflet of the cell membrane is exposed to the surface. Although the images cannot yet be used to discriminate the top from the bottom of a particular cell, it is more important to discriminate which membrane surface (outer vs inner leaflet) is being analyzed. The molecule-specific images here are consistent with what is known about these cells and can be used to identify unique sections, including the through-cell and membrane sections, which have not been previously described with a chemically specific imaging technique. These techniques will be used to identify sections of cells so they can be further examined for correlations between their molecular structure and their corresponding cellular function.

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