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SURFACE and INTERFACE ANALYSIS

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C60-ToF SIMS imaging of frozen hydrated HeLa cells

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Sample preparation continues to be a major challenge for SIMS studies of biological materials. Maintaining the native hydrated state of the material is important for preserving both chemical and spatial information. Here, we discuss a method that combines a sample wash and dry protocol followed by plunge-freezing in liquid ethane for a frozen-hydrated analysis of mammalian cells (HeLa). This method allows for the removal of the growth medium and maintains the hydrated state of the cells so that they can be prepared frozen-hydrated without the need for a freeze-fracture device. The cells, which were grown on silicon, were successfully regrown after the cleaning procedure, confirming that a significant portion of the cells remain undamaged during the wash and dry procedure. Results from preliminary SIMS measurements show that is it possible to detect a large variety of biomolecular signals, including intact lipids from the plasma membrane in the mass range of 700–900 Da from single cells, with little external water interference at the surface. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: C60; HeLa cell; frozen-hydrated; sample preparation; biological imaging

Introduction

The study of lipids and lipid metabolism is an important part of understanding both the function of cells and the role lipids play in disease progression.[1,2] Identifying the chemical signatures of lipids and other cellular species spatially on the dimensions of single cells (10–30 μm) is a challenging prospect. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) has the potential to provide this information, with several studies already showing the application for lipid identification at the surface of a material as well as for molecular identification of cellular materials into the bulk of the specimen.[2–4]Perhaps the largest obstacle for routine characterization of cells with ToF-SIMS is sample preparation. The SIMS technique obviously requires an environment in a vacuum. This reduced pressure causes several stresses to cells including molecule migration and morphological changes because of the rapid removal of volatile compounds such as water. Frozen-hydrated sample preparation has been shown to be an excellent method to closely maintain the native hydrated state and distribution of cellular materials in a high-vacuum environment.[5] The frozen-hydrated environment has also been shown to increase the ion yields for lipids and other cellular species.[6,7] However, almost all frozen-hydrated cellular studies use a freeze-fracture device, which often causes a fracture plane through the cell. Although extremely useful for subsurface characterization, it can complicate identification for an intact cell including characterization of the outer cell membrane. In this study, we discuss a preparation routine to characterize mammalian cells in a frozen-hydrated state after a sample washing procedure. The process does not require freeze-fracturing, which allows us to probe the lipid-rich region of the outer plasma membrane without interfering water–ice accumulation.

Experimental

ToF-SIMS studies were performed using a Bio-TOF SIMS instrument described previously.[8] Briefly, data were collected using a 40-keV C_{60}^+ primary ion source (Ionoptika Ltd., Chandler's Ford, UK) with an 80-ns pulse width and a beam current of approximately 30 pA rastered over a 300×300-μm^2 area while in delayed extraction mode. For depth profiling, data were collected within an etched area that was three times smaller than the raster area to avoid edge effects. Experiments were performed at ~90 K on the basis of thermocouple measurements. To achieve cryogenic temperatures, liquid nitrogen–cooled nitrogen gas was pumped from an external cylinder into cooling lines connected to the analysis stage.

The procedure for preparing frozen-hydrated cells closely follows the protocol detailed by Berman *et al.*[1] Specifically, for our experiments, cells are pipetted into a petri dish that also contained several silicon shards (smooth side up) and growth

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medium (DMEM, Cellgro, Manassas, VA). This dish was placed in a cell incubator for 24 h. Longer or shorter incubation periods and the number of cells initially deposited affect surface coverage of cells on the silicon shards. Ideally, only 30%–40% of the silicon surface should be covered with cells. Fewer numbers create difficultly in identifying the location of cells with imaging SIMS whereas too many cells exhibit large patches of overgrown areas, making isolation of single cells not possible. Once the surface coverage is appropriate, the silicon is removed from the medium and immediately dipped into a 0.15-M ammonium formate cleaning solution, in which it is gently swished around for ~5 s before repeating the process with a second clean solution. After the second wash, the edge of the silicon is placed in contact with absorbent paper, which removes much of the excess washing solution. After this procedure, a gentle stream of nitrogen gas is flowed over the silicon surface, again forcing any residual washing solution to a corner, where it is removed by contact with paper. Within less than 30 s, the surface visually appears to be dry. To examine the state of the cells after the washing procedure, the samples are >reintroduced into a new petri dish and given growth media. After 24 h, there is clear cell growth, supporting the idea that the cells are not damaged via the washing routine (Fig. 1). Results indicating the health of the cells after similar cleaning protocols have also been reported.[1,9]Immediately after the drying process, the cells are plunged into liquid nitrogen–cooled liquid ethane (99.0%; Scott Specialty Gases, Plumsteadville, PA). Although ethane and propane have similar cooling rates, ethane is used because, in several of our experiments, it has been observed to produce less chemical interference in collected SIMS data. The cells are held in liquid ethane for ~3–4 s and then are rapidly transferred to liquid nitrogen, where the silicon is attached to the SIMS sample holder. The cells are maintained in liquid nitrogen until their transfer into the instrument.It is important to maintain adequate sample temperature before and during the experiment. The most serious concern for this type of experiment is allowing the sample temperature to increase to more than ~165 K in a vacuum, at which point rapid sublimation of water from the surface begins.[10] For our procedure, the sample stub is stored in liquid nitrogen before being transferred to a sample transfer plunger, which is also cooled by emersion in liquid nitrogen.[8] The transfer device is used to place the sample in the instrument chamber while minimizing the amount of atmospheric water, which can adsorb to the surface of the sample. Once inside of the instrument, the sample is quickly transferred to a precooled analysis stage that is held at ~90 K. During the transfer, the sample stub temperature is not observed to increase by more than 20 K, well below the sublimation threshold temperature of 165 K. Therefore, properly prepared cells are maintained in their frozen-hydrated state. Water deposition onto the sample surface during transfer is a major concern for this type of preparation. Although we have occasionally observed water cluster ions from some samples, most of the sample surface area, including the cellular regions, is generally free from a thick interfering ice layer. This effect may be a consequence of the presence of a residual amount of cryogen initially retained on the sample surface. We have observed that when a sample is characterized immediately after introduction to the sample analysis stage, SIMS peaks from ethane (the cryogen) can be observed. However, under normal conditions, this ethane overlayer is not spectrally observed after a few minutes in a vacuum. It may be that the sublimation of the ethane overlayer prevents thick water-ice from adsorbing to the surface.

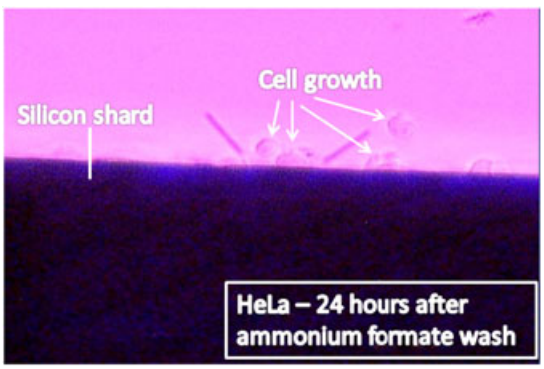

Figure 1. Optical image of a silicon shard 24 h after a wash and dry procedure reported in the experimental section. Note the presence of new cells that have grown over the edge of the silicon, indicating cell viability after sample cleaning.

Characterization of frozen-hydrated cells

The goal of this type of sample preparation is to maintain the structural integrity of the sample and to allow for proper identification of lipid membrane as well as other features at the surface. The SIMS data collected from HeLa cells grown on silicon, rinsed with 0.15 M ammonium formate, plunge-frozen in liquid ethane and investigated in their frozen-hydrated state are shown in Fig. 2. This spectrum was collected using 40 keV C_{60}^+ bombardment *without* sputter removal of any surface material and therefore describes the outermost surface of the frozen-hydrated cell (i.e. the plasma membrane). The examination of some common lipid species observed for this membrane include intact lipid molecules in the range of approximately *m/z* 700–900, cholesterol (*m/z* 369) and various fragment ions for lipids such as *m/z* 184 and 86. An individual lipid species located at *m/z* 760, which is likely 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, is also observed. As detailed in the image, these species all localize to regions localized on the HeLa cells. The size of these chemical features is slightly larger than that determined for suspended HeLa (15–20 μm). However, these cells are grown on a surface and are expected to elongate as they adhere to the silicon. Hence, lipid species from single cell regions are preserved and show ion yields useful for imaging.Spectral data collected from the same cells after receiving an etch dose of 2.0×10^{14} ions/cm^2 are presented in Fig. 3. As observed in Fig. 3, the key regions where the cells likely reside are still populated by common cellular ions. Perhaps most striking is the retention of a small but significant amount of information regarding the presence of cholesterol (*m/z* 369) and the high mass lipids (*m/z* 650–900). It is important to note that although there is no intense ion formation between *m/z* 650 and 900, integrating this area for imaging still shows localization to the cells. The intact generation of species in this region is significant because it indicates that even after sustained ion bombardment, the individual cells still yield intact lipid species, albeit with very low intensity. With a modest increase in sensitivity, we may be able to image these intact lipid species throughout a depth profile of the cell. This concept will be explored further on optimized instrumentation.[11]

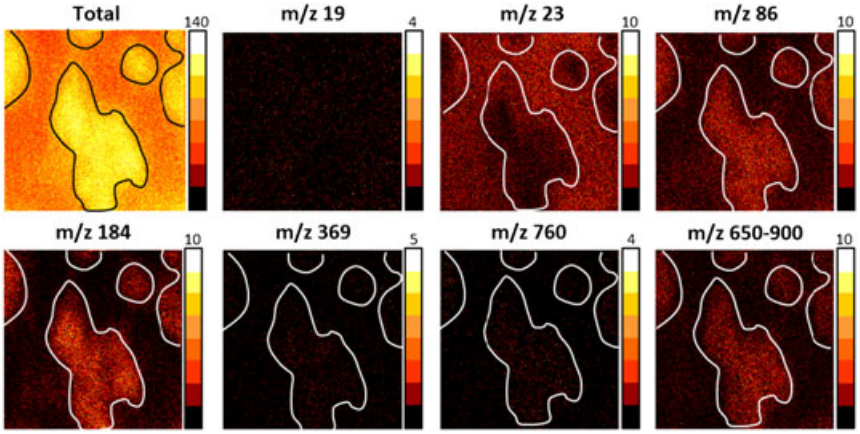

Figure 2. Mass spectral image of HeLa cells prepared frozen-hydrated for different chemical species as identified in the figure (total refers to the total counts for the spectrum and HMR corresponds to high-mass range and is the cumulative image for ions between *m/z* 700 and 900). Analysis field of view is 298 μm × 298 μm with a spectral dose of 3.5 × 1011 ions/cm^2.

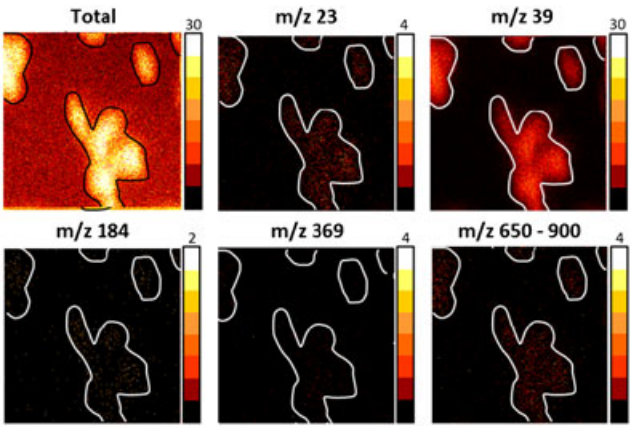

Figure 3. Mass spectral image of HeLa cells prepared frozen-hydrated for different chemical species as identified in the figure (total refers to a the total counts for the spectrum and HMR corresponds to high-mass range and is the cumulative image for ions between *m/z* 700 and 900). Analysis field of view is 298 μm × 298 μm with a spectral dose of 3.5 × 10^{11} ions/cm2 after an etch dose of 2.0 × 10^{14} ions/cm^2.

Conclusion

Sample preparation remains a key concern for biological SIMS investigations. We describe here a method to prepare cells in a frozen-hydrated state after sample washing with ammonium formate without the need for a fracturing device. The data show that cellular structure is maintained and that lipid species can be detected with useful intensities at the surface of the cells. Subsurface characterization is also possible and intact lipid species are likely detected, albeit at very low intensities.

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