SURFACE and INTERFACE ANALYSIS

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Mass spectrometry imaging of freeze-dried membrane phospholipids of dividing *Tetrahymena pyriformis*

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Time of Flight secondary ion mass spectrometry (TOF-SIMS) has been used to explore the distribution of phospholipids in the plasma membrane of *Tetrahymena pyriformis* during cell division. The dividing cells were freeze-dried prior to analysis followed by line scan and region of interest analysis at various stages of cell division. The results showed no signs of phospholipid domain formation at the junction between the dividing cells. Instead the results showed that the sample preparation technique had a great impact on one of the examined phospholipids, namely phosphatidylcholine (PC). Phosphatidylcholine and 2-aminoethylphosphonolipid (2-AEP) have therefore been evaluated in *Tetrahymena* cells that have been subjected to different sample preparation techniques: freeze drying *ex situ*, freeze fracture, and freeze fracture with partial or total freeze drying *in situ*. The result suggests that freeze drying *ex situ* causes the celia to collapse and cover the plasma membrane.
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Keywords: *Tetrahymena*; dividing cells; freeze drying; freeze fracture; phospholipids; SIMS

Introduction

Cell division is an important and complex part of the cell cycle, and several research articles suggest that small molecules, like phospholipids and sphingolipids, play important roles during cell division.[1,2] Increased membrane traffic to the cleavage site has been found[3] as well as evidence of membrane domain formations in the cleavage junction.[4]

Cell division in the single cell organism *Tetrahymena* has previously been investigated, and several proteins related to microtubules and cilia have been identified as important factors in order to complete cell division.[5–10] Being single cell organisms, *Tetrahymena* cells are good model cells to work with for single cell analysis. They are easy to culture and quite large, which is beneficial to imaging. The distribution of phospholipids in mating *Tetrahymena* cells has previously been studied with Time of Flight Secondary Ion Mass Spectrometry (TOF-SIMS) using freeze fracture. The results showed that the phospholipid composition changed at the junction of mating cells.[11,12] The abundance of the cylindrically shaped phosphatidylcholine (PC) was decreased at the site of mating in favor of the conically shaped 2-aminoethylphosphonolipid (2-AEP).[11,12] TOF-SIMS is a surface sensitive technique that enables analysis of the molecules on the top molecular layer of the sample. The TOF-SIMS analysis is performed in vacuum, and analysis of single biological cells is preferably performed after the cell sample has been freeze-dried or freeze fractured.[13–17]

In this study, we have used TOF-SIMS to investigate the phospholipid distribution in the plasma membrane of *Tetrahymena pyriformis* during cell division. We also discuss different sample preparation techniques for single cell TOF-SIMS analysis and their impacts on the distribution of phospholipids in *Tetrahymena* cells.

Experimental

Cell culture and synchronization

Tetrahymena pyriformis were cultured in a medium containing 2% proteose peptone, 0.2% yeast extract, 0.5% D-glucose and 250 ppm Fe-EDTA pH 5.5 as previously described.[18] Cell division was synchronized by six temperature cycles in diluted medium (medium : water, 50 : 50, v/v) using a thermocycler (Tpersonal thermocycler, biometra). Each cycle consisted of 30 min at 28 °C and then 30 min at 34 °C.[19] The temperature was then kept constant at 28 °C, and the cells started to divide about 75–90 min after the end of the last cycle. The dividing cells were centrifuged (400 g, 1 min using an eppendorf centrifuge 5810R) and resuspended twice. Depending on sample preparation method, the cells were resuspended in 3% of the original volume using water (freeze-dried) or HEPES (10 mM, pH 7.3) (freeze-fractured).

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Sample preparation and SIMS analysis

For freeze drying *ex situ*; 10 μl of cell solution was placed onto a clean silicon shard (SI-MAT P/Boron <100>) and quickly frozen in liquid propane (−185 °C). The sample was stored in liquid nitrogen (LN2) until it was freeze-dried for 4 h in a low pressure chamber ($\approx 10^{-3}$ mbar) on a LN2 cooled aluminum block that was allowed to slowly warm up to room temperature. The freeze-dried sample was then immediately transferred into the SIMS instrument for analysis.

To prepare freeze-fractured samples, cell solution was placed onto a Si shard mounted on the freeze fracture device, closed at room temperature, quickly frozen in liquid propane and stored in LN2. The device was kept closed until entering the main chamber of the TOF-SIMS instrument where it was fractured at controlled temperature (≈ −105 °C) and pressure ($\approx 7 \times 10^{-7}$ mbar) [16]. The freeze drying *in situ* followed the procedure of freeze fracture except that the fractured sample was allowed to slowly (3 h) warm to about −70 °C and dry in the analysis chamber.

The SIMS imaging was carried out in positive mode using a TOF-SIMS IV from ION-TOF GmbH, Germany equipped with 25 kV Bismuth (Bi) Liquid Metal Ion Gun (LMIG). High lateral resolution images were acquired in burst alignment mode with Bi_3^+primary ions (lateral resolution 200 nm, mass resolution m/Δm 300). For peak assignments, spectra were obtained in the bunched mode with Bi_3^+ primary ions (lateral resolution 4 μm, mass resolution m/Δm 7000). The analysis was kept at static conditions with the primary ion dose below 4×10^{12} ion/cm^2. All sample areas were recorded with 256 × 256 pixels.

Results and discussion

Analysis of dividing *Tetrahymena* using freeze drying *ex situ*

The division of *Tetrahymena* cells was synchronized so that 30–50% of the cells divided simultaneously.[19] After TOF-SIMS analysis, the imaged freeze-dried cells were divided into three groups according to their stage of division; pre-dividing (as shown in Fig. 1A), dividing (Fig. 1B) and post-dividing (Fig. 1C). To explore the distribution of phospholipids over the cell surface, line scans were performed over the cells as shown in the blue line in Fig. 1A, B and C. The ions that have been investigated are *m/z* 69 (C_5H_9), *m/z* 86 ($C_5H_{12}N$), representing PC and *m/z* 126 ($C_2H_9NPO_3$), representing 2-AEP. The results of the line scans are shown in Fig. 1D, E and F, respectively. Five cells per group were also analyzed using region of interest analysis. The intensities of *m/z* 69, 86 and 126 were plotted and compared between the regions of mother cell, daughter cell and junction. No significant change in intensities was detected in the different regions (data not shown).

In these analyses of freeze-dried cells, the fragment $C_5H_{12}N$ (*m/z* 86) was used to represent PC. However, to use the PC head group fragment ($C_5H_{15}NPO_4$) at *m/z* 184 would have been advantageous. Unfortunately *m/z* 184 showed a partially delocalized signal in the images as shown in Figs 1B and 2D where the signal forms a ring around the cell. This may have been caused by reasons discussed in the succeeding sections. From the images and line scan data obtained, no inhomogeneities are observed that would suggest domain formation in the plasma membrane of the cells.

Sample preparation

The *ex situ* freeze-drying sample preparation technique is easy to perform and enables analysis at room temperature; however, it seems to be associated with issues for these cells. In freeze drying, the cells will collapse as they dry, and any contaminations from the surrounding matrix will end up on top of the sample. However, by using freeze fracture, the cells are still hydrated, and the matrix on top of the cell is removed during fracture. In

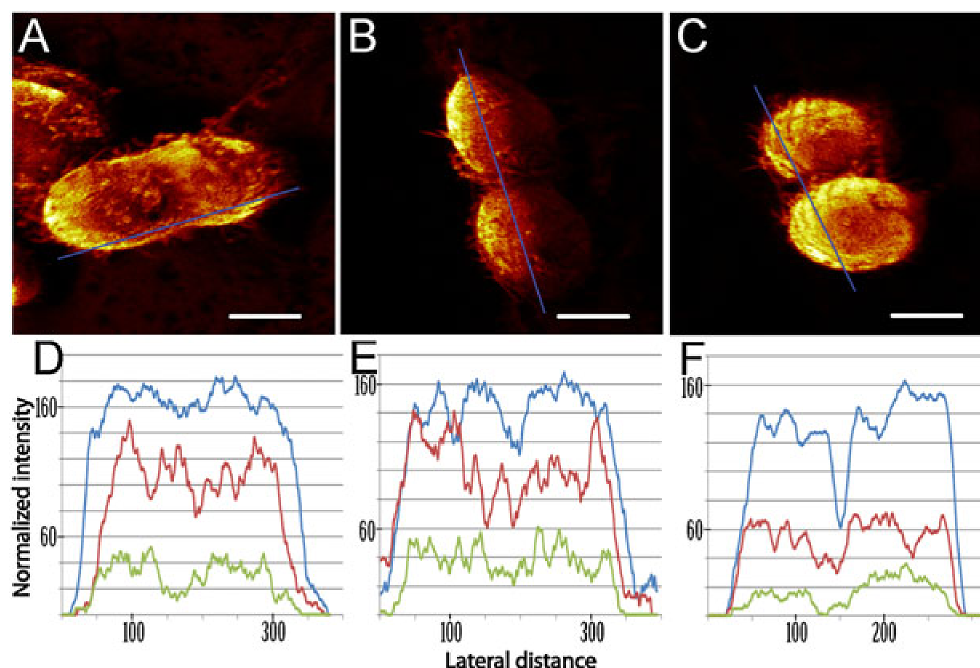

Figure 1. Total ion current (TIC) images of *Tetrahymena* cells at three different division stages and line scans (blue lines) across the cell surface. (A) Pre-dividing; (B) Dividing; (C) Post-dividing. Scale bar: 20 μm. (D) Line scan of A from left to right; (E) Line scan of B from top to bottom; (F) Line scan of C from top to bottom. The correlation between the colors and ions in the lines shown in D, E and F are blue line – *m/z* 69 (C_5H_9), red line – PC at *m/z* 86 ($C_5H_{12}N$), green line – 2-AEP at *m/z* 126 ($C_2H_9NPO_3$). The intensities are normalized to the total ion current and 15 × 15 pixels are averaged.

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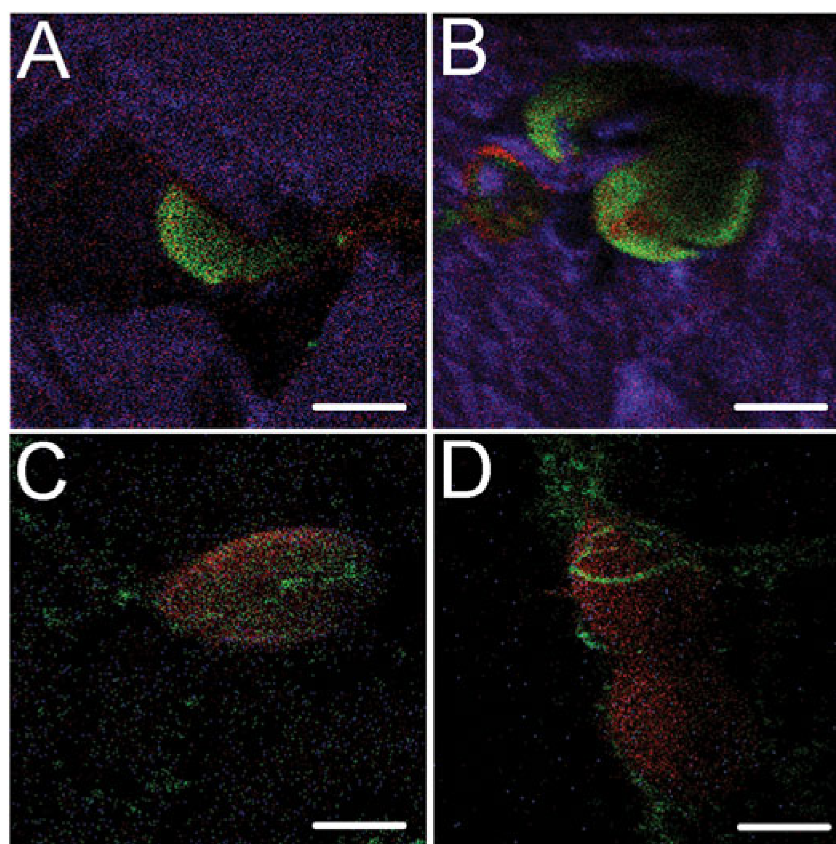

Figure 2. Ion images comparing freeze-fractured with freeze-dried preparations. Images for (A) freeze-fractured cell half covered in ice, −105 ° C; (B) freeze-fractured dividing cell where water has partly sublimed but is still present in the junction between the cells, −102 °C; (C) *in situ* freeze-dried cell, −70 °C; (D) *ex situ* freeze-dried cell, room temperature. Ions images are color coded; blue – water at *m/z* 37 (H_5O_2), green – PC at *m/z* 184 ($C_5H_{15}NPO_4$), and red – 2-AEP at *m/z* 126 ($C_2H_9NPO_3$). Scale bars are 20 μm.

the following sections, we have examined three ways to analyze a freeze fractured sample of *Tetrahymena* cells using the previously described freeze fracture device.[16]

Freeze fracture – hydrated cells

Freeze fracture has previously enabled great single cell analysis where the molecular distribution in the sample is well conserved.[11–13,16,20] Even though laborious, freeze fracture decreases the risk of contamination on the sample surface and has been used to obtain excellent images of *Tetrahymena* in previous work.[11,12] However, the large dividing *Tetrahymena* cells were associated with some difficulties for the experiments presented here. Fig. 2A shows a cell image of a freeze-fractured cell where most of the cell body is covered under a layer of ice. Other cells were fractured through different cell planes, which make them unsuitable to use for further analysis of the plasma membrane.

Freeze fracture with water sublimation in situ – hydrated cells

In this approach, the benefits of freeze fracture were utilized at the same time as a thin layer of water, which covered part of the cell, was allowed to sublime revealing the intact cell (Fig. 2B). Increasing the temperature to remove the top layer of water exposes the plasma membrane of the cell body. However, some water remains in the low-lying junction between the dividing cell bodies, which leads to shadowing and does not allow analysis of molecules in the junction.

Freeze fracture with freeze drying in situ – dehydrated cells

The water was totally removed *in situ* after freeze-fracture, as shown in Fig. 2C. Freeze drying *in situ* keeps the sample away from the atmosphere through the drying process, hence, most of the matrix contaminants are avoided. However, the elimination of water caused the cell to collapse and cell material was ejected out of the cell, shown to the left of the cell in Fig. 2C.

Different molecular distributions depend on sample preparation

Because the dehydration process seems to affect the molecular distribution in the cells, different sample preparation techniques have been studied. The images in Fig. 2 compare the different sample preparations. Here, an obvious difference in molecular distribution is observed between the images. In the hydrated cells (Fig. 2A and B), the intensity of PC is strong and localized to the cell area; however, in the dehydrated cells (Fig. 2C and D), this signal becomes delocalized. Further, in the dehydrated cells the intensities of PC (*m/z* 184 green) and 2-AEP (*m/z* 126 red) are reversed compared with that observed in the hydrated cells. This clearly indicates that the phospholipid distribution is altered in the sample during drying. The reversed intensity of PC and 2-AEP in hydrated and dehydrated cells might be due to the cilia that cover the *Tetrahymena* cells (visible in Fig. 1A–C), which enables the cells to swim freely in water. The main phospholipids in the cilia are the 2-AEP.[21–23] It is possible

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that cilia on the cell surface collapse as the sample is dehydrated causing the material from the cilia to make a layer of 2-AEP-rich material covering the plasma membrane. As TOF-SIMS is surface sensitive, it is unable to penetrate through this layer of 2-AEP leaving PC undetected.

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