Evaluation of Sample Preparation Methods for Imaging 3T3 Fibroblasts with ToF-SIMS

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Statement of Purpose: Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a powerful surface analysis technique that is increasingly being used to image tissues and cells [1,2]. High chemical specificity, along with submicron lateral and depth resolution makes ToF-SIMS imaging a great complement to other imaging techniques used in the life sciences. A key factor is sample preparation: an ideal protocol is one that keeps the cell as close to its living state as possible even when introduced into the harsh, ultra high vacuum environment of this technique. In this work multiple sample preparation protocols are investigated to determine which provides the best avenue for imaging single cells in 2D and 3D. Specifically, NIH 3T3 fibroblasts were prepared by rinsing, (0.15 M ammonium acetate or ammonium formate), flash freezing in liquid ethane, and freeze-drying or examining the samples in the frozen hydrated state. Additionally, freeze-fractured samples were also either freeze-dried or analyzed frozen hydrated and compared.

Methods: NIH 3T3 mouse fibroblasts were maintained in Dulbecco's minimum essential medium (Invitrogen) containing high glucose, sodium pyruvate and L-glutamine, and supplemented with 10% fetal bovine serum (Hyclone) at 37°C and 5% CO_2. 10^4 cells were seeded onto 1 cm^2 polished silicon and allowed to grow to 60-70% confluence, usually taking 48 hours. For rinsing experiments, 150 mM ammonium acetate (AA) or ammonium formate (AF) solutions were made immediately prior to rinsing, and brought to pH 7.4 with 1 M NH_4OH. Freeze-drying occurred for 24 hours. The cells were prepared for ToF-SIMS analysis according to the different methods described below.

ToF-SIMS experiments were performed using an ION-TOF TOF.SIMS 5-100 (ION-TOF GmbH, Münster, Germany). 3D secondary ion images were collected in the dual beam mode (imaging alternating with etching). Images with submicron lateral resolution were acquired by rastering a pulsed 25 keV Bi_3^+ beam across the sample surface. Etching was done with a 40 keV C_{60}^+ beam to minimize damage in etched sample.

Results: Figure 1 shows a sample rinsed for 10 seconds with AA and subsequently freeze-dried, a-c are before a 30 second C_{60}^+ sputter with a current of 0.69 nA, while d-f are after sputtering. The chemical integrity of the cells are maintained, as is evident by the high K/Na ratio seen in (d) and (e) [3]. Rinsing is necessary to remove a large portion of the residual media salts that will interfere with analysis. It is evident however, that rinsing does not remove all of these salts, and C_{60}^+ etching is required to remove the rest. This rinsing does provide a much cleaner surface for 3D cellular analysis than if no rinsing was used. Preliminary results indicate that AA and AF are similar in their ability to remove salts, although AA rinsed samples seem to cause less cell damage.

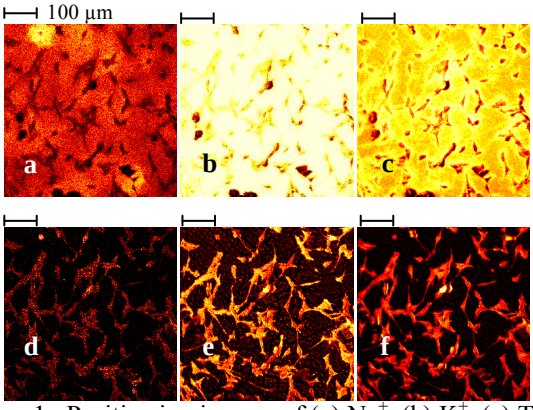

Figure 1. Positive ion images of (a) Na^+, (b) K^+, (c) Total ions before 30 seconds C_{60}^+ etching. After sputtering, (d) Na^+, (e) K^+ and (f) Total ion image.

Figure 2 presents a freeze-fractured, freeze-dried cell sample. The fracturing process is described by Chandra and Morrison [3]. Briefly, cells are grown on a silicon substrate, as before, and 10 µm polystyrene beads are added to the media. A second polished silicon piece is placed on top of the first and this "sandwich" is plunge frozen in liquid ethane. The sandwich is transferred to and fractured under liquid nitrogen. The high K/Na ratio indicates a fracture through the cell. SEM images taken of the same area (not shown) show fractured areas are smooth, as expected.

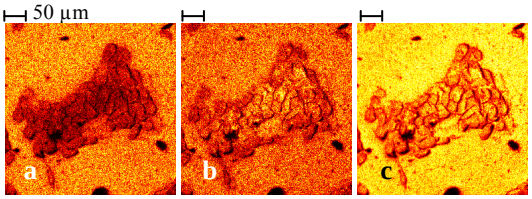

Figure 2. (a)-(c) are positive ion ToF-SIMS images of freeze-fractured, freeze-dried cells. (a) Na^+, (b) K^+, (c) Total ion image

Conclusions: NIH 3T3 mouse fibroblasts were successfully imaged using various sample preparation protocols. Some methods are better than others at providing biologically relevant results. Thus far ammonium acetate has performed better than ammonium formate in rinsing experiments. Most of the media components seem to be removed rather quickly, ~10 seconds. Additionally, it is our belief that frozen-hydrated analysis will cause less damage to the cells than freeze-drying, and thus provide more relevant results for both rinsed and fractured samples.

References: 1. Winograd N, Anal. Chem. 2005;77,143A 2. Chandra S, Ana. Chem 2000;72,104A 3. Chandra S, J. Microsc. 1986; 144, 15-37.