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Cryogenic Sample Preparation Preserves Elemental Composition for Correlative Light and X-ray Fluorescence Microscopy

Qiaoling Jin[1,] *, Barry Lai[2], Si Chen[2], Sophie Charlotte Gleber[2], Lydia Finney[2], David Vine[2], Tatjana Paunesku[3], Gayle Woloschak[3], Stefan Vogt[2], Chris Jacobsen [1,2]

1 Department of Physics &Astronomy, Northwestern University, 2145 Sheridan Road, Evanston, Illinois, United States of America
2 X-ray Science Division, Advanced Photon Source, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois, United States of America
3 Department of Radiology, Northwestern University, 303 E. Chicago Ave., Illinois, United States of America
* Presenter: Qiaoling.Jin@northwestern.edu

Synchrotron based x-ray fluorescence microscopy (XFM) is well suited to determine the detailed spatial distribution of most biologically important elements in biological cells and tissues with submicrometer resolution and attomgram sensitivity [1, 2, 3, 4]. Cryogenic sample preparation coupled with cryogenic scanning XFM systems such as the Bionanoprobe at Argonne National Laboratory (ANL) presents one of the most reliable approaches for studies of cellular elemental homeostasis [5]. However, due to the very limited availability of XFM microprobes capable of cryoscannig as well as the necessity to perform other correlative studies, different sample preparation protocols have been developed [6,7]. Aldehyde based chemical fixation and rapid freezing based cryofixation, followed by different dehydration protocols, are commonly used. Both methods have been originally developed and extensively studied in the field of transmission electron microscopy for the preservation of ultrastructure and protein antigenicity [8]. When they are adapted to prepare cultured adherent mammalian cells for XFM studies, parallel comparison among different sample preparation approaches is currently underexplored on the preservation of cellular elemental content and distribution [7].

To illustrate possible artifacts associated with each specific approach, we decided to compare how elemental content and distribution is preserved in cryofixed versus chemically fixed cells, with both of them subsequently dehydrated and scanned by XFM under ambient temperature. Mouse embryonic fibroblast NIH/3T3 cells grown on silicon nitride windows were plunge frozen in liquid nitrogen cooled liquid ethane using a FEI Vitrobot Mark IV plunge freezer. They were then cryogenically transferred and imaged using a cryo light microscope, and the cryojet XFM microprobe installed at 2-ID-D at Advanced Photon Source (APS) of ANL. Visible cryo light microscope images (Fig. 1A) indicated well-maintained cell morphology. The 2-dimensional elemental maps revealed homogeneous distribution of most freely diffusible ions K and Cl, with comparable content to that found in living cells [9]. There were more Ca ions in the cytoplasm than in the nucleus, an indication of undamaged cellular membrane before and during plunge freezing [9]. Fe has characteristic perinuclear distribution [7]. Zn concentration was higher in the nucleus than in the cytoplasm, presumably due to the presence of a large quantity of Zn binding proteins in the nucleus. We then subjected one of the plunge frozen samples to be freeze dried (PFFD) and compared to cells which were chemically fixed by 4% paraformaldehyde (PFA) and dried in the air. Both of them were scanned by XFM microprobe at APS beamline 2-ID-E. The total elemental contents in each cell were obtained through region-of-interest analysis. They were then averaged from all cells in the same sample and plotted as fractions to the content in PFFD sample. As shown in Fig. 1B, 2-dimensional maps of PFFD sample showed very similar distribution pattern of most

elements including P, S, Cl, K, Fe and Zn to the frozen hydrated cells, with some possible redistribution of Ca. Compared to PFFD sample, 4% PFA fixed cells had the severe loss of K (>99% loss) and Cl (70% loss), and higher content of Ca (200% increase). In addition, 4% PFA fixed cells showed less preserved amount of some tightly bound ions such as P, S and Fe, with about 50% S and Fe and 60% of P detected compared to PFFD sample. Although cell-to-cell variation and statistical error might factor in to this apparent reduction, the comparable level of Zn prompted us to believe that a certain degree of loss might exist for these elements. We conclude that plunge freezing followed by freeze drying preserved the contents and distributions of most elements at a level comparable to frozen hydrated cells. It should be the method of choice whenever possible. If conventional chemical fixation has to be chosen, the results on diffusible ions and certain other elements must be carefully interpreted [10].

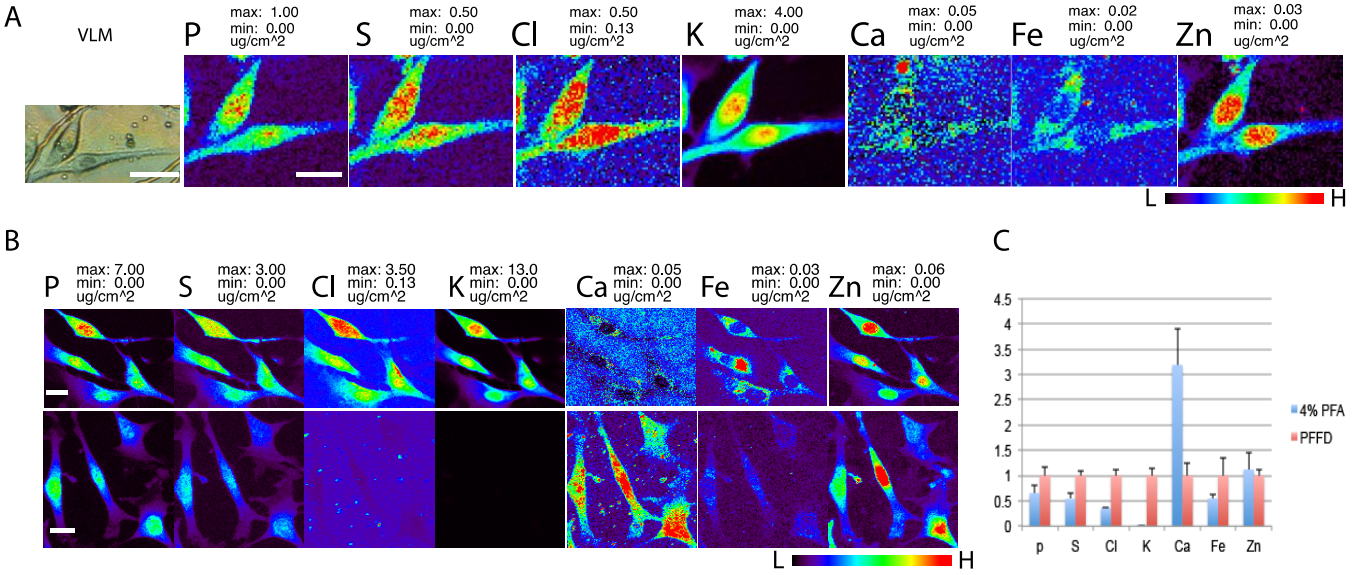

Figure 1. Comparison of different sample preparation approaches on the preservation of elemental contents and distributions. Panel A: visible cryo light microscope image (VLM) and 2-dimentional elemental maps of NIH/3T3 cells which were plunge frozen and imaged frozen hydrated by the cryojet XFM microprobe at 2-IDD at APS. Panel B: 2-dimentioanl elemental maps of plunge frozen and freeze dried NIH/3T3 cells (PFFD, 1st row) and 4% PFA fixed NIH/3T3 cells (4% PFA, 2nd row). Panel C: fractions of average elemental content in 4% PFA fixed cells to the average content in PFFD sample. Scale bars: 20 μm. Color scale in false colors spans from black (no signal) to red (maximum signal).

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