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Dye-enhanced imaging of mammalian cells with SIMS

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A major limitation of SIMS studies of cells is the limited number of molecules available for detection. This study examines the possibility of utilizing a molecular tag that is easily identifiable using SIMS and does not interfere with the mass spectra of commonly identified cellular components, to aid in the visualization of specific cellular organelles. Here, a fluorescent, nuclear stain (Hoechst 33342) was used to allow for verification of the staining protocols prior to SIMS analysis. The stain was successfully chemically imaged within the nuclear region of a glutaraldehyde-fixed bovine aortic endothelial cell. The ability to chemically map a larger variety of organelles and cellular components will allow for further possible study of a greater number of biological pathways and processes, as well as make cell–drug studies more feasible using SIMS. **Copyright © 2014 John Wiley & Sons, Ltd.**

Keywords: C60; bovine aortic endothelial cell; Hoechst 33342; glutaraldehyde fixation; biological imaging

Introduction

Cluster SIMS is beginning to have a significant impact in biology. The development of more powerful cluster ion sources has increased the amount of chemical information now obtainable from a biological sample.[1,2] However, many chemical components within a biological sample produce such small amounts of signal that they are difficult to locate and study, and as such, most studies of such systems have been limited to the detection of select components that are readily available within the cell.[1–3] The ability to enhance the signal of less abundant compounds using a molecular tag would allow for a more complete understanding of the cell itself or the cellular processes being studied. Molecular tags, in the form of organelle-specific dyes commonly used in microscopy, can be incorporated into the cluster SIMS experiment to allow for distinct localization of compounds in their expected organelles.[4,5] In addition, utilizing a dye as a molecular tag acts as a precedent for drug studies using SIMS. The methodologies applied to molecular tags can be applied to determine the location and interaction of drugs within the cell. When coupled with the additional chemical information obtainable using SIMS, these methodologies would allow for a much greater understanding of drug–cell interactions.

Hoechst 33342, the molecular tag chosen for this preliminary study, is a commonly used fluorescent biological stain that binds to nucleic acids in the minor groove of double-stranded DNA. It fluoresces most intensely when bound to adenine-rich and thymine-rich sequences, making it a useful marker for the nucleus within the cell. It is cell permeable and can be used in live or fixed cells,[6] making it compatible with the cellular sample preparation methods involving chemical fixation, freeze drying, and frozen hydration commonly used for SIMS analysis. In addition, the fluorescent nature of the stain allows for supplemental fluorescence microscopy information to be obtained to support SIMS results and to verify the integrity of sample preparation.

Materials and methods

Cell culture

Bovine aortic endothelial cells (BAOEC) cells were maintained in Bovine Endothelial Growth Medium (Cell Applications Inc. San Diego, CA, USA) at 37 °C and 5% CO_2. After trypsinizing, they were cultured onto 5 mm × 5 mm silicon chips. These chips had been cleaned by sonication in chloroform, acetone, methanol, and purified water, prior to being coated with poly-l-lysine via spin coating to promote enhanced cell growth. BAOEC were allowed to grow for 24 h.

Glutaraldehyde fixation

After the 24-h incubation period, the cell-covered silicon chips were removed from media. If the Hoechst 33342 nuclear stain was being introduced, the cells were incubated in 5 μM Hoechst 33342 in Bovine Endothelial Growth Medium (Cell Applications Inc.) for 45 min prior to fixing. Cells were chemically fixed using a glutaraldehyde solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate) for 20 min. After fixation, cells were washed five times in phosphate-buffered saline to remove excess fixing solution and then three times in 0.015 M ammonium formate (pH 7.3). Ammonium formate was used to minimize variation from accepted cellular preparation methods in which it is used to remove residual salts. The samples were allowed to air dry overnight in a laminar flow hood. A schematic of the sample preparation for Hoechst 33342 stained BAOEC can be seen in Fig. 1.

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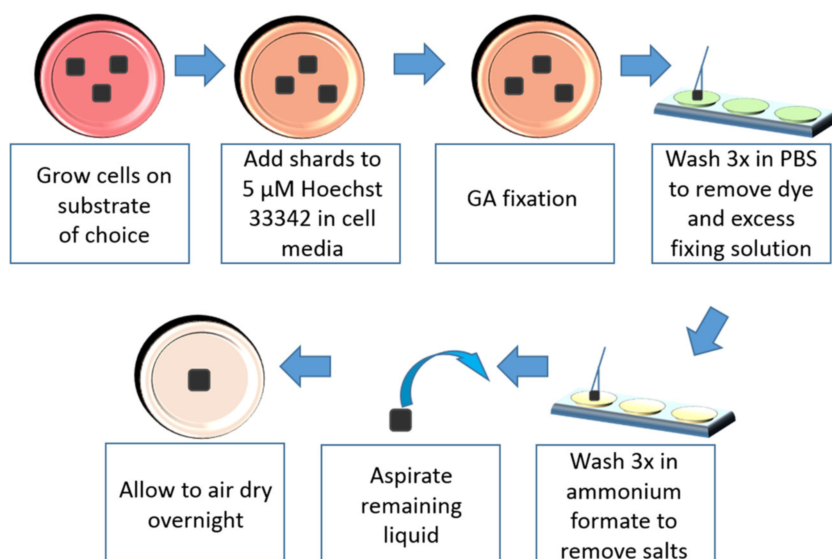

Figure 1. A schematic showing the staining and subsequent washing procedure for glutaraldehyde-fixed bovine aortic endothelial cells for SIMS analysis.

Fluorescence microscopy

A Zeiss PALM MicroBeam IV laser capture microdissection system equipped with an Xe/Hb bulb was used to capture bright-field and fluorescent images from stained BAOEC samples to verify the staining procedure. For fluorescence studies of the Hoechst 33342 stain in the cells, a DAPI/Hoechst filter set was used for selection of excitation and emission wavelengths (395 and 420 nm, respectively).

Secondary ion mass spectrometry

Secondary ion mass spectrometry measurements were performed using a *J105 3D Chemical Imager* (Ionoptika Ltd. Chandler's Ford, Hamsphire, England) equipped with a 40-keV C_{60}^{+} primary ion source, described previously.[7] Images were acquired using a 100 µM × 100 µM field of view with an ion dose of 2×10^{12} ions/cm^2 and 50% transmission.

Results and discussion

Mass spectra of Hoechst 33342

The Hoechst 33342 is used as a nuclear stain. It is important to evaluate the mass spectrum of this molecule to ensure there is no interference with well-known biological signals. As seen in Fig. 2, Hoechst 33342 produces a strong, characteristic signal at *m/z* 454. Because there are no strong biological signals from the BAOEC at this *m/z*, it can be used as an identifying signal for the stain. In addition, there are surrounding characteristic peaks that can be summed together to enhance the signal intensity in cases where signal levels are low.

Microscopy

As the dye components within the cell are to be analyzed, it is important to confirm the success of the staining procedure using

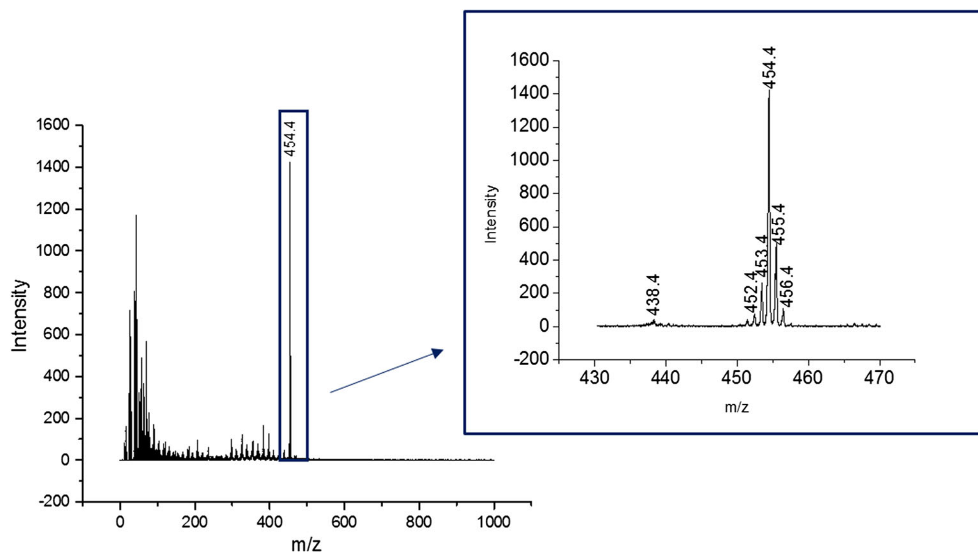

Figure 2. The positive ion spectrum of Hoechst 33342. The primary peak of interest is that at *m/z* 454, though the isotope structures, as seen in the insert, can also be used.

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fluorescence microscopy prior to mass spectrometry imaging. It is important to be sure that the sample preparation protocols leave the stain intact in the desired region of the cell, here, within the nucleus. In addition, any additional rinsing or treatment of the cells must preserve their structural and chemical integrity. Optical microscopy images of BAOEC prior to exposure to any treatment are shown in Fig. 3a, while Fig. 3b shows a fluorescence microscopy image of a glutaraldehyde-fixed BAOEC after sample preparation procedures have been completed. The location of the fluorescence shows that the Hoechst 33342 remains present in the nuclear regions of the cell, indicating that the sample preparation methods are adequate for successful fluorescence microscopy analysis and can be applied to the samples for SIMS analysis. The shape of the cell does seem to be slightly distorted and swollen when compared with cells not fixed using glutaraldehyde (Fig. 3a), which may be indicative of a collapsed cell structure; however, the cell membrane and nucleus still appear to be intact for analysis. These observations emphasize the need to carefully consider sample preparation techniques, so that one is chosen that will best highlight the results being sought.

Glutaraldehyde-fixed and stained BAOEC

The fluorescence microscopy images confirm that there is still stain present in the cell after all sample preparation methods have been completed and that the relative shape of the cell has been maintained. The chemical images in Fig. 4 show glutaraldehyde-fixed cells stained with Hoechst 33342. Figure 4a shows the distributions of several well-studied biological components. The localization of the phosphocholine (PC) head group (*m/z* 184) clearly indicates the outline of the cellular membrane. This observation is reinforced by the distribution of cholesterol (*m/z* 369) in the same region. The void left in this region is generally considered to be the location of the nucleus. The distribution of *m/z* 454, an indicator of the Hoechst 33342 nuclear stain, is also shown and can be seen to be localized within the cellular structure. Statistical methods (ion signal counting) of the dye within the nuclear region compared with the membrane region show signal intensities with a 3 : 1 ratio, favoring the nuclear region, as can be seen in Fig. 4b. An overlay of the dye signal and the PC head group signal for one

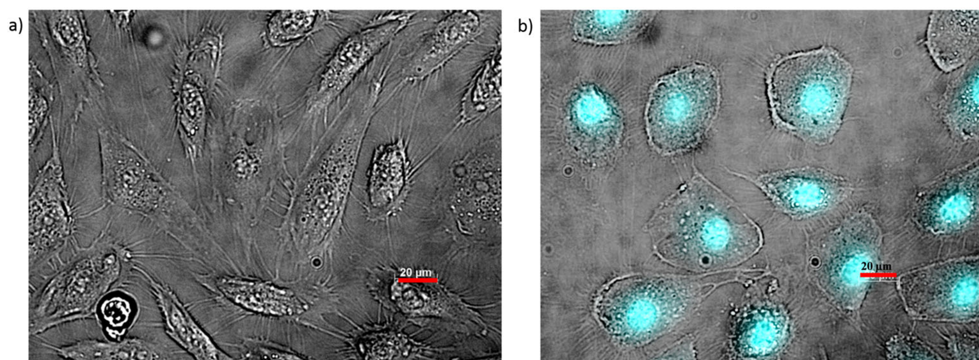

Figure 3. (a) Optical microscopy image of adhered bovine aortic endothelial cells (BAOEC) without any sample preparation. (b) Fluorescence microscopy image of glutaraldehyde-fixed BAOEC that have been stained with Hoechst 33342. The scale bar is 20 μm.

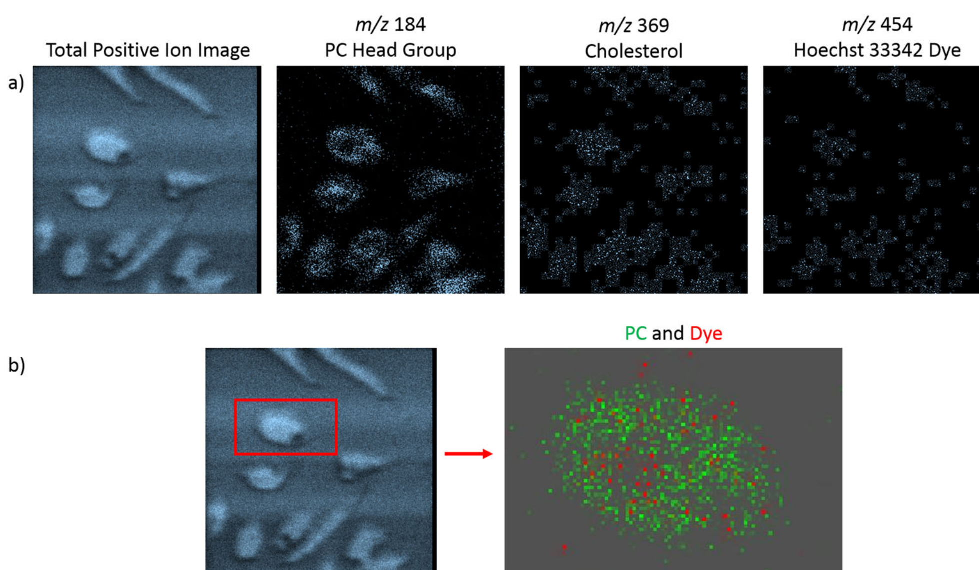

Figure 4. (a) Mass spectral images of glutaraldehyde-fixed bovine aortic endothelial cells stained with Hoechst 33342 for different chemical species as identified in the figure (total refers to the total ion counts for the spectrum). The analysis field of view is 100 μm × 100 μm with a spectral dose of 6×10^{12} ions/cm^2. (b) An overlay of the mass spectral images from (a) for *m/z* 184 and 454, representing the cellular membrane and Hoechst 33342 nuclear stain, respectively. The area shown is 35 μm × 35 μm.

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cell is shown in Fig. 4b. This leads us to believe that the stain is present in the nuclear region of the cell, as is shown in the fluorescence images.

Conclusion

It can be seen that the Hoechst 33342 nuclear stain can be seen in glutaraldehyde-fixed BAOEC cells without sacrificing signal from other commonly identifiable cellular components such as PC head group and cholesterol. Furthermore, the region in which the stain is more highly concentrated correlates to the void within the cell membrane commonly used as an indicator of the nucleus, confirming that the stain is present in the desired region of the cell. The signal intensity of the dye is limited by the number of available binding sites, and so, preparation methods that optimize nuclear signal should also result in higher levels of detectable stain.

The method of cellular sample preparation in SIMS is often based on the information being sought after the experiment, as each of the different sample preparation techniques have advantages that make them better suited for specific applications. As a result, further studies must be completed to optimize the sample staining process for all cellular sample preparation methods, so that it can be used in all cellular analysis situations. As the glutaraldehyde fixation protocol shown may indicate a collapsed cell, frozen hydrated cells should be explored to study cells in a state closer to their native morphology. Similarly, the protocols deemed successful can be applied to cellular systems exposed to a variety of pharmaceutical compounds to gain greater understanding of the cell–drug interactions.

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