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Dynamic Secondary Ion Mass Spectrometry Analysis of Boron from Boron Neutron Capture Therapy Drugs in Co-Cultures: Single-Cell Imaging of Two Different Cell Types within the Same Ion Microscopy Field of Imaging

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A co-culture, cryogenic SIMS methodology is presented for the quantitative analysis of cell type-dependent accumulation of boron delivered by BPA-F and BSH, two clinically approved drugs used in boron neutron capture therapy of cancer. T98G human glioblastoma cells were co-cultured with morphologically different normal LLC-PK_1 epithelial cells or GM3348 human skin fibroblasts. Our freeze−fracture method of cryogenic sample preparation successfully fractured the different cell types grown together in co-cultures. Quantitative observations revealed an active uptake of boron from BPA-F in both T98G and LLC-PK_1 cells but did not show cell type-dependent differences. Accumulation of BSH in all three cell types examined also did not reveal any cell type-dependent differences in co-cultures. As this method relies on the analysis, within the same field of SIMS imaging, of two different cell types that have been maintained under identical conditions of growth, drug exposure, sample preparation, and instrumental analysis, it provides the most effective approach for comparing cell type-specific differences in boron concentrations. The most effective applications of this method will be realized in testing the selectivity of experimental boronated compounds designed to specifically target tumor cells.

In areas of biology and medicine, there is often a need for an analytical technique with the capability of quantitatively measuring chemical gradients at a subcellular level. One area of ongoing medical research where a methodology for subcellular chemical analysis is required is that of boron neutron capture therapy (BNCT) for the brain cancer glioblastoma multiforme and for certain types of melanomas.[1,2] BNCT is based on the fission reaction that occurs when a boron-10 atom captures a thermal (<0.025 eV) neutron. This reaction produces a high-energy α-particle and a ^{7}Li nucleus with total kinetic energy of 2.79 MeV and path lengths of ∼10 μm, or less than one cell diameter. With this localized lethal reaction, a controllable killing of tumor cells could occur if sufficient boron (a minimum of 10−35 μg/g of wet weight) could be delivered to and accumulated in the tumor cells.[3] Furthermore, the boron delivered to the cell should ideally accumulate in the cell nucleus in order to enhance the lethal effects of the neutron capture-induced fission.[4] Thus, an ideal boronated BNCT compound will deliver boron preferentially to tumor cells in sufficient quantity for the neutron capture reaction to occur at a cell killing level, while allowing minimal boron accumulation in normal cells.

As new boronated compounds continue to be developed for use in BNCT, their boron delivery characteristics need to be examined in both tumor and normal cells in order to determine whether tumor cells are accumulating sufficient boron for a lethal neutron capture to occur while normal cells have insufficient boron for damage to be caused. These studies can be performed in animal models where one can assess the boron in normal and tumor cell samples obtained from the same animal. To obtain reliable tissue samples for determining subcellular concentrations of boron atoms, however, is not an easy task and requires special techniques and cryogenic sampling procedures.[5]

Cells growing in vitro in cultures provide ideal samples for the initial screening of BNCT drugs in order to check their efficacy. Unfortunately, the most commonly used analytical techniques for analyzing boron in biological samples require the destruction of the biological matrix of the sample prior to analysis. For example, boron analysis with direct current plasma-atomic emission spectroscopy (DCP-AES), inductively coupled plasma-atomic emission spectroscopy (ICP-AES), and ICP-mass spectrometry all require that the boron-containing biological sample be nitric acid-digested, ashed, or otherwise destroyed so that boron can be measured.[6,7] Attempts to elucidate subcellular boron gradients by chemically fractionating cells into different layers (nucleus, cytoplasm, etc.) prior to boron analysis run the risk of

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altering the native distributions of diffusible chemical species in the cell during the course of the fractionation. An analytical technique in which both chemical and morphological information is preserved is needed in order to obtain the best possible quantitative comparisons of boron distributions within a single cell and its subcellular compartments.

One technique that is capable of providing subcellular chemical information is the secondary ion mass spectrometry (SIMS)-based technique of ion microscopy.[8,9] Ion microscopy provides quantitative visual images of elemental distributions in relation to cell morphology with a spatial resolution of 0.5 μm, which provides information on chemical gradients on a subcellular scale.[10,11] Ion microscopy is capable of analyzing any element from H to U via mass-to-charge ratio (m/z) isotopic discrimination with sensitivities in the ppm to ppb range, and its broad elemental range and high sensitivity make it an ideal tool for studies in many areas of biology and medicine. The high-vacuum conditions of the technique require cryogenic sample fixation to preserve the native chemical composition of the living cell. Indeed, cell culture studies with ion microscopy on cryogenically prepared cells have provided valuable subcellular information boron accumulations in various cell types.[12−14]

The present study advances the utility of ion microscopy for directly imaging boron concentrations in cells from different cell types and growing together as a co-culture model. Co-culture models may provide the most effective way of comparing the boron accumulation response of two very different cell lines, as the cells of different types are analyzed simultaneously within the same imaging field of ion microscopy. Studies with co-culture models may provide a novel approach for checking the cell type selectivity, subcellular targeting, and time-dependent accumulation of boron from BNCT drugs. For co-culture studies, two morphologically distinct cell lines are needed so that cells in the mixed culture can be visually identified. In this paper, the feasibility of a direct SIMS analysis of co-cultured cells for subcellular boron imaging within the same field of analysis is tested by using co-cultures of normal and cancerous cell lines treated with the two drugs approved for clinical use in BNCT—the fructose complex of the amino acid analogue *p*-boronophenylalanine (BPA-F) and sodium borocaptate (BSH).

EXPERIMENTAL SECTION

Cell Lines. Two normal cell lines (pig kidney epithelial LLC-PK_1 and GM3348 human skin fibroblast) and one transformed cell line (human glioblastoma T98G) were used for co-culture studies (cell lines obtained from American Type Culture Collection; Manassas, VA). The T98G cell line was chosen because it is a human cell line derived from a glioblastoma multiforme tumor and, thus, may be quite useful as an in vitro model for glioblastoma multiforme.[15]

Preparation of Co-Cultures. For the tumor cell line, T98G human glioblastoma cells were cultured in Eagle's minimum essential medium supplemented with nonessential amino acids, 1.0 mM sodium pyruvate, Earle's balanced salt solution and 10% (by volume) fetal bovine serum. For the normal cell lines, LLC-PK_1 porcine kidney cells were grown in medium 199 and 3% (by volume) fetal bovine serum and GM3348 normal human skin fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To create the co-cultures, 60-mm-diameter Petri dishes were seeded with well-mixed normal and T98G cells at a ratio of 4:1 (~200 000 normal cells/50 000 T98G cells) due to the faster growth rate of the tumor cells. Growth of the mixed cells was carried out in the nutrient medium of the normal cell line, which did not affect the growth of the transformed T98G cells.

Growth of Cells on Silicon Substrates. Because the sample in the ion microscope is held at a potential of 4500 V relative to ground, an electrically conducting substrate is a requirement. High-purity n-type semiconductor-grade silicon (Silicon Quest International; Santa Clara, CA) provides an ideal sample substrate that is not toxic to cells.[9] Prior to seeding the Petri dishes with cells, five to six sterilized high-purity silicon chips of ~1 cm^2 surface area were placed polished side up in the dishes. The co-culture was allowed to grow to ~80% confluency prior to experimental use, typically 3 days of growth.

Treatment of Co-Cultures with Boronated Drugs. To study the uptake and intracellular distributions of boron in the mixed cultures, the co-cultured cells on chips were treated with 110 μg/mL boron equivalent concentrations of the BPA-F or BSH (Boron Biologicals, Inc., Raleigh, NC) for 1 h. The boron concentration and time of exposure for the cell culture studies were selected based on the previous observations of optimal boron accumulation.[12] The BPA-F was ^{10}B enriched (minimum 95%), while the BSH was synthesized from natural abundance boron (80% ^{11}B, 20% ^{10}B). While only ^{10}B-enriched compounds can be used in actual BNCT irradiation protocols, ^{11}B compounds are often used for laboratory experiments for economic purposes.

Cryogenic Sample Preparation and Optical Imaging of Fractured Freeze-Dried Cells. After the respective treatments, the cells were cryogenically prepared using the sandwich freeze−fracture technique developed in our laboratory.[16] This technique fractures areas of the cells grown on silicon at the apical plasma membrane, which exposes the interior of the fractured cells for ion microscopic analysis.[16,17] The apical half-membrane and the overlaying cell culture medium are removed on the nonsubstrate chip of the sandwich. The substrate chips containing the groups of fractured cells are then freeze-dried in a Tis-U-Dry freeze-drier overnight at −90 °C. To avoid rehydration, the temperature of the cold stage of the freeze-drier was slowly raised to 40 °C, the freeze-drier was vented to dry nitrogen, and the chips were rapidly transferred to a desiccator. Chips containing freeze-dried cells were then placed in airtight Teflon sample holders and screened

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in an Olympus optical microscope under reflected light Nomarski optics to locate areas of fractured cells in which the two cell types could be visually identified side by side. These areas were photographed to provide a visual record of the fractured area to aid in locating the same region in the ion microscope. In one treatment, the fractured freeze-dried cells were further imaged with a Bio-Rad MRC600/Zeiss Axiovert 10 confocal laser scanning microscope (CLSM) for checking the preservation of mitochondria by imaging the fluorescence of a mitochondrial stain, rhodamine 123.[18] In all treatments, cells were gold/palladium coated to enhance electrical conductivity prior to ion microanalysis.

Ion Microscopic Analysis. For the ion microscopic analyses, a dynamic SIMS Cameca IMS-3f ion microscope was used. A 5.5-keV mass-filtered O_2^+ primary ion beam with a beam current of 100 nA and a beam diameter of 75 μm^2 was raster scanned over an area of 250 × 250 μm^2. A contrast aperture of 60 μm with 150-μm transfer optics were employed for imaging throughout the study. Positive secondary ions were then extracted from a region centered within the rastered area. In all experiments, isotopic images of masses 10 (or 11), 12, 23, 39, and 40 were recorded from individual cells to determine the subcellular distributions of boron, carbon, sodium, potassium, and calcium, respectively. Secondary ion signal integration times were generally 0.2−0.4 s for ^{39}K and ^{23}Na, 120 s for ^{12}C and ^{40}Ca, and 150 s for ^{10}B and ^{11}B. In frozen freeze-dried cells, imaging of these isotopes does not suffer from any significant mass interferences or any matrix effects between the nucleus and the cytoplasm of the cells.[19,20]

The ion images were digitized to 14 bits/pixel directly from the microchannel plate/phosphorescent screen assembly of the ion microscope with a slow-scan charge-coupled device (CCD) camera (Photometrics; Tucson, AZ; model CH220 CCD liquid-cooled camera head equipped with a Thomson-CSF TH7882 CDA CCD). Quantitative analysis of the digitized images was carried out using DIP Station (Hayden Image Processing Group) by using $^{12}C^+$ signals as an internal standard and a relative sensitivity factor approach.[21] Final photographic images and composites for publication were prepared with Adobe Photoshop 4.0.

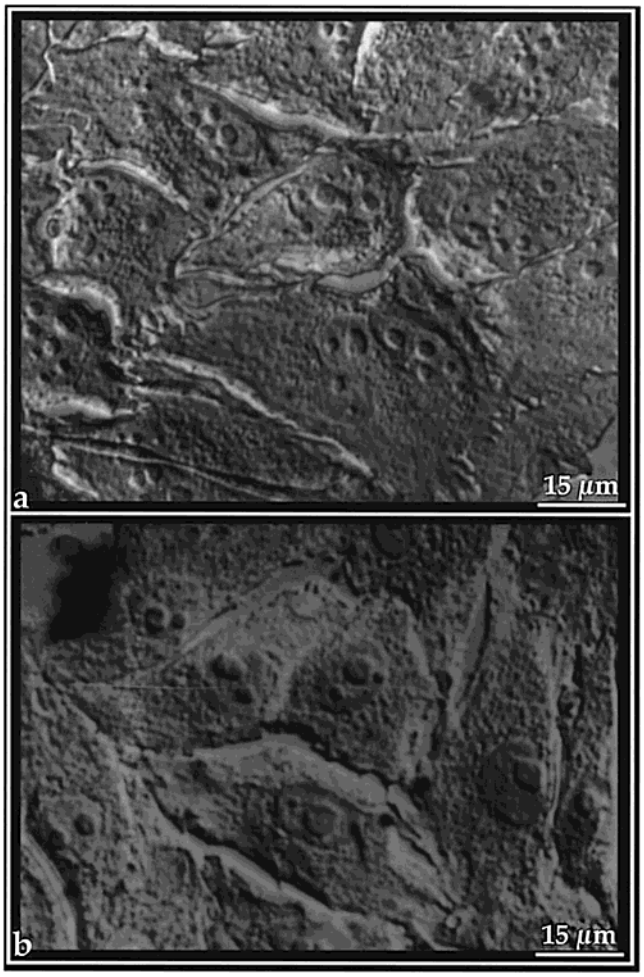

Figure 1. Reflected light Nomarski images of fractured, freeze-dried single-cell lines grown on silicon. (a) The morphology of human glioblastoma T98G cells shows characteristic multinucleation and irregularly shaped nuclei. (b) Normal epithelial LLC-PK_1 cells show large, round, single nuclei with a characteristic dome-shaped epithelium.

RESULTS AND DISCUSSION

Morphology of T98G and LLC-PK_1 Cells. Figure 1a shows an optical micrograph of fast-frozen, freeze-fractured, freeze-dried T98G human glioblastoma cells. The suitability of T98G cells for use in the cryogenic fracture method is shown in this optical micrograph, as numerous individual fractured cells are clearly visible. Two readily apparent morphological characteristics of T98G cells that can be used to identify them in mixed cultures are irregularly shaped nuclei and multinucleation, both of which can be seen in cells in this figure.

Figure 1b shows an optical micrograph of cryogenically prepared, freeze-dried LLC-PK_1 porcine kidney cells. Because of the nature of normal brain cells, it is not feasible to readily grow them in vitro; therefore, other normal cell lines must be used for co-cultured counterparts to the cancerous cell line. However, the normal cell line used in co-culturing can be selected to provide information on drug uptake in parts of the body other than the brain. The LLC-PK_1 cell line is a commonly used cell culture model for the human kidney, and the behavior of boronated drugs in the kidney, especially any toxicity effects, is of interest due to the role of the kidney in filtering the blood supply of foreign substances and excreting them from the body. Unlike T98Gs, LLC-PK_1s have large, rounded single nuclei with prominent nucleoli and a characteristically dome-shaped epithelium, which can clearly be seen in the LLC-PK_1 cells in Figure 1b. These morphological differences make LLC-PK_1 cells clearly distinguishable from T98G cells.

Identification and Morphological Evaluation of LLC-PK_1 and T98G Cells in Co-Cultures. For co-cultures to be successful, the two cell types must grow together on the silicon substrate and fracture successfully together during the cryogenic sample preparation required for quantitative ion microscopy imaging. Figure 2 shows an optical image of an area of a co-culture of T98G and LLC-PK_1 cells that has been successfully fractured with the cryogenic sandwich fracture method. The cells in this image show

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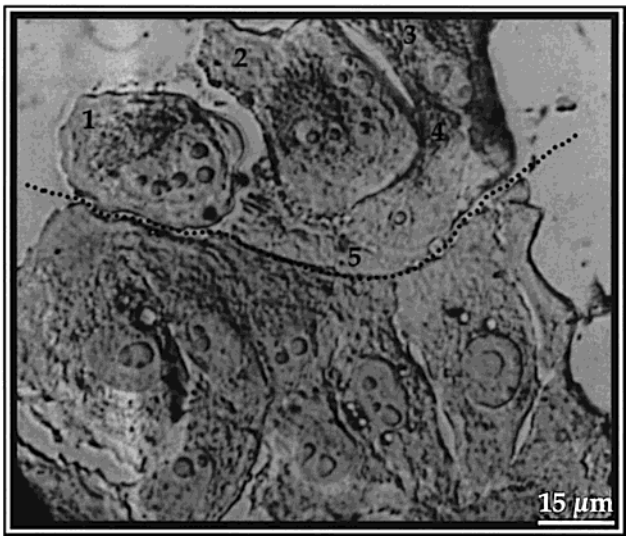

Figure 2. Reflected light Nomarski image of a fractured, freeze-dried co-culture of T98G and LLC-PK_1 cells. The LLC-PK_1 cells below the interface (dotted line) are visually distinguishable from the five neighboring T98G cells above the interface (numbered cells) based on their morphological differences.

a distinct interface (dotted line) between a group of LLC-PK_1 cells and five T98G cells (numbered). The T98G cells are clearly distinguishable from the LLC-PK_1 cells based on their specific morphological characteristics. Such areas containing fractured cells from both cell types can be analyzed with ion microscopy for a comparison of quantitative boron microlocalizations within the same field of view.

Morphological preservation in fractured freeze-dried co-culture of T98G and LLC-PK_1 cells was further characterized by the use of CLSM and imaging of mitochondria, a smaller cytoplasmic organelle reaching the spatial limits of the SIMS instrument used here. Prior to being cryogenically sampled, the co-culture of T98G and LLC-PK_1 cells was treated with rhodamine 123 for mitochondrial imaging. Correlative images on the same mixed fast-frozen, freeze-fractured, freeze-dried cells are shown in Figure 3. Figure 3a shows an optical image of a region of mixed cells. The LLC-PK_1 cells are on the right side of the dotted line that indicates the separation of the two cell types grown together. CLSM imaging of mitochondria of the identical cells is shown in Figure 3b. The brightly labeled mitochondria of LLC-PK_1 cells are uniformly distributed throughout the cell around a clearly defined, circular nucleus with dim fluorescence. T98G cells, on the other hand, show irregularly shaped multiple nuclei that have compressed the cytoplasm containing brightly labeled mitochondria with higher density in distinct regions. These characteristically distinct mitochondrial distributions observed in fractured freeze-dried co-cultures provide support to the sample preparation for preserving cellular morphology.

Ion Microscopy Imaging of Co-Cultured T98G Glioblastoma and Epithelial LLC-PK_1 Cells. Figure 4a shows an optical photo of a freeze-fractured, freeze-dried co-culture of T98G and LLC-PK_1 cells that were treated with 110 ppm boron equivalent of BPA-F for 1 h. Ion images for ^{12}C, ^{39}K, ^{23}Na, ^{40}Ca, and ^{10}B from the identical cells are shown in Figure 4b−f, respectively. Since matrix effects are not significant in fractured freeze-dried cells,[20] the level of brightness within an individual ion image is directly

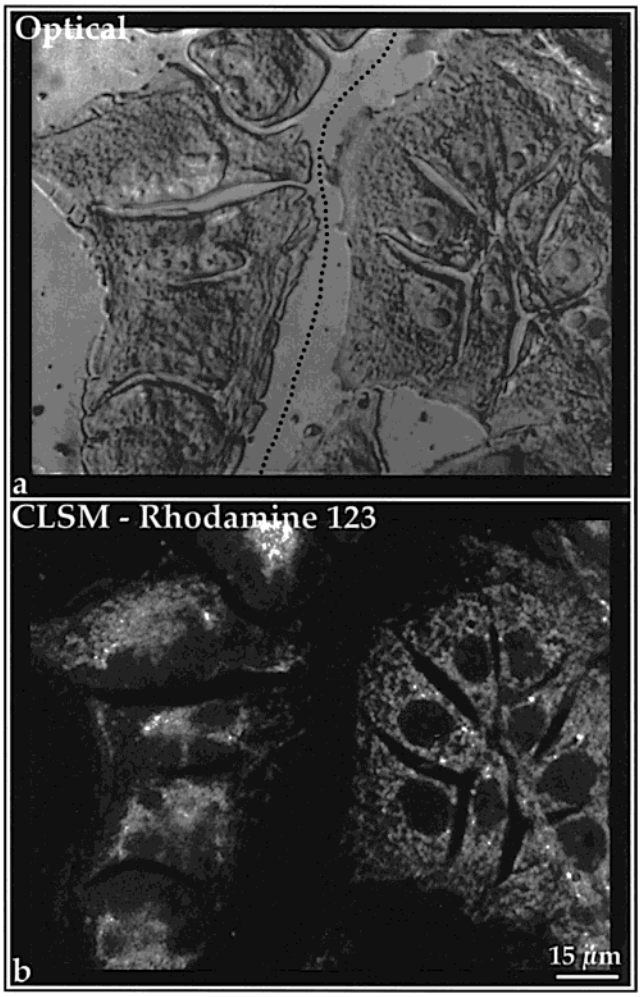

Figure 3. Correlative reflected light Nomarski and CLSM images of the same fractured, freeze-dried co-culture of T98G and LLC-PK_1 cells. (a) The Nomarski image shows clearly recognizable regions of T98G (left of dotted line) and LLC-PK_1 cells (right). (b) The correlative CLSM image of rhodamine 123 fluorescence from the identical cells reveals brightly labeled mitochondria in two cell types. Live co-cultured cells were treated with 1 μM rhodamine for 10 min prior to cryogenic sampling.

proportional to the concentration of the analyte. Two T98G cells, at the right of the field of view, are clearly recognizable in the optical photo. The distributions of potassium, sodium, and carbon indicate minor heterogeneities throughout the cells, but no recognizable gradients are observed between the nucleus and the cytoplasm of cells in either cell type. For most of the cells in either cell line, the ratio of intracellular K/Na concentrations was ∼10:1, which indicates that the mixing of cell lines during the experimental duration was not injurious to either cell type. A few cells with elevated sodium levels, as seen in the sodium image (Figure 4d), were discarded from quantitative analysis. It is not uncommon to observe a few percent of cells in cultures to be injured even in control cell lines.[12,22] The calcium distribution (Figure 4e) reveals low-intensity nuclei against higher intensities in the cytoplasm, including a bright perinuclear region. The large, rounded nuclei of the LLC-PK_1 cells are clearly identifiable and distinguishable from the smaller, more irregular multiple nuclei

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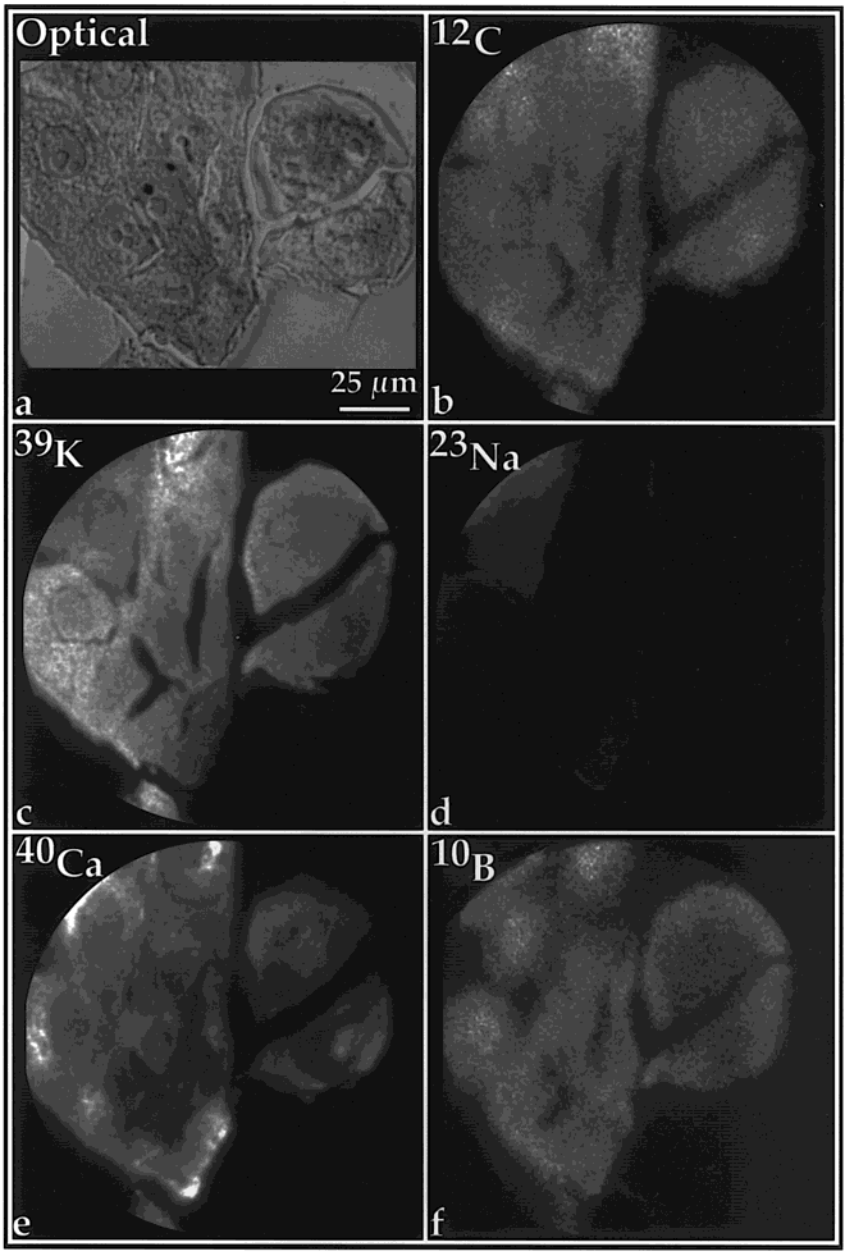

Figure 4. Correlative reflected light Nomarski and SIMS imaging of a fractured, freeze-dried co-culture of T98G and LLC-PK_1 cells treated with BPA-F. (a) Two T98G cells at the right of the Nomarski image are distinguishable from the neighboring LLC-PK_1 cells. SIMS images of ^{12}C, ^{39}K, ^{23}Na, ^{40}Ca, and ^{10}B from the same cells are shown in (b−f), respectively.

of the T98G cells. It is interesting to note in the boron image (Figure 4f) that the cytoplasm of high-sodium cells (seen in Figure 4d and discussed above) is less retentive of boron from BPA, which makes the cell nucleus appear deceptively higher in boron than the nuclei of most healthy, low-sodium cells in the field of view. Quantitative observations of boron were limited to only healthy cells. Table 1 shows quantitative observations in the cytoplasm and nuclei of T98G and LLC-PK_1 cells and will be discussed later.

Co-cultured T98G and LLC-PK_1 cells were also treated with 110 ppm boron equivalent of BSH for 1 h (SIMS images not shown). The K/Na ratios for both cell types in the BSH-treated co-cultures was also ~10, again indicating healthy, well-preserved cells. Concentrations of boron delivered by BSH to the cytoplasm and nucleus of both cell types are shown in Table 1 and discussed later.

Ion Microscopy Imaging of Co-Cultured T98G Human Glioblastoma and GM3348 Human Skin Fibroblast Cells. In Figure 5, ion microscopy imaging of a different co-culture model system is illustrated. In this model, T98G glioblastoma cells were mixed with GM3348 human skin fibroblast cells. The skin fibroblast cell line was chosen, in part, because information on the accumulation of boron in the skin can be important due to the exposure of the scalp to neutrons during irradiation. Figure 5a shows an optical photo of a fast-frozen, freeze-dried co-culture of T98G and GM3348 cells that was treated with 110 ppm boron equivalent of BSH for 1 h. Ion images of ^{39}K, ^{40}Ca, and ^{11}B from the identical cells are shown in Figure 5b−d, respectively. A

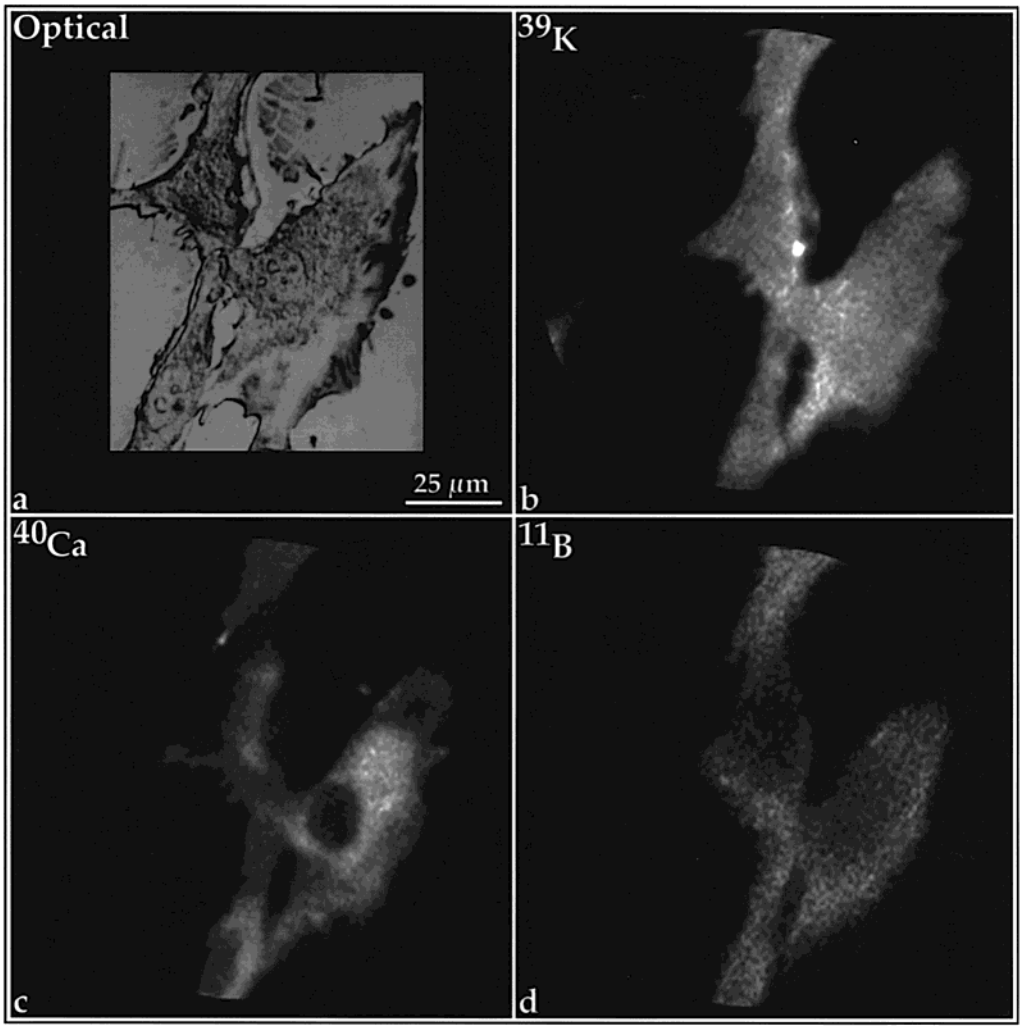

Figure 5. Correlative reflected light Nomarski and SIMS imaging of a fractured, freeze-dried co-culture of T98G and GM3348 cells treated with BSH. (a) A GM3348 skin fibroblast is clearly recognizable at the right of the Nomarski image. SIMS images of ^{39}K, ^{40}Ca, and ^{11}B from the same cells are shown in (b−d), respectively.

Table 1. Comparison of Wet Weight Boron Uptake from BPA and BSH in T98G, LLC-PK$_1$, and GM3348 Cells Treated with 110 ppm Boron Equivalent of Designated Drugs[a]

	boron concentration		
cell type	cytoplasm	nucleus	K/Na ratio
BPA treated			
T98G	155 ± 33	141 ± 15	10
LLC-PK$_1$	148 ± 31	133 ± 39	10
BSH treated			
T98G	109 ± 24	113 ± 16	10
LLC-PK$_1$	115 ± 20	118 ± 19	10
GM3348	110 ± 20	105 ± 16	8

[a] All concentrations are presented as mean μg of B/g of wet weight ± SD, assuming 85% cell water content. Average cell number examined for each data point was at least 20 cells in each treatment.

GM3348 fibroblast, at the right of the field of view, is clearly recognizable and distinguishable from T98G cells in the optical photo (Figure 5a). Potassium, sodium, and carbon are nearly homogeneous throughout the cells (sodium and carbon images not shown), and the K/Na ratio was ~10:1. The calcium distribution (Figure 5c) once again reveals low-intensity nuclei against the higher intensities in the cytoplasm. Boron concentrations for the subcellular compartments of GM3348 cells are listed in Table 1 and discussed below.

Subcellular Quantitative Analysis of Boron. Quantitative analysis of boron delivered by boronated BNCT compounds requires strict, careful cryogenic sample preparation, such as that used for ion microscopy, to minimize the risk of boron redistribution. BSH in particular has been shown to be subject to redistribution artifacts upon cell damage.[12] Any sample preparation method that might allow these species to diffuse should be avoided, lest sample preparation artifacts distort the quantitative results. All quantification of boron (Table 1) was carried out on cells showing high K/Na ratios, which signifies healthy, well-preserved cells.

In the BPA-F treated co-culture of T98G and LLC-PK$_1$ cells, the cell types did not show significant differences in uptake of boron under experimental conditions (Table 1). Furthermore, both cell types exhibited a slightly heterogeneous subcellular boron distribution. These differences, however, were not statistically significant. The fact that the cellular boron concentrations are significantly higher in both the nucleus and cytoplasmic compartments (Table 1) than the 110 ppm boron concentration they were exposed to is indicative that boron accumulation in these cells is

not merely a matter of chemical equilibrium between the cells and the BPA-F in the extracellular medium. There is some form of active boron uptake mechanism at work in these cells for BPA-F, although that mechanism is not cell type-specific in cell lines studied here. The boron concentrations in the co-cultured T98G cells are also very similar to the subcellular boron concentrations observed in BPA-F treated T98G cells alone.[12] This provides further evidence that the boron uptake characteristics of the T98G cells are not altered by co-culturing them with the LLC-PK_1 cells.

In the treatments with BSH, none of the three cell lines examined—T98G, LLC-PK_1, or GM3348—exhibited any preferential boron uptake during the time of BSH exposure. Even less heterogeneity was observed between boron in the nucleus and cytoplasm of these cells, with both compartments accumulating ~110 ppm boron in all cell types (Table 1). This indicates that the mechanism of boron delivery by BSH is not cell type-specific in the cell lines examined here. The mechanism of boron uptake from BSH by cells is unknown,[2] but these observations support the view that BSH may be reaching an equilibrium state between the cells and the extracellular medium.

The observations shown here indicate that a co-culture method used with SIMS can provide direct imaging evidence for the selectivity of a boronated drug to a specific cell type. The selection of cell types for use in co-culture models depends on the nature of the information being sought. The cell lines studied here represent a selection of very different cell types for methodological development with the use of two BNCT drugs. These two drugs are the only drugs approved for clinical use in the United States and, as such, are the only BNCT drugs readily available for experimental research. Irrespective of the mechanisms of the boron delivery from these drugs, the different cell types co-cultured together do not reveal any significant cell type differences in boron uptake. Indeed, there is some support for these observations, especially for BSH. Previous SIMS studies of four individual cell lines studied for boron accumulations from BSH did not show any significant cell type differences.[14]

SIMS analysis of co-cultured cells provides the most direct visual evidence for subcellular boron accumulations and, in doing so, presents an efficient and economical way for comparing BNCT drug selectivity in different cell types. The most effective applications of this method will be realized in the analysis of new BNCT drugs still under development, such as those designed to target membrane receptors, which are found in abundance in tumor cells.[23,24] As the cells in cultures lack the blood–brain barrier, the co-culture SIMS observations of tumor cell selectivity for glioblastoma studies must be taken as first clues on boron accumulations rather than the definitive answers. However, with the preliminary SIMS observations from co-cultures in hand, animal studies can be designed for further testing of promising drugs, and less promising drugs can be excluded from further consideration.

CONCLUSIONS

A cryogenic method is presented that allows a direct analysis of two different cell types within the same field of SIMS imaging. This method was successfully employed for the analysis of cells from various cell lines for checking boron accumulations from two clinically approved BNCT drugs. The future applications of this method may not be limited to BNCT drugs alone, as any therapeutic agent containing a detectable isotope (over the cellular background) with SIMS can be tested for cell type selectivity in a co-culture model.

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