Preparation of Single Cells for Imaging/Profiling Mass Spectrometry

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Characterizing chemical changes within individual cells is important for determining fundamental mechanisms of biological processes that will lead to new biological insights and improved disease understanding. Analyzing biological systems with imaging and profiling mass spectrometry (MS) has gained popularity in recent years as a method for creating chemical maps of biological samples. To obtain mass spectra that provide relevant molecular information about individual cells, samples must be prepared so that salts and other cell culture components are removed from the cell surface and that the cell contents are rendered accessible to the desorption beam. We have designed a cellular preparation protocol for imaging/profiling MS that removes the majority of the interfering species derived from the cellular growth medium, preserves the basic morphology of the cells, and allows chemical profiling of the diffusible elements of the cytosol. Using this method, we are able to reproducibly analyze cells from three diverse cell types: MCF7 human breast cancer cells, Madin-Darby canine kidney (MDCK) cells, and NIH/3T3 mouse fibroblasts. This preparation technique makes possible routine imaging/profiling MS analysis of individual cultured cells, allowing for understanding of molecular processes within individual cells. (J Am Soc Mass Spectrom 2008, 19, 1230–1236) © 2008 American Society for Mass Spectrometry

In the past several years, there has been an explosion in the number of researchers utilizing imaging mass spectrometric techniques for biological applications [1–6]. While many of these studies have focused on the analysis of tissue samples, there is a clear need for analysis on a single-cell level as well. Typical biochemical cell studies focus on populations, averaging the response of all of the cells assayed. This approach obscures subtle phenotypic differences among individual cells [7]. By interrogating single cells, an analysis is freed from assumptions regarding cell population homogeneity, ensuring that all individual cellular responses to environmental changes can be measured. Understanding the response of a single cell is necessary for identifying small cellular changes that may underlie many biological processes including disease development.

There are several different MS imaging systems currently being developed to analyze single cells. Although more commonly used for tissue analysis, recent advances in matrix-assisted laser desorption/ionization (MALDI) MS imaging have greatly enhanced the spatial resolution, opening the possibilities for imaging of single cells [8, 9]. To date, however, very few reports of single-cell MALDI imaging have been published [10]. Secondary ion mass spectrometry (SIMS), in both dynamic and static mode, has been widely applied to imaging analysis of single cells. Recent advances in dynamic SIMS imaging of cells have been reviewed elsewhere [4], with many examples showing excellent spatial resolution and localization of elements within cells. Very recently, the newest generation of dynamic SIMS instrumentation, NanoSIMS, has been used to obtain subcellular localization of a peptide vector [11], study diatom cell division [12], and perform nanoautography with stable isotope tracers [13]. Static SIMS has also seen wide application in cellular imaging; recent examples include three dimensional imaging of oocytes [14], relative quantification of cholesterol in cell membranes [15], and distinguishing cancerous cells of differing breast tumor phenotypes [16]. Cellular imaging has also been shown by a variety of other mass spectrometric techniques, including desorption/ionization on silicon [17] and laser post-ionization secondary neutral mass spectrometry [18].

We are utilizing time-of-flight secondary ion mass spectrometry (TOF-SIMS) to demonstrate the suitability and reproducibility of the reported cell preparation method for MS morphological imaging and chemical profiling. TOF-SIMS is a highly surface-sensitive, mass-spectral analysis technique used to detect and localize chemical and molecular information from sample surfaces. TOF-SIMS uses a finely-focused (~150 nm), pulsed primary ion beam to desorb secondary ions into a time-of-flight mass spectrometer. These secondary

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molecular and fragment ions are collected to create mass spectral images with excellent spatial resolution and good mass resolution.

To obtain high-quality MS data from single cells, the cells must be attached on a suitable substrate, free of interfering components or contaminants, and accessible to the ionization source. Traditionally for imaging mass spectrometry, cells are grown on a conductive substrate and freeze-fractured before analysis. The freeze-fracture technique was originally reported in 1957 and has been used extensively to prepare cells for membrane analysis by electron microscopy [19, 20]. Working from this concept, Chandra and Morrison developed a modified freeze-fracture method that could be used to prepare cells for imaging MS analysis [21]. Currently, the vast majority of cellular MS imaging reports utilize some variation on this method.

The freeze-fracture method, however, has several disadvantages: it requires facilities for cryogenic preparation, generally produces a low yield of suitably fractured cells, and by design tends to fracture cells between the leaflets of the membrane bilayer. Because the fracture plane is most commonly within the membrane bilayer, the cytoplasm of the cell is still obscured by a layer of phospholipids. To circumvent these disadvantages and simplify cellular preparation, several groups have reported results with simpler wash-and-dry approaches. Methods such as fixing cells in 70% ethanol [17], embedding cells in a trehalose and glycerol matrix [22], washing with sucrose and water [23], and washing with water alone [14] have all been reported.

Here, we report development of a method that allows cellular morphological imaging and chemical profiling of the molecular information from the cytosol and simplifies previous procedures by removing the need for cryogenic facilities. The simple "wash-and-dry" cellular preparation technique that we have developed not only successfully removes interferences contributed by the medium but also allows delicate cells to remain intact until just before the cells are completely dry, thus retaining the greatest reproducible molecular information from each cell.

Experimental

Cell Culture

MCF7 human breast adenocarcinoma cells, MDCK canine kidney cells, and NIH/3T3 mouse fibroblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown according to standard methods. All culture reagents and media were obtained from Invitrogen Corp. (Carlsbad, CA). Silicon substrates (~1.5 cm^2) were cleaned with 100% ethanol and sterilized by UV irradiation before experimentation. Approximately 15,000 cells were plated in 60-mm dishes containing four sterile silicon substrates using standard cell culture techniques. The cells were allowed to attach overnight on the polished side of the silicon substrates and showed no change in cellular morphology from cells grown on standard substrates. In preparation for imaging/profiling mass spectrometry, each silicon substrate with attached cells was cleaned with one of a variety of methods as described in the results and discussion. Samples were dried with argon as detailed below, kept at room temperature and atmospheric pressure and analyzed by TOF-SIMS as soon as possible after preparation.

Washing Solutions

Ammonium acetate, magnesium acetate, and Tris acid/Trizma base were purchased from Sigma (St. Louis, MO). Sucrose was purchased from J. T. Baker Inc. (Phillipsburg, NJ), sodium chloride was purchased from Mallinckrodt (Paris, KY), and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was purchased from Invitrogen Corp. (Carlsbad, CA). The chemicals were individually dissolved to a concentration iso-osmotic to cellular cytosol (300-mM sucrose, 150-mM for all others) in Millipore Milli-Q water (18.2 MΩcm), the pH was adjusted to 7.5 using a 1:9 solution of phosphoric acid and 1 M ammonium hydroxide, and each washing solution was sterile filtered using a Stericup Vacuum Filter Cup from Millipore Corporation (Billerica, MA). Phosphate buffered saline (PBS) was made to be 0.137 M NaCl, 0.002 M KCl, 0.001 M KH_2PO_4, and 0.02 M Na_2HPO_4. All solutions were stored at room temperature under sterile conditions.

Cell Proliferation

MCF7 cells were plated at 2×10^4 cells/well in a 96-well plate and grown overnight at 37 °C, 5% CO_2. Existing cell medium was removed from the cells and 100 μL of one washing solution was added and immediately removed. Fresh cell medium was then added to the wells and the plate was placed back into the incubator for 48 h to allow for cell proliferation. Cell growth was quantified using an aqueous nonradioactive cell proliferation assay (Promega, Madison, WI) with absorbance of the wells measured in a standard multi-well plate reader (Bio-Rad, Hercules, CA, Model 680 Microplate Reader) at 490 nm. Each experiment was performed at least five individual times with eight replicates per experiment. The effect of each washing buffer on cell growth was determined by comparing the washed wells with wells treated simply by removing medium and replacing with fresh medium. Seventy percent ethanol was included as a negative control. Statistical significance was tested using a one-sample Student's *t*-test (each condition compared with control) and a two-tailed *t*-test assuming unequal variances (ammonium acetate compared to water).

TOF-SIMS Analysis

TOF-SIMS measurements were conducted on a PHI TRIFT III instrument (Physical Electronics USA, Chanhassen, MN) equipped with a gallium ($^{69}Ga^+$) liquid metal ion gun (LMIG) operated at 25 kV. Data were collected over a mass range of 1 to 1850 daltons. Positive ion TOF-SIMS images were generally acquired over an area of 100 × 100 μm to 200 × 200 μm depending on the number and size of cells being analyzed. Samples were held at room temperature during the course of the TOF-SIMS measurements. All TOF-SIMS spectra were calibrated to the CH_3^+, $C_2H_3^+$, and $C_3H_5^+$ peaks before further analysis.

Use of a gallium LMIG has the advantages that the ion source is particularly stable and produces images with 150-nm lateral resolution. However, these low mass atomic primary ions generate mostly smaller molecular weight fragments of the molecules of interest. Recent developments in higher mass and cluster ion beam technologies have demonstrated significant improvements in detectable mass range and detection sensitivities [24]. The combination of higher mass range and lower fragmentation increases chemical specificity in cellular differentiation and may further improve the applicability of TOF-SIMS imaging analysis at the single cellular level. With continuous advances in the spatial resolution of larger mass and cluster primary ions, we anticipate that larger primary ion TOF-SIMS will play an increasing role in the future analyses of single cells.

Results and Discussion

Developing a reproducible and simple cell preparation method is critical for efficient mass spectral interrogation of large numbers of individual cells. When cells are simply air-dried, the cells are obscured by residual medium components, particularly salts. During evaporation, a liquid droplet collects in the area immediately around the cells. This liquid droplet evaporates last, creating a concentrated residue from the medium in the area directly around the cells. To reduce the buildup of this residue, we have developed a procedure for blowing the substrates dry with a gentle and controlled argon stream. In this process, argon gas is directed at the sample substrate through a slit nozzle of ~1 mm × 2 mm with an argon flow rate of 12 to 15 cm^3/s. The nozzle is positioned so that the argon blows the liquid across the surface of the substrate and onto an absorbent towel that is perpendicular to one edge of the silicon substrate, thus significantly reducing the collection of liquid around the cells. We expect that any nonreactive gas that is clean and can be well controlled would be suitable for the drying step. This drying process is designed to ensure that the liquid, along with any dissolved components, is pushed off of the substrate rather than evaporated, minimizing residue on the cells. During drying, care must be exercised to avoid overly vigorous or prolonged blowing on the samples, which can cause the cells to rupture with subsequent loss of molecular information from the cytosol.

While the previously described blowing technique reduces the amount of contamination observed in the area immediately around cells, simply blowing the medium from the substrate is not sufficient to render cells adequately clean for MS analysis. We have therefore undertaken an extensive search for a washing solution that will both clean the cells sufficiently and preserve the maximum possible amount of cellular information for analysis. Recent investigations [14, 23] have reported using pure deionized water to wash cells before drying and imaging MS analysis. In our laboratory, however, we have found that washing with water, even for as few as 10 s, can cause the cells to rupture during the washing step. Figure 1 shows the results of washing cells with water compared with those obtained using our optimized preparation technique described in detail below. Figure 1a–c show total ion, sodium ion, and potassium ion images, respectively, of cells washed with pure water and blown dry. In TOF-SIMS images, the localization of sodium and potassium ions can be used as a general indicator of the location of the cytosol of a cell, as cytosol is known to contain a low concentration of sodium and a high concentration of potassium compared with the surrounding medium [25]. In the water-washed cells, sodium is excluded from the cellular area as expected (Figure 1b), but there is a complete lack of potassium signal (Figure 1c), indicating that the soluble portion of the cytoplasm is not available for analysis. As cells are known to rupture upon treatment with a hypo-osmotic solution [26], the most extreme of which is deionized water, it is reasonable to assume that the cells exploded during the wash step, allowing the diffusible elements of the cell cytosol to be washed away. These results are in contrast to those seen with our sample preparation procedure, shown in Figure 1d–f, where not only is sodium excluded from the cell region, but potassium is localized in the area immediately around the cells. Our results suggest that, while water washing may be appropriate for some cell types and sufficient for analysis of the structural elements of a cell, an iso-osmotic wash solution is necessary to preserve the cellular cytosol for chemical profiling.

A wide variety of iso-osmotic washing solutions were explored for their ability both to preserve cellular contents through the washing process and to allow mass spectral analysis with the fewest imaging and spectral interferences. Possible candidates were chosen based on their use by others in the literature, (sucrose [23]) their widespread use in biological experiments (PBS, Tris, HEPES, and magnesium acetate), their simplicity (sodium chloride), or volatility (ammonium acetate). All solutions were created to be iso-osmotic with standard cellular medium. Various combinations of the above solutes were also evaluated for suitability, although none of the combinations were found to provide better results than a single solute. The washing proce-

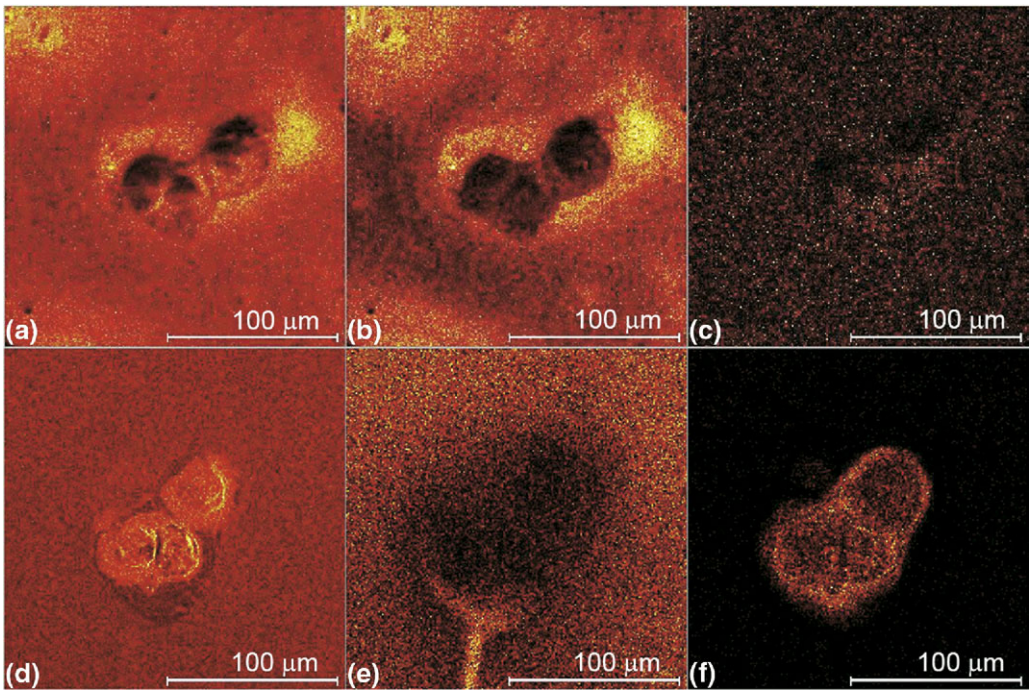

Figure 1. Row 1. TOF-SIMS images of MCF7 cells washed with deionized water: (**a**) total ion image; (**b**) sodium ion image showing exclusion from the cellular area; (**c**) potassium ion image showing a total lack of potassium localization in or near the cells. Row 2. TOF-SIMS images of similar MCF7 cells prepared with our sample preparation method: (**d**) total ion image; (**e**) sodium ion image showing exclusion from the cellular area and a small "tail" where the wash solution was blown away from the cells; (**f**) potassium ion image showing localization in the area immediately around the cells.

dure involved quickly (<15 s) dipping the sample substrate into a small beaker of fresh solution, followed by swishing in one backward/forward motion. After washing, each sample substrate was dried with a gentle stream of argon as described above.

The HEPES and magnesium acetate solutions were found to be unsuitable because the TOF-SIMS spectra of cells prepared with these solutions contained large mass spectral peaks associated with the washing solution. Washing with PBS or sodium chloride improved the cleanliness of the cells over medium alone, but still left a large number of salt crystals on the sample substrate. The sucrose wash eliminated the problem of salts interfering with MS imaging, but unfortunately left a sticky residue on the sample substrate that was clearly visible in the TOF-SIMS images. The best washing solution was found to be 150-mM ammonium acetate, which completely eliminated salt crystals and other interferences from the TOF-SIMS spectra and total ion images. The superior results obtained with the ammonium acetate wash likely are due to the relative volatility of ammonium acetate, which allows any residue remaining after the drying procedure to evaporate.

However, as was made evident by the problems created by washing with pure water, a washing procedure must maintain cell viability in addition to removing spectral and imaging interferences. We have used a standard cellular proliferation assay to assess viability by measuring the effects of the various solutions on the ability of cells to continue to proliferate after being washed. Treatment with water shows a statistically significant ($p < 0.0001$) decrease in proliferating cells. Treatment with ammonium acetate causes a minimal decline in the number of viable cells, indicating that washing with ammonium acetate is a better washing procedure both from the standpoint of keeping the cells intact during washing and for producing clean cells for imaging analysis. It is interesting that washing with water does not cause a total reduction in cell growth compared with the negative control (70% ethanol treatment). These results may indicate why this technique has been reported as successful by others [14, 23]. However, the statistically significantly ($p = 0.013$) better viability obtained with the ammonium acetate suggests that washing with ammonium acetate is preferable to washing with water for MS analysis of cells.

It is important to note that while it is critical to minimize cellular damage during any washing procedure to preserve the cytosolic contents of the cell, a dried cell will, by definition, not be entirely comparable to a living cell. Our washing and drying procedure has been designed to keep the cell intact for as long as possible throughout the washing and blowing steps. However, immediately after drying the substrate, a high-pressure differential will be created between the still-hydrated cell and the surrounding air. This pressure differential will cause the membrane to lose 3-D structure. During this process, the cytosol leaks into the

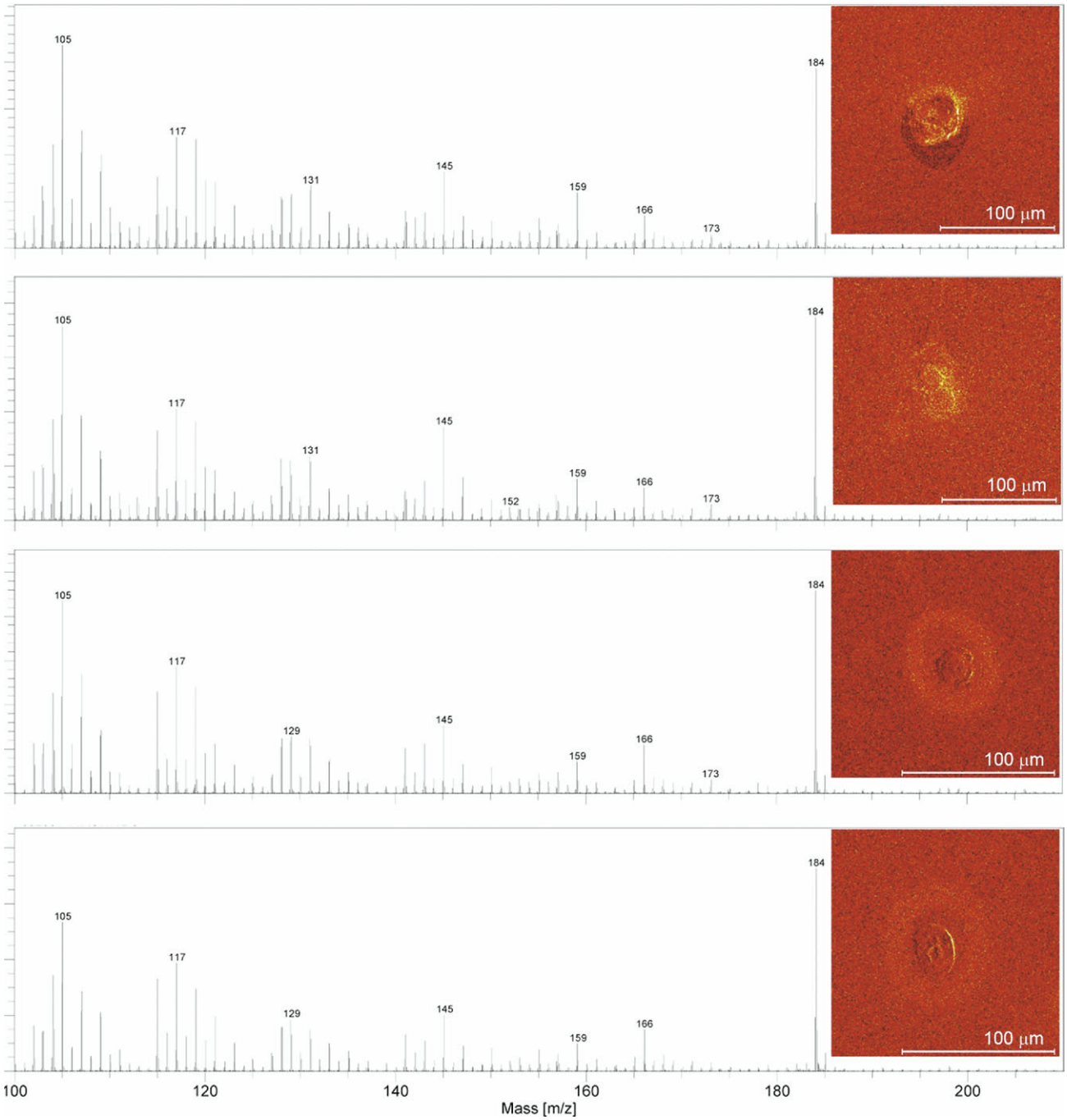

Figure 2. TOF-SIMS spectra and total ion images of MCF7 human breast cancer cells prepared on four independent substrates over a period of one month showing the reproducibility attained with the reported preparation method.

immediate vicinity of the cell. As long as this leakage occurs after all washing and drying procedures are completed, the entire cellular contents are retained in the area immediately surrounding the cell and are available for chemical profiling. Although the subcellular localization of the diffusible cellular elements is lost, our sample preparation technique maintains the structural elements and morphology of the cell, allowing mass spectral imaging of the morphological features.

A critical component of any biological sample preparation is reproducible results. To address this issue, we repeatedly prepared and analyzed MCF7 cells over a 1-month period. Figure 2 shows TOF-SIMS images and mass spectra (in the region from m/z = 100–220) for MCF7 cells prepared on four independent silicon substrates. From these results it is evident that the optimized cellular preparation procedure produces highly reproducible results.

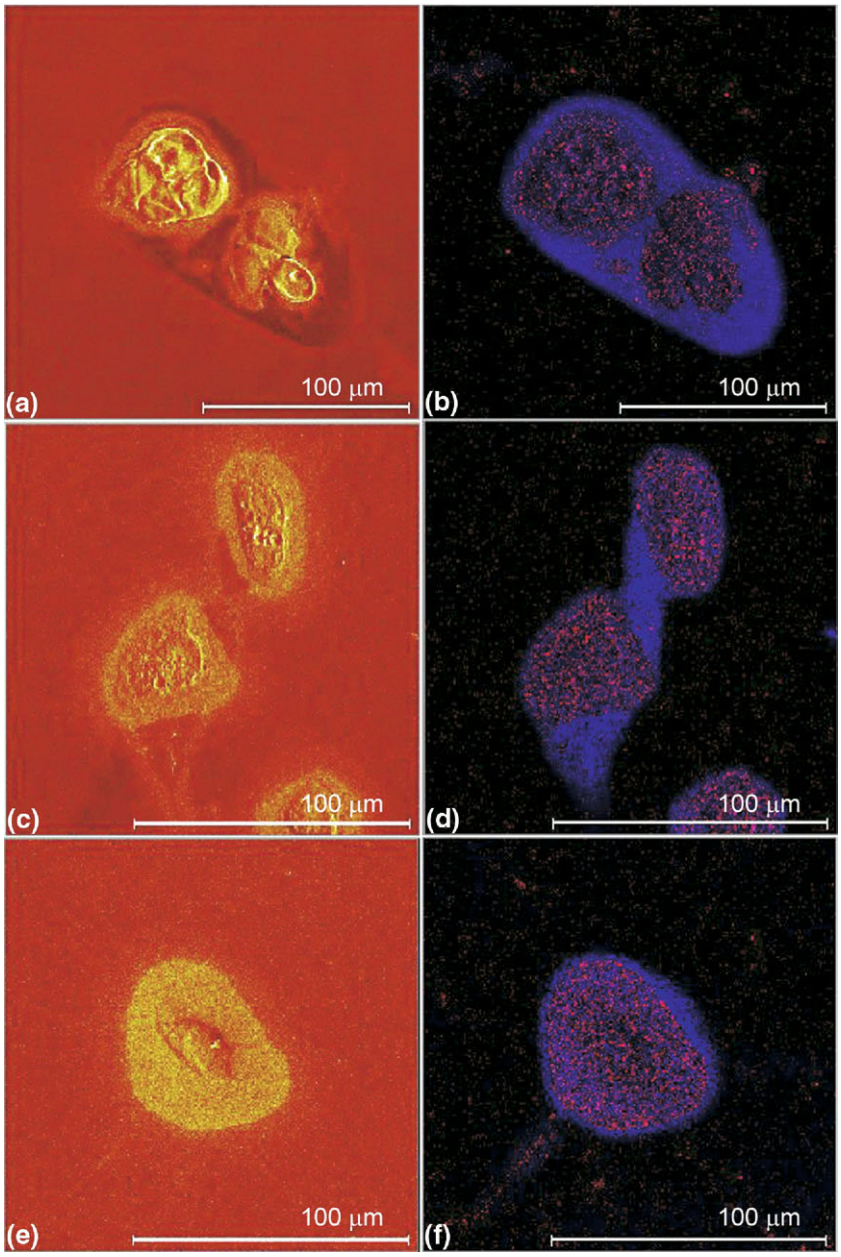

Figure 3. TOF-SIMS images of three different cell types prepared with the reported cellular preparation procedure. In the left column are total ion images, in the right column composite images of potassium ion (blue) and m/z = 184, a fragment of the phosphocholine head group (red). (**a**) and (**b**) MCF7 human breast cancer cells; (**c**) and (**d**) MDCK canine kidney cells; (**e**) and (**f**) NIH/3T3 mouse fibroblast. Note the localization of potassium in the area immediately surrounding the cells and the localization of phosphocholine on the cellular region.

In addition, this preparation procedure is applicable to a wide variety of cell types. Figure 3 shows TOF-SIMS images taken from seven MCF7 human breast cancer cells (Figure 3a and b), two MDCK cells (Figure 3c and d), and one NIH/3T3 mouse fibroblast (Figure 3e and f). These cells, which represent different species, different organs, and different states of differentiation, are widely used models for studying various biological processes. The images in Figure 3 demonstrate that, while the cells clearly differ in morphology, the preparation procedure produces cells suitable for detailed analysis for each cell type, making this a broadly applicable procedure for a variety of biological investigations.

In Figure 3, the left column shows total ion images of the cells, while the right column shows overlay single ion images of the potassium ion (blue) and m/z = 184, a fragment of the phosphocholine head group (red). The overlay images clearly illustrate the localization of phosphocholine on the membrane areas and potassium from the cytosol in the area immediately surrounding

the cell bodies. Based on the potassium localization, we believe that the membrane collapse and release of cytosol occurs just after the washing and drying procedures, thus retaining the cytosol in the area around the cell and making it available for chemical profiling.

The potassium localization in the cells can also be used as a control indicator for the washing and drying procedures. If the washing step is too vigorous or too long, the potassium signal will be lost, and if the drying step is too harsh, the potassium signal from the cytosol will be seen as a tail leaving the cell where it has been blown away by the argon stream. Most importantly, the potassium localization indicates that the cytosol of the cells has been retained and rendered available for MS analysis, while the phosphocholine signal indicates that the membrane portions are also being analyzed.

Conclusions

These experiments demonstrate a cellular preparation method that preserves the molecular information from single cells and produces sufficiently clean surfaces for unobscured mass spectral analysis. Furthermore, this preparation technique yields reproducible results over separate preparations, is applicable to a wide variety of cell types, removes the need for cryogenic facilities, and produces a high yield of cells suitable for morphological imaging and chemical profiling. Importantly, the quick drying step also allows for the interrogation of both membrane and cytosolic molecular contents, as evidenced by the localization of both phosphocholine and potassium in the mass spectral images. The reported preparation technique allows routine imaging/profiling MS analysis of individual cells, opening the possibilities for a large variety of experiments aimed at furthering scientific understanding of chemical processes within cells.

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