ToF-SIMS of tissues: “Lessons learned” from mice and women

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The ability to image cells and tissues with chemical and molecular specificity could greatly expand our understanding of biological processes. The subcellular resolution mass spectral imaging capability of time of flight secondary ion mass spectrometry (ToF-SIMS) has the potential to acquire chemically detailed images. However, the complexities of biological systems combined with the sensitivity of ToF-SIMS require careful planning of experimental methods. Tissue sample preparation methods of formalin fixation followed by paraffin embedding (FFPE) and OCT embedding are compared. Results show that the FFPE can potentially be used as a tissue sample preparation protocol for ToF-SIMS analysis if a cluster ion presputter is used prior to analysis and if nonlipid related tissue features are the features of interest. In contrast, embedding tissue in OCT minimizes contamination and maintains lipid signals. Various data acquisition methodologies and analysis options are discussed and compared using mouse breast and diaphragm muscle tissue. Methodologies for acquiring ToF-SIMS 2D images are highlighted along with applications of multivariate analysis to better identify specific features in a tissue sections when compared to H&E images of serial sections. Identification of tissue features is necessary for researchers to visualize a molecular map that correlates with specific biological features or functions. Finally, lessons learned from sample preparation, data acquisition, and data analysis methods developed using mouse models are applied to a preliminary analysis of human breast tumor tissue sections. © 2015 American Vacuum Society. [<http://dx.doi.org/10.1116/1.4907860>]

I. INTRODUCTION

Imaging the chemical composition within specific regions (or microenvironments) of cells and tissues can provide insight to biologically relevant problems. The ability to image cells and tissues with chemical and molecular specificity could increase our understanding of chemical changes in cells and tissues as a result of a variety of biological stresses. Chemistry of tumor microenvironments, lipid metabolomics in tissues, and tissue regeneration are examples of the types of biological processes that could benefit from visualization on a cellular and subcellular level. Chemical mapping could also enable tracking the spatial distribution of metabolites, lipids, or nanoparticles in cells and tissues. The subcellular resolution mass spectral imaging capability of time of flight secondary ion mass spectrometry (ToF-SIMS) holds the potential to achieve these possibilities.

The use of imaging ToF-SIMS in medical and biological research has increased in recent years. The high yield of lipid based ions, combined with the subcellular lateral resolution imaging capabilities of ToF-SIMS makes it an excellent tool for studies of lipids,^{1–4} cells,^{5–11} and tissues.^{12–16} Studies have been carried out in a variety of areas including the

analysis of cancer cells,^{17–19} cancer tissues,^{20,21} muscular dystrophy,^{22,23} and drug permeation into skin.²⁴ Several groups have discussed sample preparation methods for cells^{25–28} and tissues.^{14,29} However, the use of ToF-SIMS is still currently in early stages of development for answering biologically related questions.

Here we discuss some of our work in ToF-SIMS sample preparation, data collection, and analysis for tissue sections with an emphasis on practical considerations required when carrying out such analyses. Preliminary work on mouse tissue is shown comparing SIMS spectra for different tissue block preparation methods as well as their effect on image clarity. Specific chemical regions within both mouse mammary tissue and mouse diaphragm muscle tissue are compared to traditional imaging methods for tissue including H&E stained tissue images and immunofluorescence labeled confocal images. Due to the complexity of the data, principal component analysis (PCA) is applied to various data sets to aid in determining differences and similarities between tissue samples.

To further illustrate the importance of careful sample preparation and handling for ToF-SIMS tissue analysis, we show that one can analyze multiple slices from the same tissue block on different days and generate reproducible results. For this test, ToF-SIMS is used to analyze

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differences in the chemical makeup of two estrogen receptor positive (ER+) and one estrogen receptor negative (ER-) breast cancer tumor tissue samples.

II. EXPERIMENT

A. Time-of-flight secondary ion mass spectrometry

ToF-SIMS experiments were performed by using an ION-TOF ToF-SIMS 5-100 (ION-TOF GmbH, Münster, Germany) spectrometer. A liquid metal ion gun was used to generate a pulsed 25 keV Bi_3^+ beam for spectra and image acquisition, whereas an electron impact gun was used to generate a 20 keV C_{60}^{2+} beam for sputtering. Both beams are oriented at the sample at an angle of 45° . Primary ion currents were measured before each data set with a Faraday cup. In the high spatial resolution (HSR) mode, the Bi_3^+ current was typically 0.04–0.06 pA using a 100 ns pulse width. In the high mass resolution (HMR) mode, the Bi_3^+ current was typically 0.05–0.15 pA. Low energy electrons were flooded onto the sample to compensate for charge accumulation for all samples. For all analyses, the primary ion dose for a given analysis spot was kept below 1×10^{12} ions/cm².

For cancer tissue analysis, spectra were acquired from several 1 mm $\times$ 1 mm “patches” on thin sections of optimal cutting temperature (OCT)-embedded tissue. Each patch contained 25 200 $\mu\text{m} \times 200 \mu\text{m}$ “tiles.” The Bi_3^+ dose was limited to 5.0×10^{11} ions/cm² for each tile in both the positive and negative ion modes in order to keep the total dose per tile below 1×10^{12} ions/cm². All data for each tile was acquired with a 256×256 pixel density, giving each 1 mm $\times$ 1 mm patch a total pixel count of 1280×1280 . As described in Sec. II A, areas that contained OCT were removed from the images in order to more clearly see tissue borders and more readily identify holes in the tissue.

The SurfaceLab 6 software (ION-TOF GmbH, Münster, Germany) was used to calibrate all spectra, generate peak lists, and export peak area tables or binary image files that could be further processed using the NBToolbox (Dan Graham, NESAC/BIO, University of Washington) in MATLAB (MathWorks, Natick MA). Peaks were chosen manually if their maximum intensity was at least twice that of the surrounding background. Substrate peaks were included in the peaks list, and no additional pixels were removed from the images prior to PCA.

B. Principal component analysis

Image data sets were not normalized, but were Poisson-scaled³⁰ and mean centered before PCA was applied. Spectral data sets were normalized to the total counts of the respective spectra, square root transformed and mean centered before PCA. More information about PCA can be found in the literature including good general overviews,^{31,32} overviews specific to PCA of ToF-SIMS data,^{33–35} and information about working with image data sets.^{36,37} For the tissue section data described in Sec. II C, the spectra from each 200 $\mu\text{m} \times 200 \mu\text{m}$ tile was extracted using the NBToolbox and treated as individual spectra in the PCA analysis.

C. Tissue preparation

1. Tissue embedding

Formalin fixed paraffin embedded (FFPE) tissue: Tissue was harvested, fixed in 10% neutral buffered formalin for a minimum of 72 h, processed (dehydrated in alcohols and cleared in xylenes), embedded in paraffin, and stored at room temperature.

Cryoembedded tissue: Tissue was harvested and embedded in Tissue-Tek[®] OCT Compound for Cryostat Sectioning (Fisher Scientific), cryopreserved using liquid nitrogen (LN_2), and stored in a -80°C freezer.

2. Tissue cutting and transport

OCT-embedded blocks were removed from the freezer and sectioned inside a cryostat-microtome held at -23°C . For human breast cancer tissue, 5 μm thick sections were cut at the Fred Hutchison Cancer Research Center (FHCRC) and placed onto 1 cm $\times$ 1 cm silicon chips that were previously cleaned with $2 \times$ successive sonications in dichloromethane, acetone, and methanol. The silicon chips were placed in a petri dish and transported to the ToF-SIMS where the data were immediately acquired. The time from cutting to analysis was not more than 90 min for any sample. A similar protocol was used for mouse mammary OCT and FFPE embedded sections, with the exception that FFPE samples were cut at room temperature. Both types of samples were analyzed in the ToF-SIMS at room temperature.

Mouse diaphragm muscle tissue was embedded in OCT and prepared in a similar manner to the breast tissue with the exception that the tissues were frozen in LN_2 cooled isopentane and cut at -18°C into slices between 5 and 20 μm thick. Mouse muscle sections were either vacuum dried for 15 min or taken directly to the lab for ToF-SIMS analysis.

III. RESULTS AND DISCUSSION

A. Mice: Sample preparation

As with any ToF-SIMS study, sample preparation is critical to generating meaningful results. Factors such as embedding material, substrate, and timing of analysis are all important for consistent analysis, especially if a comparison between two different tissue slices is the goal. For example, while H&E samples used for stained tissue images are typically placed on glass slides, we have found using silicon wafers as substrates for ToF-SIMS eliminates issues with charge compensation and substrate signals that can occur when using glass. Timing of analysis is particularly important when dealing with frozen tissue since components start to degrade upon removal from the freezer. Furthermore, since most tissue studies will be carried out in collaboration with medical researchers, it is important that a clear sample preparation method is agreed upon including details such as what embedding materials and substrates should be used, and how and when samples should be prepared for analysis.

Initial studies on tissue sample preparation, ToF-SIMS imaging parameters, and data analysis were performed using

mouse mammary and diaphragm muscle tissue. One of the first considerations to make when preparing tissue samples is how to process the sample. FFPE is a common preparation method used for preparing histological samples (i.e., for H&E stained tissue images). This type of processing can be used to prepare the tissue samples for ToF-SIMS analysis. Initial studies using FFPE mouse mammary tissue with imaging ToF-SIMS show that the underlying structure of the tissue can be seen but is covered with a layer of paraffin (Fig. 1). The layer of paraffin, shown as red in Fig. 1(a), is seen across the entire image. To investigate if the paraffin material could be removed, a short sputter with C_{60} cluster ions was carried out and found to be sufficient to remove most of the paraffin overlayer. Figure 1(b) shows a three color overlay ToF-SIMS $500\ \mu\text{m} \times 500\ \mu\text{m}$ image of the same spot as Fig. 1(a) after a 10 s sputtering of C_{60} over a $400\ \mu\text{m} \times 400\ \mu\text{m}$ region. Before the sputter, two peaks are seen at the nominal mass 86 for a paraffin related peak at $m/z\ 86.14^+$ and an amino acid related peak at $m/z\ 86.07^+$ [Fig. 1(c)]. While the peaks can be readily separated using the high mass resolution mode of imaging, as can be seen in Fig. 1(a), there are no regions that are only blue (for the amino acid peak) and only purple areas (mixture of blue and red) can be seen indicating that paraffin covers the amino acid regions. However, after the short sputter cycle, blue areas in the tissue image (amino acid peak areas) can be clearly seen, indicating that the paraffin was mostly removed by the sputter cycle. As seen in the spectra reconstructed from the sputter region [see Fig. 1(c)] the paraffin peak is reduced significantly more than the amino acid peak in the sputtered region, indicating the paraffin was primarily on the surface. In addition, the sputter region qualitatively shows a sharper image from the underlying tissue areas. Even though the sputtering removed much of the paraffin, it would be best to avoid sputtering the sample since any type of sputtering will induce sample damage. Additionally, it may be difficult to remove all of the paraffin residue from the tissue, which would add random variation between samples.

Another issue noted with FFPE was that areas of the underlying silicon substrate could be seen [shown in green for both Figs. 1(a) and 1(b)] indicating that large areas of the tissue were missing (i.e., not present or displaced). Formalin

fixation is a well known lipid extraction method³⁸ and it is likely that these missing areas were lipid containing regions. It is known that one of the strengths of ToF-SIMS is its ability to detect a wide range of lipids from biological samples and lipid peaks are not typically seen in the FFPE samples. Therefore, FFPE tissue is not optimal for ToF-SIMS imaging analysis of lipids. However, since many lower mass peaks, such as those related to amino acids are still present, FFPE samples could be useful for SIMS analysis of tissues to reduce interference of lipid-related peaks in the lower mass region and focus on differences in amino acid content or other low mass peaks.

OCT is another embedding material that is used when sectioning frozen tissues, though it is not as common as FFPE in clinical settings. Tissues embedded in OCT are cut using a cryomicrotome and mounted on Si wafers for ToF-SIMS analysis at room temperature. Positive ion spectra from FFPE mouse mammary tissue as well as cryopreserved mouse mammary tissue embedded in OCT are shown in Fig. 2 for the $m/z\ 540\text{--}590$ region, a region that potentially includes peaks from diacylglycerol species (for example, DAG 32:2, 32:1, and 34:1).^{39,40} Control spectra for paraffin as well as OCT are included as well for comparison. Each spectrum has been scaled so that all peaks can easily be seen.

As can be seen in Fig. 2, the FFPE tissue and the paraffin control ToF-SIMS spectra have many peaks in the $m/z\ 540\text{--}590$ region and show similar fragmentation patterns. This is due to the fact that a thin layer of paraffin is likely covering the tissue and the paraffin process extracts lipids,³⁸ as mentioned above. After FFPE embedding the high mass region of the FFPE embedded tissue spectrum is almost identical to a paraffin spectrum. OCT is commonly made from polyglycols and polyvinyl-alcohol^{41,42} as well as other additives, so it is not surprising that the OCT control also has peaks in the higher mass region. These higher mass peaks from OCT are likely polyethylene glycol (PEG) oligomers. Some of the OCT peaks are very close to those for DAG peaks seen in some tissues (Fig. 2), but they typically do not overlap entirely. One method to limit interference of the OCT-related peaks in the data analysis is to use the most intense peak for a given batch of OCT to identify

FIG. 1. ToF-SIMS of FFPE embedded mouse mammary tissue. (a) Three color overlay image ($500\ \mu\text{m} \times 500\ \mu\text{m}$) of “as cut” sample with red: $C_6H_{14}^+$ ($m/z\ 86.14$) (paraffin); blue: $C_5H_{10}O^+$ ($m/z\ 86.07$) (tissue); and green: Si^+ . (b) Image A after a 10 s C_{60} sputter. (c) ToF-SIMS spectra showing the $m/z\ 86$ region before (red) and after (blue) sputtering. Spectra reconstruction was carried out from the same $325\ \mu\text{m} \times 325\ \mu\text{m}$ region centered where the sputtering occurred for both pre- and postsputtering.

FIG. 2. ToF-SIMS positive ion spectra in the m/z 540–590 region for paraffin control and FFPE mouse mammary tissue (presputter) as well as OCT control and mouse tissue in OCT. Spectra have been scaled so that all peaks are easily visible.

the presence of OCT in the tissue images. Areas with high signal for OCT are then excluded from the image. This can be done by thresholding the image for the chosen peak and then using the thresholded image as a ROI to reconstruct the non-OCT regions. This results in a data set that minimizes the influence of the OCT. Potential candidate peaks for selecting areas to remove OCT-related signal can be determined from analysis of control OCT. Common key OCT peaks include m/z 304.3⁺, 332.2⁺, 360.3⁺, and 388.4⁺, which represent the benzalkonium additive molecules with C₁₂H₂₅⁺, C₁₄H₂₉⁺, C₁₆H₃₃⁺, and C₁₈H₃₇⁺ side chains, respectively.

In addition to using regions of interest to exclude areas that may contain OCT, we have also initiated work on creating additive-free OCT materials that can be used for embedding. One of the common additives to OCT are benzalkonium salts.⁴¹ These compounds are known to suppress ionization in MALDI,⁴³ but it is unclear if they have any effect on ToF-SIMS spectra. However, these additives produce many peaks that could overlap with signals of interest. Initial work using polyvinyl alcohol and PEG or polypropylene glycol have shown that additive free OCT like materials can be produced and further development is underway to produce optimal compositions that can be used for cryosectioning.

As with any sample preparation method, care should be taken to assure that the embedding medium and cutting method are not introducing artifacts into the data. This includes making sure that the embedding medium used for tissue preparation is not contaminating the sample, such as the thin layer of paraffin that covers FFPE samples presputter, and that it enables creation of consistent samples. The OCT embedding medium was not found on the surface of cryosectioned tissues in this investigation and was the preferred method chosen for the breast cancer biopsy sample comparison discussed in Sec. III C. Regardless of the method used, consistency in sample preparation is important to minimize variation between samples if a sample-to-sample comparison is a research goal.

B. Mice: Image data acquisition and analysis

Once samples have been prepared, one must consider how to collect the necessary data from each sample in a way that is timely, consistent, and adequate to answer the question at hand. For example, most ToF-SIMS systems operate using a pulsed primary ion beam. This makes it difficult to acquire images with both high mass and spatial resolution at the same time within a reasonable time. However, one would like to have both high mass and high spatial resolution data for each sampled area. In order to circumvent this issue, we used a protocol of collecting high mass resolution data in both polarities and then HSR in both polarities from the same analysis region. For this, the HMR data are acquired at 5.0×10^{11} ions/cm² for each polarity to avoid going over the static limit. HSR data are collected last since this data are used mainly for alignment with other imaging methods and preservation of all chemical information is not necessarily critical for this use.

Another consideration is selecting the area to be analyzed and whether to analyze several small areas or scan the entire sample. This should be considered carefully since scanning large areas can be time intensive, which can lead to sample degradation during analysis or lipid migration.¹⁵ It is also important when working with clinically relevant samples to consult with personnel with expertise in identifying tissue features since tissue often looks fairly uniform under the low magnification microscopes often found in ToF-SIMS instruments. Finally, it is useful if one has an optical image such as an H&E stained section from an adjacent tissue slice that can be used for comparison during data collection. As shown by some researchers, it is also possible to acquire ToF-SIMS images of lipids from the same or serial section where an H&E stained image is acquired.^{21,44}

The typical image field of view for ToF-SIMS is in the submillimeter range. However, tissue sample sizes are often many millimeters and even centimeters in size. As discussed previously, it can be challenging to identify where on the tissue to acquire data with ToF-SIMS if specific regions of interest are needed to answer a biological question. By stitching a series of images together a larger image can be acquired that can be more easily compared to a histology sample as shown in Fig. 3. Other ToF-SIMS studies have also used this approach of using large area scans for studying other tissues.^{15,45–47} A comparison of the ToF-SIMS stitched image to an H&E stained serial tissue slice (nearby slice from same tissue block) shows that some histologic features can be correlated between the two images. As shown in Fig. 3, some features in the ToF-SIMS image can be distinguished as tissue structures even without the H&E image for comparison. Figure 3(a) shows that the m/z 184⁺ peak (red) matches well with the stromal features in the H&E image. The m/z 184⁺ peak is attributed to the phosphocholine head group and has been noted to be a strong ToF-SIMS peak in many types of biological specimens. Additionally, other chemically distinct regions can be identified in the ToF-SIMS image. The green color, which is a summation of

FIG. 3. Lactating mouse breast tissue images. (a) Positive ion ToF-SIMS stitched image showing three color overlay of red: Phosphocholine, m/z 184⁺; green: DAGs 22:0, 24:0, 26:0, 28:0 at m/z 439⁺, 465⁺, 495⁺, and 523⁺; blue: DAGs 34:0 and 36:0 at m/z 579⁺ and 607⁺. Image is an excerpt from the original image, which is 3.2 mm $\times$ 4.0 mm and contains 80 (8 $\times$ 10) 400 μ m $\times$ 400 μ m tiles. Image contrast was enhanced for clearer presentation of colors for publication. (b) H&E of serial slice from the same mouse tissue block.

signals from relatively ‘shorter’ chain length diacylglycerol (DAGs), is found in regions that correspond to milk ducts in the H&E image. The blue color is a summation of signals from relatively longer chain DAGs and is found in areas containing adipocytes in the H&E image from the serial section. By using the larger stitched image, regions of interest can be identified and then analyzed in further detail either as a HSR image, a summed depth profile (sum of all depth profile slices to enhance contrast), or perhaps just imaged with higher pixel resolution to enhance feature sharpness.

When specific ToF-SIMS peaks do not map directly to specific biological features in the image, imaging PCA can be used as an aid. A study of a muscular dystrophy (*mdx*) mouse diaphragm muscle was carried out by comparing PCA score images with immunofluorescence-labeled confocal sections (Fig. 4). The imaging PCA highlighted regions from the ToF-SIMS data that appear to correspond with fibrotic regions in the muscle tissue (negative imaging PCA scores, light blue) and the cellular nuclei and a fat globule (positive PCA scores, yellow). The corresponding loadings

are shown in Fig. 4(c). Briefly, the positive ion loadings show phosphate peaks (from DNA and phospholipids) and other DNA related peaks that have a high density in the cellular nuclei. Some fatty acid peaks are also seen in the positive loadings. The negative ion loadings show low mass peaks (m/z 16[−], 26[−], 42[−]) that could be from proteins in the fibrotic area (such as fibronectin and collagen). Since some peaks may have contributions from various components, such as phosphate peaks which could be derived from phospholipids or DNA, imaging PCA is helpful in distinguishing features by identifying all peaks that show similar trends. By looking at the combination of peaks that correspond with a given feature, one can better determine the source of the sample differences.

By carefully planning the data collection parameters and including information from other imaging methods, more information can be extracted from a ToF-SIMS data set. By using the ability of most ToF-SIMS instruments to stitch together many smaller images into a larger raster area, one can capture the areas of interest across large tissue sections. Acquiring both high mass and high spatial resolution data

FIG. 4. Muscular dystrophy (*mdx*) mouse diaphragm muscle tissue images. (a) PCA scores image from ToF-SIMS data (200 μ m $\times$ 200 μ m) showing: Positive scores (yellow) and negative scores (cyan) from negative ion data of mouse muscle. (b) Confocal microscopy image (20 $\times$ magnification, 250 μ m $\times$ 250 μ m) of mouse muscle tissue (different area) showing: red: fibrotic regions—fibronectin antibody; blue: cell nuclei—DAPI (general nuclei stain); green macrophages—CD68 antibody (macrophage/inflammatory cells). (c) Loadings plot corresponding with (a).

enables maintaining chemical specificity between sample areas while having sufficient spatial resolution to compare ToF-SIMS images with optical imaging methods that can help identify regions of interest within a sample.

C. Women: Preliminary study for application of developed procedures and methods

The sample preparation and data analysis methods developed on model mouse tissues discussed in Secs. III A and III B were critical in implementing a plan for the analysis of human breast cancer tissue biopsies. Cancer is a heterogeneous disease that manifests itself in a variety of morphological types and clinical outcomes. It is of interest to assess regional changes in tumor metabolism as a function of tumor microenvironment. However, there are few techniques that can specifically interrogate the tumor microenvironment. In preparation for a larger clinical trial study investigating the chemistry of treatment-resistant breast cancers the sample preparation, data acquisition, and data processing methods developed using the mouse model studies were applied initially to OCT embedded, frozen breast cancer biopsy samples from two different patients, labeled below as tissue block 1 and tissue block 2. The receptor status for the two tissue blocks was estrogen-receptor positive (ER+) for tissue 1 and estrogen-receptor negative (ER-) for tissue 2. The second section of the tissue block 1 sample was analyzed two months later to test for sample-to-sample reproducibility of the analysis method. All tissue sections were cut immediately before ToF-SIMS analysis and all tissue blocks were previously stored frozen at -80°C . Analysis patches of $1\text{ mm} \times 1\text{ mm}$ were acquired on each sample using the stitched image method described previously. At least two areas were acquired for each tissue section. This means that at least 50 spectra were generated for each tissue section by extracting the spectra from individual raster tiles within the analysis patches as described in Sec. II. Spectral data reconstructed from those regions was then analyzed with PCA using the NBToolbox.

PCA was used to determine the peaks that were the largest sources of variance within the data and to determine if the duplicate samples from tissue 1 were spectrally similar. For PCA, all peaks two times the background signal within the mass range m/z 0–920 were selected, excluding known salt, salt adduct, substrate, embedding material, and inorganic peaks. The resulting PCA scores plot of PC1 vs PC2 is shown in Fig. 5 along with the associated loadings for PC1. Tissue blocks 1 and 2 separated across PC1 and the two sections from tissue block 1 overlap in the Scores plot demonstrating reproducibility in the analysis. Some of the peaks with significant loadings on PC1 include m/z 184⁺, a phospholipid-related peak, vitamin E (m/z 430⁺), and cholesterol (m/z 369⁺).

While it is promising that these two breast cancer subtypes separate in a PCA analysis, it is not conclusive that the separation is directly related to the ER status rather than interindividual variation. Further studies of a larger set of

FIG. 5. ToF-SIMS spectral PCA analysis of human breast tumors from two different patients. (a) Scores plot showing separation by patient and overlap of patient 1 samples. (b) Loadings plot for PC1.

human breast tumor biopsies are required to test the utility of ToF-SIMS to investigate whether different chemistries correlate with different tumor subtypes.

After analyzing a sample using large area rasters, one can also choose smaller areas to investigate in more detail. A closer view of the tumor tissues shows that there are many different potential types of chemical “regions” such as highly cellularized epithelial regions, stromal regions, fatty deposit regions, etc. Examples of these different regions can be seen in Fig. 6 showing a ToF-SIMS summed depth profile of a breast tumor sample taken at high spatial resolution mode as well as the H&E image of a serial section in a similar region. The CNO^- summed signal is most intense in the stromal areas [shown red in the ToF-SIMS two color overlay and also red (eosinophilic) in the H&E image Figs. 6(a) and 6(b)]. The cellular epithelial nuclei, identified by the intense PO_2^- and PO_3^- signals [shown in green in Fig. 6(a)] and basophilia (blue) in the H&E image [Fig. 6(b)], are more dense and larger in size in the highly cellularized regions and less dense and smaller in size in the stromal regions. The areas shown in black in the SIMS images and clear (white) in the H&E image are the fatty regions, analogous to those shown in the mouse mammary tissue image (Fig. 3), but the representative fatty acid peaks are not shown in the two color overlay image to allow an easier comparison with the H&E

FIG. 6. Human breast tissue with tumor. (a) ToF-SIMS high spatial resolution summed depth profile two color overlay image: red represents CNO^- , green represents $\text{PO}_2^- + \text{PO}_3^-$ ($400\ \mu\text{m} \times 400\ \mu\text{m}$ image with $700\ \mu\text{m} \times 700\ \mu\text{m}$ C_{60} sputter crater). (b) H&E image of serial tissue section from similar region (not to scale).

image. The ability to see individual nuclei and in some cases cytoplasm and other features within a tissue enable alignment of ToF-SIMS images with optical images and potentially other imaging methods. This has been demonstrated by other groups using MALDI and ToF-SIMS images.^{45,48}

The high spatial resolution of ToF-SIMS enables the ability to extract spectra from very specific regions within a tissue and then compare that data using PCA or other analytical methods. Spectra that are reconstructed from those regions can be used for comparison between tissue types or between the regions themselves. Isolation of specific regions

within tissues can enable more chemically meaningful separation between different tissue samples in a spectral PCA such as that shown in Fig. 6 since one can compare specific regions directly and eliminate variation in the tissue that is not due to the area of interest.

IV. SUMMARY AND CONCLUSIONS

There are many factors to consider when analyzing tissue samples. These include how to prepare the sample, how to collect the data, and how to analyze the data collected. Each of these can affect the outcome of a study, particularly when multiple tissue samples are being compared. Biological samples are complex and ToF-SIMS is sensitive to many types of sample variation (composition, contamination, topography, etc.). It is important that all collaborators develop and utilize a consistent sample preparation protocol in order to ensure consistent sample handling, including factors such as which embedding material and substrate are to be used as well as the timing of cutting and analysis. Due to the complexity of ToF-SIMS data it is also important to learn as much as one can from collaborators (biologists, physicians, biochemists, etc.) and to communicate with them often particularly when deciding what areas to analyze and focus on within a given tissue section. The ToF-SIMS large area stitched images are helpful when comparing to H&E tissue image areas and PCA can also be utilized to identify specific tissue regions for comparison that might not otherwise be easily identified in the original ToF-SIMS images. The most important lesson learned from the comparison of ToF-SIMS analysis of two human breast tissue samples is that consistency is critical. Once developed, a methodology that produces consistent results should be followed carefully. For the analysis of breast cancer tissue biopsies in this study, the combination of OCT embedding, using a silicon wafer substrate, consistent timing between cutting and analysis, and using stitched image data collection resulted in minimal variation from sample-to-sample of the same tissue type.

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¹M. J. Baker, L. Zheng, N. Winograd, N. P. Lockyer, and J. C. Vickerman, *Langmuir* **24**, 11803 (2008).

²C. Heim, P. Sjovall, J. Lausmaa, T. Leefmann, and V. Thiel, *Rapid Commun. Mass Spectrom.* **23**, 2741 (2009).

³E. Keating, A. J. Waring, F. J. Walther, F. Possmayer, R. A. W. Veldhuizen, and N. O. Petersen, *Biochim. Biophys. Acta, Biomembr.* **1808**, 614 (2011).

- ⁴S. Keskin, A. Piwowar, J. Hue, K. Shen, and N. Winograd, *Surf. Interface Anal.* **45**, 244 (2013).
- ⁵J. S. Fletcher, N. P. Lockyer, S. Vaidyanathan, and J. C. Vickerman, *Anal. Chem.* **79**, 2199 (2007).
- ⁶I. Lanekoff, M. E. Kurczy, K. L. Adams, J. Malm, R. Karlsson, P. Sjoval, and A. G. Ewing, *Surf. Interface Anal.* **43**, 257 (2011).
- ⁷I. Lanekoff, N. T. N. Phan, C. T. Van Bell, N. Winograd, P. Sjoval, and A. G. Ewing, *Surf. Interface Anal.* **45**, 211 (2013).
- ⁸M. K. Passarelli, A. G. Ewing, and N. Winograd, *Anal. Chem.* **85**, 2231 (2013).
- ⁹M. K. Passarelli, A. G. Ewing, and N. Winograd, *Surf. Interface Anal.* **45**, 298 (2013).
- ¹⁰A. M. Piwowar, S. Keskin, M. O. Delgado, K. Shen, J. J. Hue, I. Lanekoff, A. G. Ewing, and N. Winograd, *Surf. Interface Anal.* **45**, 302 (2013).
- ¹¹M. A. Robinson, D. J. Graham, and D. G. Castner, *Anal. Chem.* **84**, 4880 (2012).
- ¹²J. S. Fletcher, N. P. Lockyer, and J. C. Vickerman, *Mass Spectrom. Rev.* **30**, 142 (2011).
- ¹³M. K. Passarelli and N. Winograd, *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **1811**, 976 (2011).
- ¹⁴M. K. Passarelli and N. Winograd, *Surf. Interface Anal.* **43**, 269 (2011).
- ¹⁵P. Sjoval, B. Johansson, and J. Lausmaa, *Appl. Surf. Sci.* **252**, 6966 (2006).
- ¹⁶D. Touboul, O. Laprevote, and A. Brunelle, *Curr. Opin. Chem. Biol.* **15**, 725 (2011).
- ¹⁷M. J. Baker, E. Gazi, M. D. Brown, N. W. Clarke, J. C. Vickerman, and N. P. Lockyer, *Appl. Surf. Sci.* **255**, 1084 (2008).
- ¹⁸E. Armitage, H. L. Kotze, J. S. Fletcher, A. Henderson, K. J. Williams, N. P. Lockyer, and J. C. Vickerman, *Surf. Interface Anal.* **45**, 282 (2013).
- ¹⁹K. S. Kulp, E. S. F. Berman, M. G. Knize, D. L. Shattuck, E. J. Nelson, L. Wu, J. L. Montgomery, J. S. Felton, and K. J. Wu, *Anal. Chem.* **78**, 3651 (2006).
- ²⁰J. W. Park, H. K. Shon, B. C. Yoo, I. H. Kim, D. W. Moon, and T. G. Lee, *Appl. Surf. Sci.* **255**, 1119 (2008).
- ²¹L. G. Wu, X. H. Lu, K. S. Kulp, M. G. Knize, E. S. F. Berman, E. J. Nelson, J. S. Felton, and K. J. J. Wu, *Int. J. Mass Spectrom.* **260**, 137 (2007).
- ²²N. Tahallah, A. Brunelle, S. De La Porte, and O. Laprevote, *J. Lipid Res.* **49**, 438 (2008).
- ²³D. Touboul, A. Brunelle, F. Halgand, S. De La Porte, and O. Laprevote, *J. Lipid Res.* **46**, 1388 (2005).
- ²⁴A. M. Judd, D. J. Scurr, J. R. Heylings, K. W. Wan, and G. P. Moss, *Pharm. Res.* **30**, 1896 (2013).
- ²⁵M. A. Robinson and D. G. Castner, *Biointerphases* **8**, 15 (2013).
- ²⁶M. E. Kurczy, P. D. Piehowski, S. A. Parry, M. Jiang, G. Chen, A. G. Ewing, and N. Winograd, *Appl. Surf. Sci.* **255**, 1298 (2008).
- ²⁷E. S. F. Berman, S. L. Fortson, K. D. Checchi, L. Wu, J. S. Felton, K. J. J. Wu, and K. S. Kulp, *J. Am. Soc. Mass Spectrom.* **19**, 1230 (2008).
- ²⁸J. Malm, D. Giannaras, M. O. Riehle, N. Gadegaard, and P. Sjoval, *Anal. Chem.* **81**, 7197 (2009).
- ²⁹E. A. Jones, N. P. Lockyer, and J. C. Vickerman, *Anal. Chem.* **80**, 2125 (2008).
- ³⁰M. R. Keenan and P. G. Kotula, *Surf. Interface Anal.* **36**, 203 (2004).
- ³¹S. Wold, K. Esbensen, and P. Geladi, *Chemom. Intell. Lab. Syst.* **2**, 37 (1987).
- ³²J. E. Jackson, *J. Qual. Technol.* **12**, 201 (1980).
- ³³D. J. Graham, M. S. Wagner, and D. G. Castner, *Appl. Surf. Sci.* **252**, 6860 (2006).
- ³⁴M. S. Wagner, D. J. Graham, B. D. Ratner, and D. G. Castner, *Surf. Sci.* **570**, 78 (2004).
- ³⁵D. J. Graham and D. G. Castner, *Biointerphases* **7**, 49 (2012).
- ³⁶P. Geladi, H. Isaksson, L. Lindqvist, S. Wold, and K. Esbensen, *Chemom. Intell. Lab. Syst.* **5**, 209 (1989).
- ³⁷B. J. Tyler, G. Rayal, and D. G. Castner, *Biomaterials* **28**, 2412 (2007).
- ³⁸M. Elleder and Z. Lojda, *Histochemie* **34**, 143 (1973).
- ³⁹D. Debois, M. P. Bralet, F. Le Naour, A. Brunelle, and O. Laprevote, *Anal. Chem.* **81**, 2823 (2009).
- ⁴⁰P. Sjoval, B. Johansson, D. Belazi, P. Stenvinkel, B. Lindholm, J. Lausmaa, and M. Schalling, *Appl. Surf. Sci.* **255**, 1177 (2008).
- ⁴¹K. A. Z. Berry, B. L. Li, S. D. Reynolds, R. M. Barkley, M. A. Gijon, J. A. Hankin, P. M. Henson, and R. C. Murphy, *J. Lipid Res.* **52**, 1551 (2011).
- ⁴²C. Cocco, G. V. Melis, and G. L. Ferri, *Appl. Immunohistochem. Mol. Morphol.* **11**, 274 (2003).
- ⁴³S. A. Schwartz, M. L. Reyzer, and R. M. Caprioli, *J. Mass Spectrom.* **38**, 699 (2003).
- ⁴⁴C. Bich, S. Vianello, V. Guerinneau, D. Touboul, S. De La Porte, and A. Brunelle, *Surf. Interface Anal.* **45**, 260 (2013).
- ⁴⁵F. Benabdellah, A. Seyer, L. Quinton, D. Touboul, A. Brunelle, and O. Laprevote, *Anal. Bioanal. Chem.* **396**, 151 (2010).
- ⁴⁶H. Y. Nie, A. R. Taylor, J. T. Francis, M. J. Walzak, W. M. Lau, and D. F. MacFabe, *Surf. Interface Anal.* **43**, 358 (2011).
- ⁴⁷P. Sjoval, J. Lausmaa, and B. Johansson, *Anal. Chem.* **76**, 4271 (2004).
- ⁴⁸G. B. Eijkel, B. Kükrer Kaletas, I. M. van der Wiel, J. M. Kros, T. M. Luijck, and R. M. A. Heeren, *Surf. Interface Anal.* **41**, 675 (2009).