A Novel Histology-directed Strategy for MALDI-MS Tissue Profiling That Improves Throughput and Cellular Specificity in Human Breast Cancer*

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We describe a novel tissue profiling strategy that improves the cellular specificity and analysis throughput of protein profiles obtained by direct MALDI analysis. The new approach integrates the cellular specificity of histology, the accuracy and reproducibility of robotic liquid dispensing, and the speed and objectivity of automated spectra acquisition. Traditional methodologies for preparing and analyzing tissue samples rely heavily on manual procedures, which for various reasons discussed, restrict cellular specificity and sample throughput. Here, a robotic spotter deposits micron-sized droplets of matrix precisely onto foci of normal mammary epithelium, ductal carcinoma in situ, invasive mammary cancer, and peritumoral stroma selected by a pathologist from high resolution histological images of sectioned human breast cancer samples. The location of each matrix spot was then determined and uploaded into the instrument to facilitate automated profile acquisition by MALDI-TOF. In the example shown, the different lesions were clearly differentiated using mass profiling. Further, the workflow permits a visual projection of any information produced from the profile analyses directly on the histological image for a unique combination of proteomic and histological assessment of sample regions. The higher performance characteristics offered by the new workflow promises to be a significant advancement toward the next generation of tissue profiling studies. *Molecular & Cellular Proteomics* 5:1975–1983, 2006.

The inherent heterogeneity of whole tissue sections presents a unique challenge for the biomarker discovery platform. This is especially true when the goal includes acquisition of cell-type-specific protein profiles by MALDI mass spectrometry and their subsequent analysis to identify disease-specific biomarkers (1–6). Many factors both biological and experimental can affect the measured abundance of protein ions, and these may in turn correlate to aspects of the sample unrelated to the disease itself. Distinguishing protein ions as disease-specific requires isolating those abundance changes symptomatic of disease from those changes influenced by experimental conditions. From this perspective, various studies have explored techniques for pre-processing spectra as a means of minimizing overall variance (7–11) prior to statistical analysis, but these procedures vary and do not completely eliminate inconsistent classification results (7, 11–15). Nevertheless, the existing body of work is strong evidence that biomarker discovery by tissue profiling is possible and may one day benefit the clinical diagnosis and treatment of disease.

If tissue profiling is ever to achieve this promised potential it will require substantial improvements not only to back-end data processing but also to front-end sample handling and data collection as a means of promoting higher profile specificity and data throughput. At present, the methodologies employed for collecting and analyzing tissue profiles rely heavily on manual procedures that impose undue limitations on quality and quantity of profile spectra produced. Technologies are available to address many of the needed improvements, but they are not integrated into a single coherent analysis workflow.

The quantity and identity of ions observed in the tissue profiles are directly influenced by factors such as the volume of matrix solution deposited and the locations at which it is applied. Traditional protocols for tissue profiling employ pipettes to deposit submicroliter volumes of MALDI matrix solution onto thin sections (16, 17). As carrier solvent evaporates proteins are extracted from the tissue and incorporated into growing matrix crystals. In previous studies pipetting volumes as small as 100 nL produced matrix spots on the order of 1-mm diameter (18), quite large on a cellular scale. Any cell covered by the matrix spot can, in principle, be a source of protein ions observed in the profile spectrum and contribute to a greater variability within the collection of profile spectra. It follows that specificity of the profile spectra can be im-

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proved by reducing the diameter of the matrix spot and de-
positing it very accurately onto the cells of interest. Signifi-
cantly smaller matrix spots are produced by dispensing 100
pl–10 nl of matrix solution from finely pulled capillaries (19).
However, delivering them in large numbers with the degree of
accuracy and reproducibility necessary for profiling heteroge-
neous tissue sections requires considerable skill and patience
because all work must be carried out using microscopic
visualization.

Laser capture microdissection (LCM)1 is an alternative
technique that provides very high profile specificity by ex-
tracting the cells of interest from the sample section. Data
variability is therefore not as sensitive to the size of the matrix
spot because potentially interfering cells have been removed
and consequently cannot contribute ions to the measured
profile (19, 20). A secondary benefit of LCM is that cells of
interest, even those widely dispersed throughout a large re-

gion of the tissue section, can be collected within a relatively
small area of the polymeric cap and covered by a single
droplet of matrix. For these reasons LCM has become the de
facto benchmark for high-specificity profiling, but there are
considerations for its use. Exact protocols differ according to
LCM apparatus, but in general, sections must be rigorously
dehydrated using ethanol and/or xylene for as long as 10 min
prior to cell collection. We have generally observed that these
treatments can adversely affect the number and identity of
protein ions observed in the profile spectra compared with
profiles acquired directly from an intact section. Additionally,
the non-conductive nature of the polymeric caps used by
some LCM systems can affect mass spectrometer perform-
ance because of localized surface charging.

A number of groups, including our own, are exploring ro-
botic devices as tools for increasing the reproducibility of
submicroliter volumes of matrix solution deposited onto tis-

eue sections (21–25). Micropots of matrix with diameters on
the order of 200–300 μm can be generated with greater speed and
precision than manual methods, allowing arrays of
hundreds of matrix micropots to be deposited in a matter of
minutes. In many cases, however, samples contain only a few
discrete clusters of the cells of interest, and these are often
not readily visible without proper magnification and, in some
cases, histological staining. Rather than blanketing the sec-
tion with hundreds of matrix spots in hopes of coating a few
cells of interest by random chance, it is more advantageous
from the perspective of both profile specificity and analysis
time to deposit fewer spots that are targeted precisely onto
the cells of interest. The strategy we present here accom-
plishes this by integrating histopathology with tissue profiling
via digital imagery. In addition, the nature of the workflow
allows one to interactively visualize results from the profiling
analyses directly on the histology image for better exploration
of possible links between molecular signatures and pathol-
ogy. We demonstrate this strategy with initial results from
primary human breast cancer specimens that contain invasive
mammary cancer (IMC), ductal carcinoma in situ (DCIS), be-
nign epithelial elements, stroma, microvessels, or inflamma-
tory cells, often within the same region of tissue. The unique
combination of LCM-like specificity and high throughput of
the histology directed profiling methodology is necessary to
produce accurate profiles of each distinct element within such
heterogeneous samples.

**EXPERIMENTAL PROCEDURES**

Sample Processing—Human breast tumor specimens from four
patients (females, 40–60 years) and three tumor samples from a
transgenic mouse model that spontaneously develops metastatic
mammary tumors (MMTV PyVmT) were flash frozen in liquid nitrogen
immediately after sampling and stored at −80 °C. For standard
MALDI profile analysis, two serial 12-μm sections were cut from each
sample. One section was thaw-mounted directly onto a gold MALDI
target plate followed by fixation in graded ethanol (70, 90, and 99%;
30 s each) baths. The adjacent section was mounted onto histology
slides and stained with hematoxylin and eosin (H&E) following stand-
ard protocols. Sections were prepared for the PixCell II LCM system
(Arcturus, Mountain View, CA) following a published protocol of
graded ethanol and xylene dehydration followed by cell collection
onto polymeric caps (19). After collecting the cells, the polymeric films
were removed and adhered to standard MALDI plates using conduc-
tive double-sided tape. MALDI and LCM sample plates were stored in a
dry oxygen-free atmosphere until the analysis.

Photomicrographs and Graphical Processing—Photomicrographs
of stained tissue sections were acquired with an Olympus BX-50
microscope at a magnification of ×100. MALDI plates with mounted
tissue sections were imaged before and after application of matrix
using a 4800-dpi scanner. To facilitate this study the microscope was
upgraded with a Biopoint II motorized x-y stage (Ludl, Hawthorne,
NY) and 3.3-megapixel digital camera (QImaging, Burnaby, British
Columbia, Canada) to generate multi-frame mosaic images for high
magnification detail of whole tissue sections. Computer control of the
stage movement and image capture is accomplished using ImagePro
and the plug-in ScopePro module (Media Cybernetics, Silver Spring,
MD). The Biopoint stage can also be controlled manually using a
joystick with the software providing real-time positional feedback.
Digital images were processed using combinations of Adobe Photo-
shop, functions native to ImagePro, and custom Matlab scripts.

Robotic Matrix Spotting and MALDI-MS Acquisition—The robotic
matrix spotter used in this study was a prototype device (LabCyte,
Sunnyvale, CA) that uses a focused acoustic ejector to produce
matrix droplets of ~120 pL. A detailed report of the operational
characteristics has been presented elsewhere (21). Briefly, a precision
2-axis stage translates the sample plate over a droplet ejector for
accurate deposition of matrix spots. A digital video system integrated
into the device provides a magnification range of ×0.5–4 for viewing
the mounted sample sections. The optical center of the video system
is aligned with the droplet ejector and calibrated to a joystick for
manual control of the stage. For all samples, matrix was deposited in
cycles of 13 drops dispensed at 10 Hz at each designated coordinate.
A total of 6 cycles were found to provide optimal matrix crystals.
Sinapinic acid matrix solution was prepared to a concentration of 25
mg/ml in 1:1 acetonitrile:0.2% TFA. The diameters of crystalline ma-
trix spots were typically 200–250 μm. Profile spectra were acquired

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1 The abbreviations used are: LCM, laser capture microdissection;
IMC, invasive mammary carcinoma; DCIS, ductal carcinoma in situ;
H&E, hematoxylin and eosin; NTE, non-transformed epithelium; MDS,
multidimensional scaling
using an Ultraflex II (Bruker Daltonics, Billerica, MA) MALDI-TOF mass spectrometer equipped with the SmartBeam laser and run using standard an automated linear-mode acquisition method optimized for 2–70 kDa. The laser beam was focused to a diameter of 125 \( \mu m \) allowing the acquisition of data from multiple locations within each matrix spot. A total of 750 laser shots were collected for each profile spectrum in increments of 75 shots beginning at the center of each spot and spiraling outward as crystals were depleted. Laser energy was optimized at the beginning of the analysis for a few randomly selected spots, and no further adjustments were made during the course of data collection. The registering of matrix spots to the instrument for automatic acquisition is explained further in the results section.

**Data Processing and Statistical Analysis—**Whole spectrum analysis was performed using a methodology described previously (26). All mass spectra were converted to ASCII text and imported into ProTS-Data (Efeckta Technologies, Steamboat Springs, CO) for baseline correction, normalization by total ion current and realignment/recalibration of individual spectra. For the supervised class validation analysis, a standard weighted means averaging algorithm was applied. In this manner, \( m/z \) values were filtered according to the highest weight which best differentiated adjacent non-transformed epithelium (NTE) versus cancer groups. Further filtering was carried out to exclude values with a weighted means averaging of less than 1.0 (similar in respect to 2\( \sigma \) from the mean control value) and to exclude mean intensity differences that fell below 2-fold (experimentally derived cut-off valued often applied for tissue profiling, data not shown). The filtered values were then used for peak detection and further evaluated by plotting the whole spectra as compared with the difference spectra in Origin v7.0 (OriginLab Corp, Northampton, MA). Intravariable error and graphs depicting variance throughout the spectra were prepared and plotted with tools provided by Efeckta. Euclidian distance matrices were calculated on supervised and non-supervised data using Ward’s method in Statistica v.6.0 (Statssoft, Tulsa OK). Multidimensional scaling (in 3 dimensions) of the Euclidian distance matrices and three-dimensional plotting of the results was carried out using standard Matlab functions.

**RESULTS**

**Selection of Profile Sites from Digital Histology Images—**Digital photomicrographs acquired from histology and MALDI sections are used to identify and designate sites of interest for profiling. Because the H&E stain interferes with MALDI ionization, serial sections are needed for MALDI and histology analysis. Fig. 1, A and B show adjacent sections that were collected from one of the breast cancer samples and prepared for MALDI and histological analysis, respectively. This tumor was unique among the 4 specimens analyzed in that it exhibited foci of both DCIS and IMC in addition to other cell types of interest. Using Photoshop all histology images were annotated with a small circular shape or mark positioned at locations of interest to graphically designate them as sites on which to deposit matrix. Although the exact shape of the annotation marks is not critical, circular marks as shown in Fig. 2A were used because they can be scaled to the shape and size of the matrix spot and provide a degree of spatial perspective that helps avoid overlapping spots. From the illustration in Fig. 2B different cell types of interest may be in such close proximity that they would otherwise be indistinguishable by traditional profiling. Color coding the annotation marks by cell type provides an extra layer of meta-information that facilitates later assignment of the profile spectra to a particular histology classification. Fig. 2A shows the digital image of the example section that has been annotated with circles of four different colors to designate profile sites consisting of DCIS, IMC, and stroma cells along with adjacent NTE. In a final step, annotation marks are placed onto at least three distinctive features that are visible in the MALDI image,
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A) to be used as fiducial landmarks for registering the MALDI plate to the robotic spotter. Both annotated images are then stored separately in tiff format.

Fig. 2. A, H&E section from Fig. 1B annotated with circular marks placed at sites to be profiled and colored according to histopathology: red, peritumoral stroma; black, IMC; blue, DCIS; and green, NTE. B, illustration of the different surface areas profiled by the histology directed strategy (colored spots) and traditional profiling (100 nL of matrix deposited with mechanical pipette, shaded area). C, overlay of aligned H&E image and MALDI image. D, MALDI section after robotic deposition of matrix onto designated sites.

Microspotting Matrix onto Selected Profile Sites—The relative positions, in pixel coordinates, of the profile sites and landmarks are extracted from the annotated images and registered to the robotic spotter. First, the annotated MALDI and H&E images were aligned along internal and external contours as illustrated in Fig. 2C to register the annotation marks designating the profile sites and the annotation marks designating the landmarks. With all annotation marks referenced to a common pixel coordinate system the cell counting function in ImagePro was used to identify the respective shape and color of the marks and determine their center x-y pixel coordinate. A table of respective pixel coordinates for each mark was stored as a text file.

A transformation function was computed to convert the selected pixel coordinates into coordinates usable by the matrix spotter. The transformation function requires that the fiducial landmarks be located in both pixel and spotter coordinate systems. These were located in spotter coordinates by transferring the MALDI plate to a holder affixed to the translational stage of the robotic spotter. Using the joystick control each landmark was, in turn, positioned so that the exact feature element that was designated with the annotation mark was aligned to the droplet ejector and the stage coordinate recorded. After completing this step, coordinates for the fiducial landmarks were known for both pixel and spotter coordinate systems. An affine transformation function was generated to interconvert the two coordinate systems and was then applied to the pixel coordinates of all profile sites to translate these into coordinates for the spotter. Fig. 2D shows the example section after matrix has been applied, and Fig. 3A shows an image of the MALDI plate after applying matrix to all eight sections.

Automated Profile Acquisition—Automated profile acquisition required the generation of a custom plate geometry file. This was generated by processing a digital image of the prepared MALDI plate to automatically locate all graphical features that appear to be matrix spots. To accomplish this, a small subimage of a single matrix spot was defined from the plate image and used as a reference, which was then rastered over all pixels in the plate image. At each position a two-dimensional cross correlation was computed generating a correlation image in which the pixel intensity reflects similarity to a matrix spot (high intensity → high correlation). Additional histogram filtering of the correlation image removed spurious non-matrix pixels. An algorithm similar to the one used earlier for locating the annotation marks converted the grayscale correlation image to binary (Fig. 3B) and computed the center pixel of each feature. These x-y pixel coordinates (Fig. 3C) were then written to disk in the native target geometry format of the mass spectrometer control software. Once the target was loaded into the instrument and the custom geometry file loaded, each matrix spot became the equivalent of a distinct sample well. Three spots nearer the outermost corners of the spot distribution were selected for final plate alignment using the standard procedure in the instrument control software. Fig. 3D shows the matrix positions after loading the custom geometry file into the control software. Automated acquisition of profile spectra was then carried out using a standard acquisition method optimized for proteins.

Comparing Histology-directed Profiling with LCM Profiling—Reproducibility of histology-directed profiling was compared against profiles obtained from samples collected by LCM. Tumor specimens from the transgenic mouse model were selected for this experiment because of a relatively high degree of cellular homogeneity. Sections were prepared for LCM according to traditional protocols including xylene washes before the equivalent of 10,000 cells were collected onto the polymeric LCM cap. Matrix was then deposited onto the collected cells via manual pipette. To address concerns that the different sample treatments (i.e. ethanol washes versus xylene washes) introduce bias into the comparison, a second serial section was washed according to the LCM protocol and profiled using the histology-directed methodology. The average spectrum was calculated from each data set and shown in Fig. 4. In all, 30 and 29 histology-directed
profiles were acquired from the ethanol and xylene washed tissue, respectively. The manual LCM spotting process limited the number of distinct sample spots to 4. To evaluate each preparation technique we determined the number of features common to at least 80% of the spectra and computed the average % coefficient of variance (CV) for measured intensity. For histology-directed profiling, the data from the xylene treated sections exhibited greater variability than the data from the ethanol treated sections, CVs of 42% compared with 32%. Correspondingly, the xylene treated sections produced fewer common features compared with the ethanol treated sections, 10 versus 79. Variability within the LCM profiles was not computed because of the small sample set, but 59 common features were found. This seemingly contradicts the expectation that LCM profiles would be similar to histology-directed profiles when both are acquired from xylene washed sections. However, other studies in our lab suggest that LCM profiles may have a secondary dependence on the matrix-to-cell ratio.

A similar comparison of the two techniques was made by profiling IMC cells present in sections of human breast tissue. Profiles were collected from three tumor sections processed according to the standard protocols for each respective technique. As before, the number of discrete LCM profiles was limited to two profiles per tumor. The number of histology directed profiles was limited to 10 profiles per tumor to avoid widely disparate sample set sizes. The average spectrum was computed from each sample set and is shown in Fig. 5.
Profiles from these two data sets exhibit 80% similarity with more than 60 features common across both data sets. This was in line with observations from the mouse tumors and confirms that the specificity and reproducibility of histology-directed profiling can be similar to LCM profiling when appropriately evaluated by expert histopathology.

Accurate profiling of distinct cell types within the same tissue section is critical for obtaining characteristic molecular signatures. Profiles collected from the example section shown in Fig. 1 were analyzed by unsupervised classification followed by multidimensional scaling (MDS) of the distance matrix in three dimensions. A plot of the MDS results, Fig. 6A, shows that NTE, stroma, and DCIS/IMC profiles acquired from this section separate very well into three distinct groups. The profiles within the DCIS/IMC cluster were analyzed further to determine whether there were sufficient spectral differences to distinguish the two disease stages. Histology-directed profiles from 30 IMC and 7 DCIS spots acquired from this section were processed and analyzed using supervised classification to identify distinguishing features. MDS in three dimensions was subsequently computed from the 30 top weighted features, and the results were plotted in Fig. 6B. From the plotted position of the DCIS and IMS profiles no clear class separation was observed. However, after coloring the data points according to the histopathology of the profile sites some separation was apparent with the 7 DCIS profiles (shown in red) localizing to one region of the MDS distribution indicating that, while small, molecular differences can be observed.

The histology-directed approach offers an additional benefit in the form of the metadata that is created in the course of the workflow and that provides direct links between physical tissue locations, as represented in the histology image, to individual profile spectra. Using these links ion intensities, analysis results, or any other information produced from the profile analyses can be visually projected onto the cellular sites from which the data were obtained. This aspect is illustrated in Fig. 6C, in which the color of the annotation marks has been changed to reflect their respective position along a red-blue similarity scale derived from the supervised classification. Such an overlay of molecular profiling results with histopathology permits better visual exploration of correlations between molecular profiles and histopathology.

**DISCUSSION**

If tissue profiling is to reach its promised clinical potential, next-generation tools are needed to overcome limitations inherent to traditional experimental techniques. Manual protocols impose undue limits not only to experimental reproducibility but also to the number of profiles that can be acquired from a sample set in practical time frames. Effective integration of the robust throughput capabilities of matrix spotters with automated data acquisition increases significantly the quantity and quality of profile spectra with very little additional effort. As future developments in matrix deposition technology will undoubtedly focus on the deposition of smaller drops at faster rates, there will be a corresponding need for greater control over the placement of matrix spots. Commercial spotting devices typically incorporate a digital imaging system for viewing sample plates, and these systems are often calibrated to the drop ejector to allow the user to interactively designate sites to deposit matrix simply by clicking the mouse at the appropriate location in the image. However, although the capabilities of
such on-board imaging systems are sufficient, in terms of magnification and resolution, for visualizing gross morphological features, they fall short of providing microscope-quality images that allow one to easily discern cellular detail.

Using breast cancer samples, we have demonstrated a novel strategy for combining digital histological images with automated matrix deposition, and data acquisition. Photomicrographs are standard for visualizing the pathology of tissues, and they are generally used in traditional tissue profiling workflows. However, their use has largely been one of visual aid for guiding the manual deposition of matrix solution onto regions of interest. Although it may not be immediately apparent, the rectangular array of pixels comprising an image projects a Cartesian coordinate system onto the physical objects depicted in the image. This is particularly true when the objects depicted in the image are planar in nature, as is the case for tissue sections. Spatial relationships of objects depicted within the image can therefore be accurately described using respective pixel positions within the image. The main challenge is then to develop algorithms for automatically recognizing and locating features of interest, namely matrix spots or cells of interest, from background pixels. Basic image processing procedures are ideal for scanning images for particular morphological shapes and colors. Once their location has been determined in pixel coordinates, the objects can be registered to other coordinate systems.

Two factors, outside of the inherent limitations of the spotter, potentially limit the ability to place matrix precisely onto the

Fig. 6. A, results of an unsupervised classification of profiles of specific cell types acquired from one breast tumor section as determined by multidimensional scaling of results from an unsupervised classification on whole spectra. Colors indicate histopathology of the profile site. B, spatial plot representing profile similarity of DCIS versus IMC as determined by multidimensional scaling of the top ranked markers identified by supervised classification. Each profile is colored according to histopathology of the profile site. C, H&E section with annotation marks colored to represent results of classification analysis. A gradient color scale derived from the supervised classification indicating a degree of similarity to DCIS or IMC characteristics.
chosen locations. These are: (a) the accuracy of locating the fiducial landmarks within the spotter coordinate system and (b) the accuracy of aligning the images before combining annotation marks for the profile sites with the marks designating the fiducials. The selection of distinctive landmarks minimizes the contribution of the former. The latter, however, can be significant because of difficulties in cutting and handling serial sections from fresh-frozen tissue without some deformation of one or both sections. Presently the 200-µm size of the matrix spot allows for some misalignment, but as dispenser technology evolves toward smaller droplets this will become a limitation. One approach around this issue can be to utilize MALDI-compatible histology stains such as cresyl violet (16). In this case only one section, hence only one image, is needed for both MALDI and histology analysis, eliminating errors associated with image alignment. However, for some applications such as immunohistochemistry this is not an option.

The final step of the directed profiling procedure is the acquisition of profile spectra using automated instrument control. Operating a MALDI mass spectrometer in automated mode requires that the control software know a priori where each sample spot is located on the target. This is usually facilitated by depositing spots onto prescribed wells on the target that are already registered to the instrument. Tissue sections and their associated matrix spots, however, often do not conform to any prescribed pattern and are therefore not amenable to existing target templates. Uploading of custom sample coordinates is allowed by most commercial instruments, but this requires the determination of the relative positioning of all matrix spots on the plate. Automatic feature recognition of matrix spots from an actual image of the spotted plate accomplishes multiple tasks. First, it determines the accurate relative positioning of all spots in pixel coordinates which is required to effectively sample a 200-µm matrix spot with a 100–150-µm laser beam. Secondly, variations in spot shapes and sizes can be determined and used to enhance the sampling of each spot with the laser. Finally, the approach is not limited to matrix spots generated robotically. Matrix spots deposited manually can also be located and uploaded to the instrument.

The heterogeneity within the breast cancer samples used in this study makes them ideal for demonstrating the capabilities of the new profiling strategy. Accurate profiling of the various cell types dispersed throughout such samples demands a precision and specificity usually reserved for protocols involving LCM. The present methodology provides similar results, but directly from tissue, thus eliminating the need for rigorous LCM sample processing except in cases that demand a cellular specificity greater than our current matrix spot size. From the perspective of analysis throughput, more than 800 profile spectra were acquired from 4 samples in only a few hours. Extrapolating such acquisition rates to larger sample sets one can easily see the potential benefit for improving statistical confidence, especially when compared with more traditional profile data sets comprised of only 3–5 profiles per section.

The ability to specifically target and distinguish molecular signatures from different cell types within a single tissue section is, to our knowledge, a first for tissue profiling. Further, the preliminary MDS analysis of DCIS and IMC profiles acquired from the section containing both cell types is especially exciting and warrants further study. DCIS does not always progress to IMC, and there is presently no method for accurately determining which patients will progress. Identifying molecular markers that characterize the transition from DCIS to IMC (27–29) may provide novel therapeutic targets.

CONCLUSIONS

The procedures described here offer significant improvements in the cellular specificity and throughput of tissue profiling by MALDI-MS. The clinical impact realized from profiling studies will ultimately reflect how well the profile spectra actually represent cells specific to a disease pathway. Traditional sample preparation techniques involve nonspecific deposition of matrix solution into the general vicinity of diseased cells. Problems become more acute when the cells of interest are dispersed throughout the tissue and the protein profiles become contaminated with proteins originating from neighboring or infiltrating cells. Robotic microspotters address this problem to a great degree, but their capability for visualizing and targeting specific regions of the sample can be limited, particularly when profile sites are selected from a section other than the one being spotted. Our approach integrates the visual specificity of histology (via H&E and/or immunohistochemistry) with the positioning accuracy of the microspotter stage to direct placement of matrix onto specific cells. Automated acquisition of profile spectra from these randomly distributed matrix spots is then facilitated by processing a digital image of the spotted sample plate to extract relative pixel positions of all spots and transform these into values readable by the instrument. Together, these represent important advancements to the tissue profiling methodology. Additionally the demonstrated capability of visually integrating analysis results directly within the histopathology image may prove beneficial to developing a better understanding of disease development. With this capability many barriers to analyzing large clinically significant data sets are minimized, and tissue profiling moves one step closer in the path from bench to bedside by optimizing how clinicians diagnose and treat disease.

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