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Abstract

Purpose: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used to analyze tumor sections and can determine tumor type, nodal involvement, and survival, and shows promise in predicting therapeutic response. Our purpose was to develop a method compatible with MALDI-TOF MS that allows selective analysis of cancer cells in mixed clinical samples such as fine-needle aspirates.

Experimental Design: Lung cancer cell lines were cytocentrifuged onto metal-coated, transparent glass slides and used for optimization of fixation, staining, and RBC lysis protocols. Fine-needle aspirates from human tumors and mouse model tumors were used to provide fresh tissue samples for determining the feasibility of this method.

Results: The MALDI-TOF MS compatible fixation and staining techniques provided high-resolution cellular morphology, which allowed identification and selective spotting of tumor cells. The RBC lysis step efficiently removed contaminating RBC yielding spectra nearly free from hemoglobin peaks. Protein profiles of fine-needle aspirates were found highly reproducible and similar to the profiles of the tissue from which they were obtained. Using this method, we were able to differentiate between xenograft tumors derived from two different human cell lines, A549 and H460.

Conclusion: This procedure results in the production of high-quality, cancer cell–specific protein profiles. This highly reproducible technique could be applied to many other types of mixed clinical samples and has the potential to be very useful in the clinical diagnosis, classification, and, potentially, the individualized treatment of cancer patients.

Whereas the treatment of lung cancer has seen significant advances recently, the methods used for lung cancer diagnosis have not changed in decades. After identifying a suspicious mass by some form of imaging, either chest X-ray or computed tomography scan, a tissue sample from the suspect lesion is stained with H&E and examined by a pathologist under a light microscope. Based on the observed cytologic features, the tumor is classified as non–small-cell lung carcinoma or small-cell lung carcinoma. Whereas non–small-cell lung carcinoma can be further subcategorized into three main groups, adenocarcinoma, squamous cell carcinoma, or large cell carcinoma (1), all of these subtypes are treated the same in spite of a large variability in clinical outcomes. Clearly, practical new technologies need to be investigated for improving diagnosis, optimizing treatment efficacy, and reducing treatment toxicity.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a technology that can be used to generate protein signatures from tissue sections that can contain several hundred protein signals (2). Indirect tissue profiling experiments, thin tissue sections are cut from fresh frozen biopsies, thaw-mounted on a flat, metal MALDI target plate, and spotted with matrix (typically sinapinic acid). With this method, protein profiles have been obtained from tumor tissue sections and successfully analyzed with a class prediction model developed for handling the large data sets produced by MALDI (3–5).

Many clinical samples are fine-needle aspirates that result in a liquid suspension of cells highly contaminated with blood,
normal cells, and noncellular debris that are smeared or spun down on slides for analysis without adjacent sections to analyze. To accomplish light microscopic and mass spectrometric analysis of the cells of interest in such a clinical sample, there are two essential requirements that must be met: first, it is necessary for the sample plate to be optically transparent and conductive (for use in the MALDI apparatus); second, a stain must be found that is simultaneously revealing of cellular architecture and compatible with MALDI-TOF MS.

Recently, indium-tin oxide–coated transparent glass slides have been used to mount tissues for MALDI analysis (6, 7). These slides are coated with a 160- to 240-Å-thick layer of indium-tin oxide, which provides the surface necessary for maintaining stable accelerating potential within the source of the time-of-flight mass spectrometer. These conductive glass slides have been found ideal to mount tissue sections with the benefit that the sections can be viewed by light microscopy before MS analysis. Unfortunately, the most common staining methods used in histology, DiffQuik and H&E, have been found to be incompatible with MALDI MS (8, 9). To improve visualization of features, several staining protocols have been successfully used, which include methylene blue and cresyl violet (7). These provide excellent histology with high resolution of tissue architecture and allow high-quality mass spectra to be obtained.

MALDI-TOF MS protein profiling combines speed, simplicity, reproducibility, and sensitivity, making it a very powerful technology. The technological innovation introduced by the use of indium-tin oxide–coated glass slides and MALDI-compatible stains increases this versatility. We have sought to take advantage of these features in designing a new diagnostic tool for the direct analysis of low-abundance cancer cells contained within fine-needle aspirates. We show here that by combining a very simple existing technology, the cytocentrifuge, which is present in every clinical setting, with the simplicity and power of MALDI-TOF MS, a cutting edge diagnostic for lung cancer can be created that should provide a wealth of information and may ultimately have a significant impact on treatment decisions, and potentially, patient outcomes.

Materials and Methods

Cell culture. Human non–small-cell lung carcinoma cell lines (NCI-H1703, NCI-H2009, NCI-H1299, NCI-H460, and A549) and MC26 cells, a murine colon carcinoma cell line, were grown in RPMI (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (HyClone, Logan, UT), 2 mmol/L glutamine, with 10,000 units/mL penicillin and 10,000 units/mL streptomycin (Invitrogen). Lewis lung carcinoma cells were grown in MEM, 10% fetal bovine serum, 2 mmol/L glutamine, with 10,000 units/mL penicillin and 10,000 units/mL streptomycin.

Cytocentrifugation, fixation, and staining. Cells were washed twice with 10-μL PBS, detached from the bottom of the dish by scraping, and counted. Cells were diluted with PBS to 200,000 cells/mL and, using a double cytunnel (Thermo Electron, Waltham, MA), 200 and 400 μL of the cell suspension were cytocentrifuged (described below) onto indium-tin oxide–coated glass slides (Delta Technologies Ltd., Stillwater, MN) in a Cytospin3 (Thermo Electron) at 800 rpm for 5 minutes. Erythrocyte lysis buffer (Qiagen USA, Valencia, CA) was used to remove RBC from 60,000 lung cancer cells in 200 μL of normal saline alone, or with 2, 5, and 10 μL of whole blood. Initially, cells were fixed in acetone (Sigma, St. Louis, MO), methanol (VWR, West Chester, PA), or 95% ethanol for 5 minutes after cytocentrifugation. It was later determined that fixation in 95% ethanol provided high-quality cell morphology and was used for all later studies. The cells were stained with 0.5% cresyl violet or 0.15% methylene blue for 1 minute, followed by two 1-minute washes in 70% ethanol (7). Slides were air-dried before the addition of matrix. Samples not analyzed immediately were stored at −80°C until the day of analysis when matrix was added. The H&E-stained slides were prepared using standard techniques.

Simulated and clinical fine-needle aspirates. Tumors were generated by injection of 1 × 10^6 MC26 murine colon carcinoma cells into the flanks of BALB/c mice or by injection of 1 × 10^4 NCI-H460 cells into the left flanks and 1 × 10^4 A549 cells into the right flanks of nude mice. After 30 to 35 days, resulting tumors were removed and placed into ice-cold saline. A 23-gauge needle attached to a 5-μL syringe was inserted into the tumor. Aspirate was placed into 0.2 mL of ice-cold saline. For human samples, a 25-gauge needle attached to a 5-μL syringe was inserted into surgically resected tumor tissue or into adjacent normal tissue contained within the resection specimen immediately after removal of the tumor. As above, the aspirate was placed into ice-cold saline. If the volume of saline was >0.2 mL, the sample was centrifuged at 400 × g and the pellet resuspended in 0.2 mL of ice-cold saline. True fine-needle aspirates were obtained from the clinic in 1 to 2 mL of saline on wet ice without identifiers. Samples were spun down and resuspended in 0.2 mL of ice-cold saline for further processing as described. All samples were processed at the time they were received.

Red cell lysis. Cells with whole blood or fine-needle aspirates in 200 μL of normal saline were placed on ice. Five volumes of erythrocyte lysis buffer (Qiagen USA) were added and the samples were mixed by inversion and placed on ice for 15 minutes with occasional mixing every 3 to 4 minutes. Cells were centrifuged at 400 × g for 5 minutes at 4°C and the supernatant removed with a pipette. The samples were then resuspended in 200 μL of normal saline for cytocentrifugation.

MALDI-TOF MS. For the cell lines, 300 nL of matrix, sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid; Fluka, Buchs, Switzerland) at 20 mg/mL diluted in a solution of 50% acetonitrile, 0.1% trifluoroacetic acid (Fisher Scientific, Pittsburgh, PA), were deposited in a randomly chosen spot within the circle of cells laid down on the slide by cytocentrifugation. This was followed by a second 300 nL of matrix placed directly on top of the first spot to increase crystal density. For the simulated fine-needle aspirates, clusters of cancerous cells, 100 cells (or fewer) to several hundred cells, identified by a pathologist were hand-spotted while visualizing the cells under a microscope at ×10 or ×20 magnification. MALDI MS analyses were done in an Applied Biosystems Voyager DE-STR time of flight mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a 337-nm nitrogen laser operated at 20 Hz. Samples were analyzed in the linear mode geometry in delayed extraction conditions under 25-kV accelerating potential. The delayed extraction variables were optimized for maximum resolution at −m/z 15,000. Data were recorded in the m/z range of 2,000 to 70,000 by averaging signals from four independent acquisitions of 250 laser shots each (1,000 shots total). The MALDI MS acquisition method was first calibrated using a mixture of standard calibrating proteins: insulin (MW 5,777.6), cytochrome c (MW 12,360.1), apomyoglobin (MW 16,951.6), and trypsinogen (MW 23,982). Individual spectrums were then internally calibrated using previously identified mass signals. These are, for mouse samples: cytochrome c oxidase (MW 5,443.4), histone H4 (MW 11,306), histone H2A.2 (MW 14,004), and β hemoglobin when detected (15, 617); and for human samples: cytochrome c oxidase (MW 5,356.2), histone H4 (MW 11,306), histone H2A.2 (MW 14,005), and α and β hemoglobin when detected (MW 15,126). Tissue section analysis. Ten-micron-thick tissue sections from fresh-frozen tumor biopsies were cut at −15°C using a cryostat (CM3050 S, Leica Microsystems AG, Wetzlar, Germany) and thaw-mounted on the conductive glass slides. Serial sections were also cut for subsequent H&E staining. The sections were dried at room temperature before being successively rinsed in 70% and 95% ethanol for 30 seconds,
respectively. MALDI matrix was hand-deposited on the sections by double spotting two 300-nL drops using an automatic pipette. MALDI MS analyses were done as described above.

Statistical analysis. Whole spectrum analysis was done as previously described (10) with the exception that classification was not carried out here. Briefly, all mass spectra were exported as ASCII text files and imported into ProTS Data version 1.1 (Biodexis, Steamboat Springs, CO) for baseline correction, normalization by total ion current, and aligning in batch mode. The processed text files were then imported into a script written in Matlab (Jeremy Norris, PhD, Vanderbilt University) to be aligned according to a single m/z column. A standard weighted mean averaging algorithm was then applied (11). In this way, m/z values were filtered according to the highest weight that best differentiated the normal versus cancer groups. Further filtering was carried out to exclude values with weighted mean averages <1.0 (ref. 10; similar in respect to 2 a from the mean control value) and to exclude mean intensity differences that fell below 2-fold (ref. 2; experimentally derived cutoff value often applied for tissue profiling; data not published). 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 7.0. Invariant error and graphs depicting variance throughout the spectra were prepared and plotted with tools provided by Biodexis. The hierarchical clustering analysis and subsequent icicle plot were carried out with Statistica 6.0 (StatSoft, Tulsa, OK).

Results

MALDI-TOF MS on cytopsins generates a high-quality signal from fine-needle aspirate-sized clusters of cancer cells. Due to the limited sample size and high degree of nontumor contamination of most fine-needle aspirates, it is imperative that the method be sensitive enough to detect small numbers of cells. The sensitivity of the technique has been shown in one recent study where protein signals were obtained from as few as 10 cells isolated by laser capture microdissection (9). In our pilot experiments to determine the feasibility of the technique, we found that it was possible to get abundant, high-quality MS signals from 50 to 100 cells when very low volumes of matrix (~50 nL) were deposited on the cells using pulsed glass capillaries. Given the quality of the MS data recovered with so few cells and considering that cancer cell clumps contained in needle aspirates are frequently in this range, we decided that pursuing the development of methods for analysis of cancer cells in fine-needle aspirates was feasible.

Development of cell fixation and staining techniques compatible with MALDI-TOF MS of cytopsins. For optimal morphologic identification, fixation and staining is required. Therefore, we tested three standard fixation techniques including 95% ethanol, methanol, and acetone for compatibility with MALDI MS. Forty thousand A549 lung cancer cells were cytocentrifuged onto an indium-tin oxide–coated slide, fixed in organic solvent, air-dried, and profiled by MALDI MS (Fig. 1A). Ethanol and methanol showed improved MS data quality with respect to a nonfixed control with higher ion yield (on average, by a factor of ~2 in the m/z range 4,000-60,000) and slightly better resolution (e.g., see signal cluster at ~m/z 18,000 in Fig. 1A). Acetone fixation gave somewhat lower yield and resolution spectra with respect to the control. It was determined that 95% ethanol fixation was the simplest procedure that produced high-quality spectra and this was used for subsequent experiments.

Staining is necessary to visualize the cells using light microscopy and for the identification of cell types in complex mixtures from clinical fine-needle aspirates. Following cytocentrifugation onto indium-tin oxide–coated glass slides, NCI-H2009 cells were fixed in ethanol and stained with cresyl violet or methylene blue. Examination of the cells by light microscopy revealed that the nuclei could be easily seen with well-defined nucleoli using either stain, but the resolution of nucleoli was somewhat better with cresyl violet (Fig. 1B). Unlike the staining of unfixed tissue where cresyl violet and methylene blue were comparable (ref. 7), the spectra obtained from the fixed cells stained with cresyl violet showed slightly better resolution and signal-to-noise ratio than spectra from cells stained with methylene blue (Fig. 1C). Therefore, cresyl violet was chosen for further studies.

Erythrocyte removal is a critical step for obtaining MALDI-TOF MS spectra from clinical needle aspirates. Contamination of clinical samples with blood and other cell types, such as polymorphonuclear cells, macrophages, and dendritic cells, is common and a potential problem for MALDI MS protein profiling. The presence of large amounts of hemoglobin from RBC suppresses any other protein signatures in the sample. To reduce the hemoglobin content in fine-needle aspirate samples, an erythrocyte lysis step was added to the protocol before cytocentrifugation. Mass spectra obtained from NCI-H1299 cells treated with erythrocyte lysis buffer showed that cells exposed to erythrocyte lysis buffer generated the identical spectra as untreated cells (data not shown). To test the efficiency of erythrocyte lysis buffer in removing varying amounts of contaminating blood, 6 × 10⁷ NCI-H1299 cells in 0.2 mL of saline were mixed with 2, 5, or 10 μL of whole blood. The effectiveness of erythrocyte removal is seen when comparing cytocentrifuged, erythrocyte lysis buffer–treated samples to untreated samples by light microscopy (Fig. 2). In the untreated samples, the areas between stained H1299 cells are filled with RBC (Fig. 2A and B). Treated samples are devoid of RBC but do contain polymorphonuclear cells after lysis (Fig. 2C and D). The H1299 cells with blood were also monitored by MALDI MS before and after the erythrocyte lysis buffer treatment. The efficacy of the erythrocyte lysis step is revealed by examining the resulting spectra from cells that were contaminated with 10 μL of whole blood (Fig. 2E). In untreated samples (blue spectrum), dominant α and β hemoglobin peaks at m/z 15,127 and 15,668, respectively, doubly charged species at m/z 7,564 and 7,835, and hemoglobin dimers at m/z 30,253 (2α), 30,793 (α + β), and 31,335 (2β) are detected. After the erythrocyte lysis step (green spectrum), in samples that contained 10 μL of blood, hemoglobin signals were attenuated to a level below that of abundant cellular proteins, such as histone H4 (at m/z 11,307 and 11,349), with almost complete elimination of peaks due to the doubly charged hemoglobin species and the hemoglobin dimers. Several intense peaks at m/z of 3,372, 3,443, and 3,487 are present in samples that have been contaminated with blood and have previously been identified as defensins, which are proteins present at high levels in neutrophils (12, 13). The intense defensin peaks are indicative of the high abundance of neutrophils in the samples after removal of large numbers of RBC. To determine the extent to which WBC affect the spectra, 10 μL of whole blood in 0.2 mL of saline were treated and the MALDI MS spectra acquired without attempting to avoid matrix deposition on white cells. Whereas the defensins were again very abundant, the overall intensity, and therefore the complexity, of the protein profile is lower than in spectrums from untreated samples. In a recent study where proteins signals were obtained from as few as 50 to 100 cells when very low volumes of matrix were used for peak detection and further evaluated by plotting the whole spectra as compared with the difference spectra in Origin 7.0. Invariant error and graphs depicting variance throughout the spectra were prepared and plotted with tools provided by Biodexis. The hierarchical clustering analysis and subsequent icicle plot were carried out with Statistica 6.0 (StatSoft, Tulsa, OK).
Fig. 1. Optimization of MALDI-compatible fixation and staining techniques. A, spectra produced from tests of organic fixation of A549 cells revealed that 95% ethanol and methanol generated high-quality spectra with signal-to-noise ratio ~ 2-fold higher than unfixed cells. Acetone fixation induced markedly lower resolution spectra with lower signal-to-noise ratio than unfixed cells. B, photomicrographs of cresyl violet – stained and methylene blue – stained NCI-H2009 cells that were ethanol fixed show that cresyl violet – stained cells have better resolution of nuclear structures such as nucleoli. H&E-stained cells are presented for comparison. C, MALDI-TOF MS spectra for unstained control NCI-H1703 cells, cresyl violet – stained cells, and methylene blue – stained cells.
was not significant when compared with that of the non–small-cell lung carcinoma cell line alone (data not shown).

Spectra from simulated fine-needle aspirates accurately reflect the spectra of the matched tissue. The technique outlined in Fig. 3A is rapid and technically simple. With the exception of the erythrocyte lysis step and the change of stains from H&E to cresyl violet, this procedure is very similar to that routinely used in clinical laboratories. To test the usefulness of the method, we used a “simulated fine-needle aspirate” model that mimics a clinical fine-needle aspirate, a model used in several previous studies (14, 15). Our initial experiments used aspirates derived from mouse allograft tumors and resected human tumors. An example of an aspirate obtained from a resected human tumor and adjacent nontumor tissue is shown in Fig. 3B. The MALDI MS spectra obtained from the aspirates are of high quality, showing >300 distinct peaks, and comparison of tumor and nontumor shows clear differences between the two (Fig. 3C).

Human tumors are very heterogeneous with large areas of stromal tissue, blood vessels, considerable numbers of inflammatory cells, as well as areas of necrosis (Fig. 4; Supplementary Fig. S1). In addition, changes in the tumor cells themselves occur as molecular changes create various subclones within the tumor. Therefore, we wanted to confirm that spectra obtained from simulated fine-needle aspirates of resected human tumors resembled those of the tumor tissue. Examination of the spectrum for a simulated fine-needle aspirate and the corresponding tissue section revealed that the simulated fine-needle aspirate and the tumor section share many of the same peaks (~70%; Fig. 4). Almost all of the fine-needle aspirate samples have predominant histone signals that are present in the tissue, but at a lower intensity. Qualitatively, fresh-frozen tissue samples seem to contain more peaks, presumably as a result of additional proteins derived from normal stromal tissue, infiltrating polymorphonuclear cells, macrophages, dendritic cells, or necrotic tissue. Interestingly, the spectrum of a fine-needle aspirate from a tumor with little necrosis and very few infiltrating neutrophils, unlike the tumor analyzed in Fig. 4, gave very low defensin peaks (m/z 3,372 and 3,443) as would be expected (Supplementary Fig. S1). Therefore, due to the large number of signals shared between the tumor and the fine-needle aspirate and the correlation of defensin peaks with numbers of neutrophils, we are confident that spectra obtained from a fine-needle aspirate will be highly informative and accurately reflect the make-up of the tumor from which it is derived, although spectra derived from fine-needle aspirates and tumor sections are clearly not completely equivalent.

MALDI-TOF MS from fine-needle aspirates is reproducible. To test reproducibility between different samples obtained on the
same day, two Lewis lung carcinoma tumors were isolated from mice and processed as described in Materials and Methods. From each of these tumors, 10 needle passes were made to obtain aspirates from which 15 and 21 protein profiles were acquired, respectively. To test the variability of samples obtained on different days, two Lewis lung carcinoma tumors were isolated from a pair of mice 3 days later. Following the same protocol, the number of protein profiles acquired from these tumors was 19 and 16, respectively. The size of the clusters varied with estimates ranging from just over a hundred cells to several thousand. Figure 5A represents three randomly pulled MALDI MS spectra obtained from distinct clumps of cancer cells isolated from the same tumor. As can be observed, these profiles were found to be highly reproducible with little variation. The mean spectrum from the four tumors analyzed at two separate time points (71 spectra in all) is shown in Fig. 5B. Confidence intervals (1 SD above and below the mean) are shown in the form of dotted lines. The coefficient of variance over the entire mass range studied (i.e., 2-30 kDa) was consistent across any one experiment, ranging from 0.46 to 0.69 (25th-75th inner quartiles; Supplementary Fig. S2). The analysis of single-day versus day-to-day experiments increased the geometric mean coefficient of variance value from 0.53 to 0.57. The above-mentioned results indicate that we can reproducibly harvest and prepare fine-needle aspirates for MALDI-TOF MS.

**MALDI-TOF MS of simulated fine-needle aspirates can be used to discern tumor types.** It was then tested whether this technique can generate spectra of sufficient quality to distinguish between different tumor types. To this end, in two separate experiments, two xenograft tumors, derived from A549 (adenocarcinoma) and H460 (large cell carcinoma) cell lines, were simultaneously grown in opposite flanks of the same mouse. After tumor removal, 10 needle passes were made to obtain aspirates from each tumor. The aspirates were processed and matrix added as described above. In the first experiment, 28 and 20 protein profiles were obtained from visually identified cancer cell clumps from the A549 and H460 derived tumors, respectively. In the second experiment, a smaller set of 14 and 9 spectra were obtained for the A549 and H460 derived tumors, respectively.

From the first experiment, the resulting average spectra shown for each tumor type clearly illustrate distinct differences between the protein profiles (Fig. 6A). Within the same figure, a difference plot, shown in blue, highlights those peaks that have been found to be statistically different between the two tumor types. The inset in Fig. 6A illustrates an isolated peak that was found to be one of the major discriminators between the two tumor types. This peak is identified in the difference plot by the black arrow. The average peak height is shown as a solid line with 1 SD above and below the average spectra depicted as dotted lines. It should be noted that there is no overlap between the SE plots, indicating a high level of significance between these two tumors with just a single marker.

The icicle plot (Fig. 6B) depicts a qualitative supervised Hierarchical Cluster Analysis carried out on the top 18 markers and shows a clear separation between the H460 and A549 aspirates. It should be noted that two spectra taken from the same H460 tumor were shown to fall into the A549 group. These two samples came from the same pass of the needle. Considering the high level of separation observed between the two tumors analyzed, this result was unexpected. Therefore, it is possible that spectra from these two samples reflect a peculiarity in the region from where this pass was done or a spotting error, which will be discussed further. Results of the supervised Hierarchical Cluster Analysis of the second experiment gave very similar results with 100% of the smaller set of spectra (14 from A549 and 9 from H460) being classified correctly.
Quality spectra from clinical samples. True fine-needle aspirates were obtained from the clinic without identifiers and prepared as described (Fig. 3A). Cresyl violet–stained slides were examined by a pathologist for the presence of tumor cells. Two of the samples were positive for tumor cells and analyzed further by MALDI-MS. The spectra obtained were rich with peaks and of high quality (Fig. 7).

Discussion

We have developed a technique based on MALDI-TOF MS for the analysis of cancer cells contained within fine-needle aspirates, which is suitable for the clinical setting. Specimens can be processed easily and rapidly using common reagents and a cytocentrifuge, which can be found in any cytology laboratory. By removing contaminating blood with a simple procedure, tumor cells can be identified by light microscopy and selectively analyzed by MALDI-TOF MS without ion suppression from contaminating hemoglobin.

Reproducibility is prerequisite for any technology if it is to be used for clinical decision-making. From this perspective, we found that multiple samples derived from the same tumor, different tumors, or tumors analyzed on different days generated nearly identical spectra after processing with our novel approach. In two separate experiments, samples generated from tumors that were derived from two different cell lines, H460 and A549, neatly segregated into two distinct groups following a supervised Hierarchical Clustering Analysis. This qualitative analysis clearly shows reproducibility within any single group and further illustrates how this level of reproducibility allows the clear separation between differing groups of samples such as in our case two distinct tumor types. However, in one of the experiments, 96% (46 of 48) of the samples were classified correctly. Of three cell clusters spotted from the same needle pass into the H460 tumor, two were misclassified. One likely explanation for the misgrouping is that this particular pass of the needle came from an area that contained cells undergoing necrosis, which gave some signals, unlike H460 or A549. Difficulties arising from sampling errors are unfortunately intrinsic to blind needle aspirates, but emphasize the importance of attempting to obtain samples from the periphery of the tumor and avoiding potentially necrotic areas. Alternatively, signal contamination from other cell types could possibly come from our current method of manually spotting clumps using a pulled capillary. Although small spots can be achieved, the placement accuracy may in some cases be relatively imprecise. Automated matrix dispensing robots are currently being developed with placement...
accuracies in the order of 20 μm or better (16), which we intend to use as we refine the technology. Regardless, the overall results were quite encouraging as all other spectra clustered tightly within their respective groups. More work is currently being carried out to further refine this unique and very promising approach and to translate it into the analysis of real clinical specimens.

Many technologies are currently being investigated for refining our ability to diagnose cancer, define cancer type, and predict prognosis and response to treatment. The most developed of these is gene expression profiling using microarray analysis. Indeed, microarray studies have been done using fine-needle aspirates from several types of solid tumors without purification of tumor from nontumor cell types (14, 15, 17–20). However, problems have been encountered in isolating RNA of sufficient quality for microarray analysis and most clinical samples will be too heterogeneous to provide meaningful results.

Additional technologies include multiplex analysis of known biomarkers by bead-based multiplexing, where antibodies are coupled to bead matrices, and high-density single-nucleotide polymorphism arrays (21, 22). As previously mentioned, the heterogeneity of most clinical samples complicates the analysis of either technology and could affect the result. By using the combination of erythrocyte lysis buffer to remove RBC and the indium-tin oxide–coated glass slides with MALDI-compatible stains, we have circumvented the problem of potentially interfering contaminants. Directly spotting clusters of cancer cells with matrix by visualizing them under a microscope, with few exceptions, leaves the large majority of contaminating cells untouched and the analysis free from such complications. The equipment to enable image-guided, robotic spotting and spectrum acquisition is currently being developed and should further enhance our ability to precisely target tumor cells.

A major obstacle to progress in this field is the difficulty in obtaining suitable numbers and types of tumor samples from patients necessary for training the specialized algorithms designed to find differential peaks. As current collection protocols in clinical trials change, with requirements that tumor biopsies be taken before and after treatment, the numbers of samples available for analysis should increase dramatically, particularly if fine-needle aspirates are usable for both defining and testing these patterns. With a large number of diagnoses at most institutions being accomplished by fine-needle aspirates, the ability to use these specimens with the technology we describe here should speed our progress. Standardization of the technique will allow collaborations between medical centers and allow a more rapid accumulation of the necessary data.

The MALDI-TOF MS technology has the potential to fill a significant need in the area of diagnostic medicine. The sensitivity makes it especially suited for small sample sizes. Data can be generated with minimal training due to the ease of the technique. The speed of the sample preparation and data acquisition makes the processing of large numbers of samples

![Fig. 6. MALDI-TOF MS of fine-needle aspirates can be used to discriminate between tumor types. A, average spectra are shown comparing A549 and H460 along with an associated difference plot in blue depicting the discriminate biomarkers used to discern differences between the two tumor types (the statistical approach used to discern markers of interest is discussed in Materials and Methods). Inset, an isolated peak that was found to be one of the major discriminators used to classify the two tumor types. The average peak height is shown with 1 SD above and below the mean for each spectrum (dotted lines). This peak is identified in the difference plot by the black arrow. B, the icicle plot shown depicts a qualitative supervised Hierarchical Cluster Analysis using the top 18 markers and shows a clear separation between the H460 and A549 aspirates.](image1.png)

![Fig. 7. MALDI-TOF MS analysis of clinical fine-needle aspirates is of high quality. Clinical fine-needle aspirates were obtained and processed as described in the text. The spectra are still rich in peaks throughout the presented mass despite the presence of the hemoglobin still significantly observed in these samples even after the erythrocyte lysis step.](image2.png)
possible. Finally, the cost of the method, including materials, technician time, and mass spectrometer time, which can be provided by specialized reference institutions for a nominal fee, would be a fraction of the cost of other methods being developed for cancer diagnosis and response prediction. All of these attributes make the use of the cytospin-MALDI-TOF MS technology for the processing of fine-needle aspirates well suited for potential translation into the clinical laboratory.

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