Direct Analysis of Laser Capture Microdissected Cells by MALDI Mass Spectrometry

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Laser capture microdissection (LCM) has become an important tool in biological research, permitting isolation of specific cell populations from frozen tissue samples containing a mixture of cell types. Cells obtained by LCM can be directly analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). We report here methodology for the preparation and analysis of LCM captured cells with MALDI MS, giving high sensitivity and mass resolution. Comparison of the spectra obtained from cell populations of interest can identify unique disease or function-related protein markers. Using this approach, mass spectra obtained from human breast tissue containing invasive mammary carcinoma and normal breast epithelium using LCM were compared. Over 40 peaks were identified that significantly differed in intensity between invasive mammary carcinoma and normal breast epithelium. In addition, mass spectra are presented that show protein patterns from mouse liver and mouse colon crypts. The reported tissue preparation procedure and subsequent analysis by MALDI MS provide a new methodology for protein discovery involving LCM captured cells. (J Am Soc Mass Spectrom 2002, 13, 1292–1297) © 2002 American Society for Mass Spectrometry

Isolation of pure cell populations from healthy and diseased tissues by laser capture microdissection (LCM) has had a significant impact in biological research. In this technique, a proprietary thermoplastic membrane placed directly on a tissue section melts and adheres selectively to the cells of interest after irradiation with a focused infrared laser pulse. Subsequent removal of the membrane with the adhered cells provides a target that can be used for molecular analysis. In previously reported proteomic studies using cells captured by LCM, proteins were isolated from these cells using a protein-extraction buffer. The resultant proteins were then analyzed by two-dimensional gel electrophoresis and MS analysis [1–3] or chemiluminescent immunoassays [4]. While informative, these approaches are time consuming and require large sample sizes, and are therefore impractical for routine use. Alternatively, MALDI MS may be used for analysis of cell preparations obtained by LCM [5, 6]. Following microdissection, the thermoplastic film is placed directly on a target analysis plate and introduced directly into the mass spectrometer after the matrix is applied.

We describe here new methods and sample preparation procedures that improve the sensitivity and resolution of the MALDI MS analysis of LCM captured cells. Results are presented for several types of tissues, including mouse liver, mouse colon crypts, and human breast tissue. Using optimized tissue preparation techniques, mass spectra obtained from laser microdissected cells show high sensitivity and good mass resolution, permitting identification of unique protein peaks in each specimen type.

Materials and Methods

Tissue Collection and Preparation

Mouse and human tissues were used in this study, in compliance with federal, state, and university regulations. Mice were sacrificed in a CO_2 gas chamber, the abdominal cavity was opened, and the colon and liver were removed. The lumen of the colon was opened longitudinally and rinsed three times with phosphate buffered saline. Sections of colon and liver were then snap frozen in liquid nitrogen and stored at −80 °C until use. Human breast tissue was obtained from surgical specimens received in the Department of Sur-

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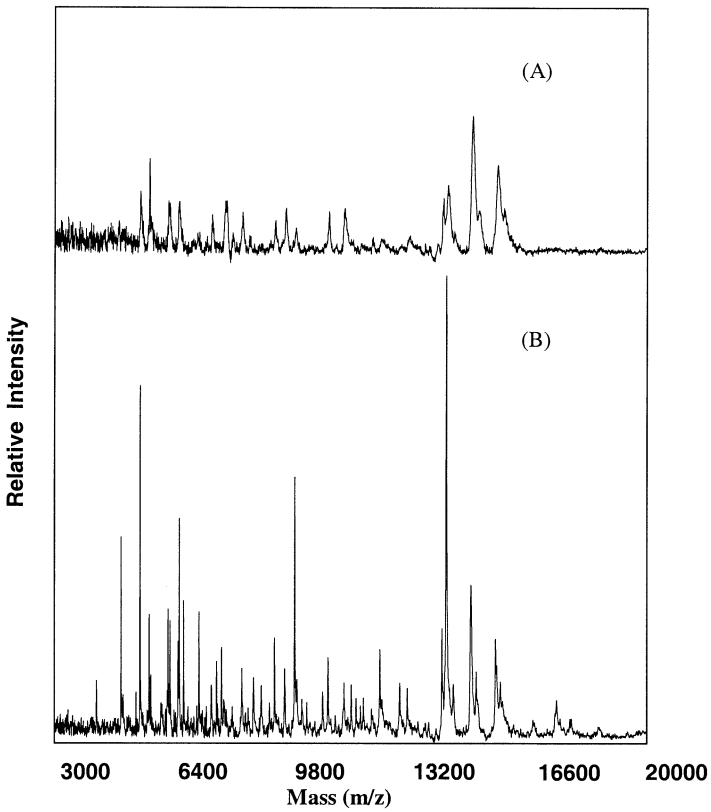

Figure 1. The histologic staining of tissue prior to LCM reduces spectrum quality. (**a**) MALDI MS spectrum obtained from haematoxylin and eosin (H&E) stained LCM captured mouse liver cells. (**b**) MALDI MS spectrum obtained from unstained LCM captured mouse liver cells.

gical Pathology, Vanderbilt University. Tissues were snap-frozen in liquid nitrogen in a small plastic container without OCT less than 15 min after removal from the patient. Samples were then stored in a liquid nitrogen tank or a −80 °C freezer prior to use. These studies were approved by the Institutional Review Board of Vanderbilt University Medical School.

Tissue Preparation

The LCM tissue preparation protocol that has been used for various applications has been described elsewhere [1]. Our procedure, optimized for mass spectrometric analysis, is as follows: 5 μm frozen tissue sections were prepared using a cryostat; these were then mounted on uncoated glass slides without the use of embedding media. The sections were placed immediately in 70% ethanol for 1 min. Subsequent dehydration was achieved using graded alcohol and xylene treatments as follows: 95% ethanol for 30 s (times 2), 100% ethanol for 30 s (times 2), and xylene for 5 min (times 2). Slides were then dried in a laminar flow hood for 5 to 10 min prior to microdissection. With this technique we were able to obtain excellent visualization of the majority of tissue sections without staining. In our studies comparing the quality of MALDI MS spectra obtained from stained and unstained frozen sections, standard hematoxylin and eosin stains were employed. The staining method used the procedure described above that was then modified after 1 min in 70% ethanol as follows: 20 s in water, 30 s each in hematoxylin followed by eosin, 20 s in water, followed by the previously described alcohol and xylene wash steps. To address the possibility of soluble protein loss from tissue sections as a result of the wash steps, spectra from sections of mouse liver washed in 70% ethanol or water were compared to untreated liver. Twelve micron sections of mouse liver were cut using a cryostat and placed directly on three gold-coated MALDI plates. The plates were submerged in 70% ethanol for 1 min, water for 3 min, or no treatment, respectively. The plates were subsequently dried in a vacuum desiccator and 1 μl of sinapinic acid (Sigma, St. Louis, MO) in 6/4/0.03 vol/vol/vol acetonitrile/water/TFA was deposited on each of the tissues. Spectra generated from the alcohol and water treated tissue were compared to spectra generated from tissue placed directly on the gold plate without exposure to alcohol or water.

Laser Capture Microdissection

Laser capture microdissection was performed using the PixCell II LCM system (Arcturus, Mountain View, CA) by a board-certified pathologist (M.E.S). For dissection of human breast tissue and mouse liver tissue, 100 to 1000 shots using either a 7.5 or 15 μm infrared laser

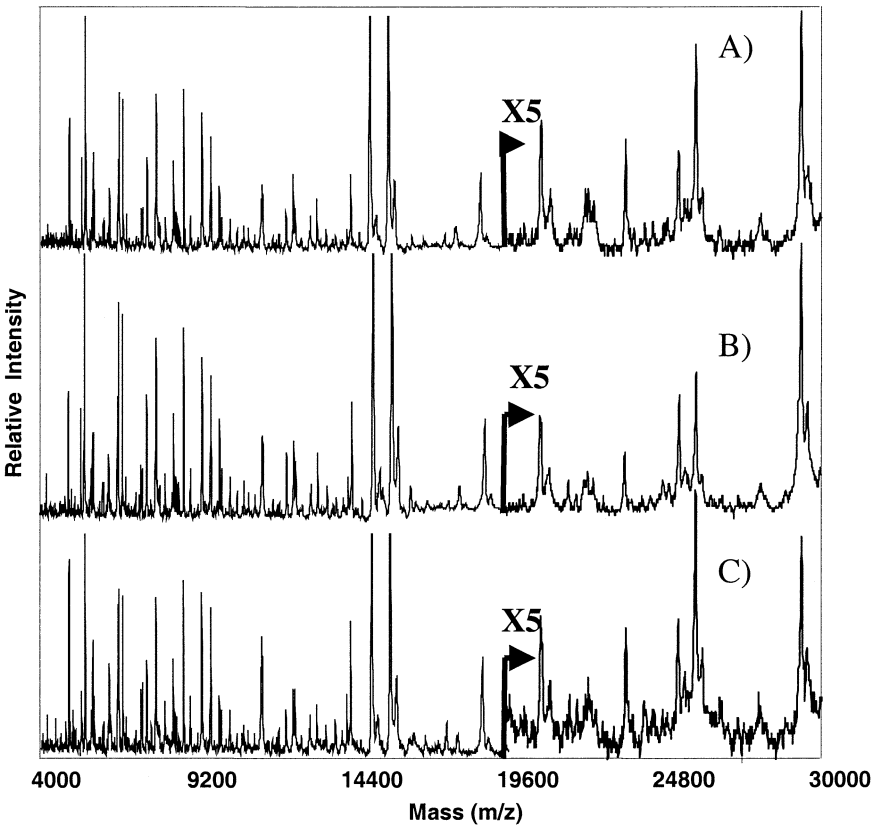

Figure 2. The tissue dehydration process required in preparation for LCM does not result in significant protein loss from the tissues. MALDI MS spectra of (**a**) untreated mouse liver, (**b**) mouse liver after exposure to 70% ethanol for 1 min, and (**c**) mouse liver after exposure to water for 3 min.

beam were used to obtain approximately 200 to 2000 cells of interest on CapSure LCM caps (Arcturus). All samples were prepared in duplicate. To investigate the number of cells required for a successful MALDI MS analysis, a single shot using the 30 μm diameter spot adjustment on the Arcturus PixCell II was performed on a section of unstained mouse colon capturing 10 crypt cells.

Preparation of Microdissected Cells for MALDI-MS

After LCM, the thermoplastic film was removed from the LCM cap using forceps and placed on the MALDI plate using conductive double-sided tape. To restrict matrix volume to the minimum amount needed to coat the microdissected cells, a finely pulled glass capillary

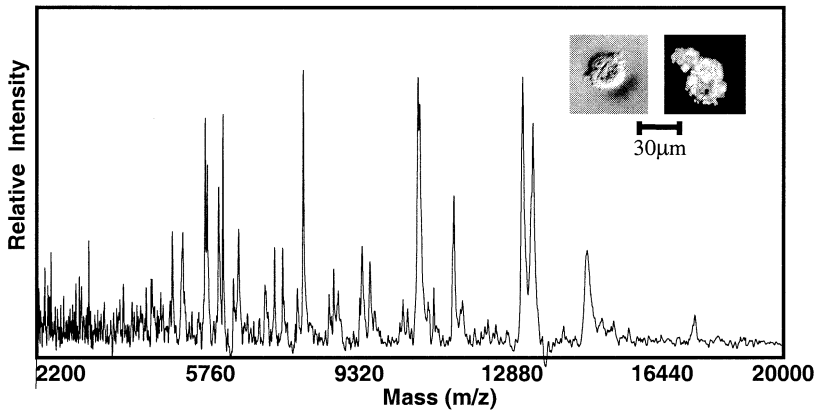

Figure 3. MALDI MS spectra acquired from 10 mouse colon crypt cells. The optical images on the top show the laser microdissected cells before (left) and after (right) matrix deposition.

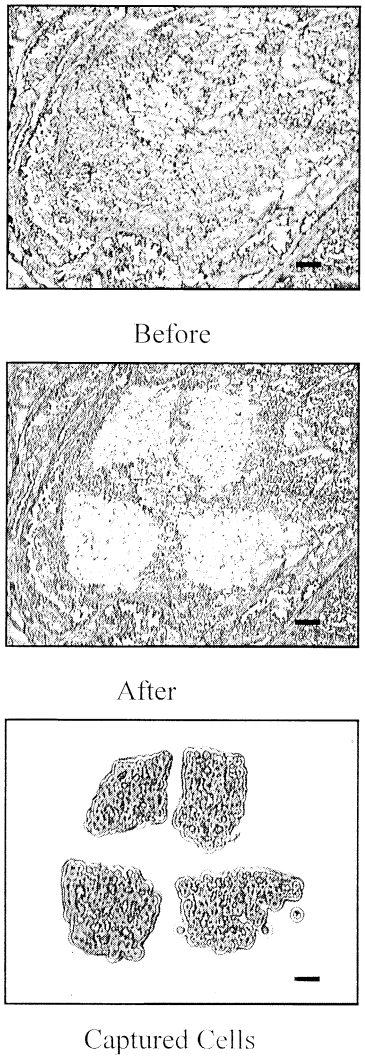

Figure 4. LCM capture of unstained human breast ductal carcinoma in situ cells. The bar represents a length of 30 μm.

was employed to deposit matrix solution on the captured cells under microscope visualization. The volume of matrix ranged from 100 pl to 10 nl depending on the number of microdissected cells. The matrix solution consisted of sinapinic acid at 20 mg/ml in 6/4/0.03 vol/vol/vol acetonitrile/water/TFA to which a mixture of proteins including bovine insulin (*Mr.* 5733.6) and horse skeletal apomyoglobin (*Mr.* 16951.6) were added to serve as internal standards, both obtained from Sigma Chemical Co.

MALDI-MS Analysis

MALDI-MS analysis was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) with a 337 nm nitrogen laser. Acquisition was achieved in the linear positive ion mode under optimized delayed extraction conditions. Seven hundred and fifty laser shots were averaged to create a single spectrum from the captured cells. Standards placed in the matrix solution were used for internal calibration. Spectra were also generated from LCM samples spotted with matrix lacking the internal standard to document the absence of peaks in the region containing the standards. The spectra were baseline subtracted by software written in our laboratory.

Results and Discussion

Methodology

The goal of this project was to optimize sample preparation procedures to obtain high quality mass spectra acquired from LCM samples. First, we compared spectra obtained by placing tissue directly on the MALDI plate versus LCM captured samples. Initially we noted that the spectra obtained from LCM captured cells were of somewhat poorer quality to those obtained directly from tissue on the MALDI plate in terms of both signal intensity and mass resolution. We assessed the effect of several parameters on MALDI signal acquisition, i.e., tissue staining, alcohol treatment required for tissue section preparation, matrix application, and the use of a non-conductive thermoplastic film. Sections of mouse liver were used to simultaneously examine the effects of tissue staining during tissue section preparation for LCM and microspotting of matrix solution for MALDI-MS on the subsequent mass spectra generated from these samples. We compared hematoxylin and eosin stained tissue versus unstained tissue, each prepared by dehydration in graded alcohol and xylene washes. Following LCM, the samples were microspotted with matrix for MALDI-MS analysis. We found that the hematoxylin and eosin stain severely interfered with spectral quality obtained from the subsequent LCM samples. Moreover, spectra obtained from unstained LCM cells were nearly identical in quality to those generated by placing tissue directly on the MALDI plate. Figure 1 compares the spectra of mouse liver cells with and without haematoxylin and eosin (H&E) staining followed by chemical dehydration. The spectra obtained from unstained cells clearly show more signals and higher resolution when compared to those of stained cells. Once the staining step was eliminated from the sample preparation, the quality of the spectra from tissue placed directly on the MALDI plate and analysis from microdissected cells were quite similar, suggesting that possible charging effects from the LCM film were not a significant problem. Spectra obtained on LCM film achieved better than 100 ppm mass accuracy below 30,000 *m/z* with internal calibration. In addition, the color filter from the Arcturus PixCell II provided excellent visualization of tissue morphology for the majority of unstained tissue sections. In this way, elimination of the staining step dramatically improved the quality of the mass spectra without compromising histological visualization.

Since the desorbed proteins are predominantly soluble proteins, we evaluated the extent to which the wash

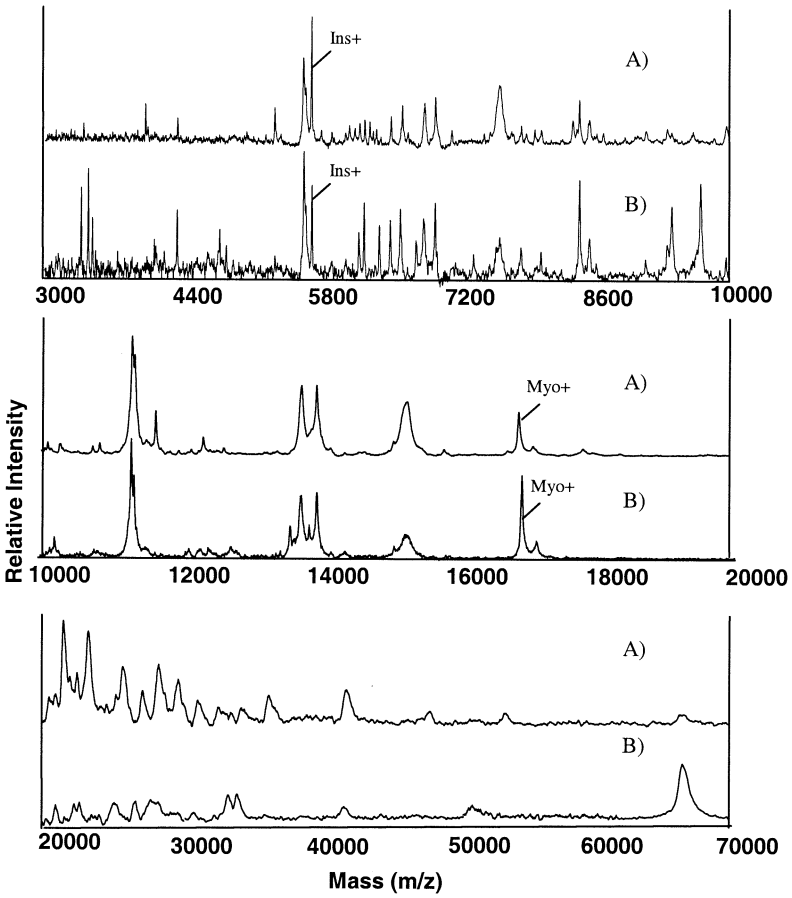

Figure 5. Protein profiles obtained by MALDI MS over three different *m/z* ranges from (**a**) microdissected invasive mammary carcinoma and (**b**) normal breast epithelial cells. Internal standards were used for calibration. Ins^+, $[Insulin + H]^+$ internal standard; Myo^+, $[Apomyoglobin + H]^+$ internal standard.

steps containing graded alcohol removed some of the soluble proteins from the cells. To evaluate this, spectra generated from whole tissue sections of mouse liver washed in 70% ethanol or water were compared to untreated liver tissue. We found no significant differences in the spectra generated following the three treatments (Figure 2). This shows that the preparation steps did not result in significant loss of protein from the samples.

In prior studies [5], the entire LCM sample was coated with 1 μl of matrix solution. Although good quality spectra were produced, such a large volume of matrix solution could potentially dilute and mix the proteins associated with each cell. In order to concentrate a small volume of matrix on specific cell clusters, the sinapinic acid matrix solution was micro-spotted on the captured cells under microscope visualization. A finely pulled glass microcapillary was used to deposit sub-nanoliter volumes of matrix solution on the top of the captured cells. Limiting placement of the matrix to the captured cells also provided for an accurate location of the captured cells by the camera system on the MALDI instrument. We found this microspotting procedure to greatly increase the number and intensity of peaks recorded in the spectra as well as to enhance the spectral quality.

We also wanted to investigate the number of cells required for a successful and representative MALDI MS analysis. Using the 30 μm diameter spot adjustment on the LCM instrument, a single shot of the infrared laser was performed on a section of unstained mouse colon. Based on an average diameter of 9 μm per dehydrated crypt cell, we calculated that approximately 10 cells had adhered to the LCM film per laser shot. Although technically we possess the ability to obtain a single cell by LCM, ten cells were chosen for this analysis to ensure that a minimally representative cell population was examined. These cells were microspotted with approximately 100 pl of matrix solution and analyzed. Figure 3 presents the MALDI MS spectrum obtained from this sample acquired with 150 laser shots. This clearly demonstrates that relatively abundant protein signals may be recorded from a relatively small number of microdissected cells.

Breast Tumor Analysis

To illustrate the LCM process, Figure 4 shows optical images of unstained human breast ductal carcinoma in situ before and after the LCM process, and the isolated cells. Further studies were performed with human

breast tissue containing invasive mammary carcinoma and normal breast epithelium. The MALDI MS protein profiles of these tissues are given in Figure 5. Comparison of these spectra shows significant differences in peak intensities between the two tissues. Further work involving tandem mass spectrometry is underway to identify a number of these specific proteins. From sample sectioning through MS data acquisition, less than 2 h was needed for processing these samples, making the procedure amenable to clinical use.

Conclusion

LCM combined with MALDI MS is a sensitive, accurate, fast, and reliable method to acquire protein profiles from a specific cell population in a heterogeneous tissue. We have described sample preparation methodology to provide high quality mass spectra. The procedure utilizes microspotting of matrix solution, internal calibration, and elimination of histologic staining. In addition, it was determined that abundant signals can be obtained from as few as 10 or less LCM captured cells. The dehydration of tissue samples using organic solvent washes, a normal part of the LCM preparation procedure, did not result in significant protein loss from the samples.

LCM combined with MALDI MS has numerous potential applications for proteomic studies using a wide range of tissue types. Its unique ability to quickly and accurately generate protein profiles from small numbers of cells of interest in a heterogeneous tissue is of extraordinary utility in biological research. This simple sample preparation procedure can be easily adapted to different applications, and may be useful in a routine clinical setting.

Acknowledgments

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