In situ proteomics

Researchers are applying MS directly to tissue sections to determine the localized expression patterns of molecules.

BY MICHELLE L. REYZER AND RICHARD M. CAPRIOLI

Protein analysis has traditionally involved tissue homogenization, protein extraction (typically by multiple steps of liquid–liquid extraction and centrifugation), protein separation by liquid chromatography or gel electrophoresis, and molecular weight determination by mass spectrometry (MS) or gel electrophoresis. Unfortunately, this method suffers from many limitations, not the least of which is that once a tissue is homogenized, all spatial information is lost. Direct tissue analysis, however, makes it possible to localize proteins in a tissue section and, compared with multiple liquid–liquid extractions, minimizes analyte losses.

Matrix-assisted laser desorption/ionization (MALDI) MS is a powerful discovery tool that involves minimal sample preparation. The tissue is frozen, sliced, and put on the MALDI plate. By analyzing every protein signal that emerges from a given tissue—whether or not its identity is known—and comparing the protein profiles obtained for diseased and nondiseased states, the changes in protein expression between the two different states can be ascertained. This can rapidly lead to promising protein candidates that are useful as biomarkers and/or therapeutic targets.

But putting a piece of excised tissue directly into a sensitive and expensive time-of-flight (TOF) mass spectrometer seems impractical at best and destructive and costly at worst. Yet, for the past several years, Richard Caprioli and his colleagues at Vanderbilt University (Nashville, TN) have been doing just that to gain insight into differences in protein localization and expression between cancerous and noncancerous tissues. They have obtained protein profiles showing differences between normal mouse colon tissue and adjacent cancerous tissue. They have also acquired mass spectral images from a human glioblastoma showing differential localization of several proteins in different areas of the tumor, including thymosin β-4 on the proliferating periphery. This emerging technology takes advantage of the molecular specificity and sensitivity of MS to obtain protein maps of different tissues, enabling rapid discovery of protein expression differences between tissues and the distribution of proteins within a tissue.

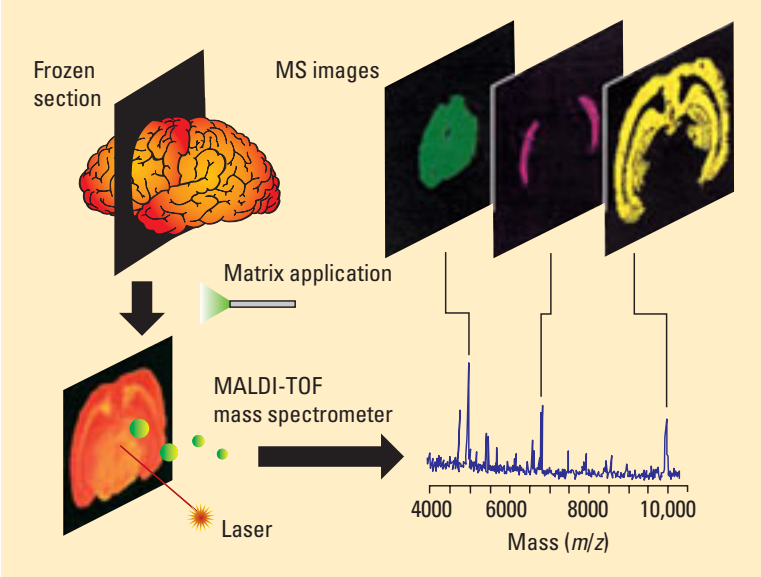

Figure 1. In situ methodology. A frozen tissue section is coated with matrix either evenly or in spots and is inserted into the MALDI mass spectrometer. To image the slice, the laser moves across the surface, and spectra are collected at each spot. The intensity of a protein's signal is plotted as a function of its location, and the resulting image displays the localization of that protein in the tissue. (Reprinted with permission from Stoeckli, M.; et al. *Nat. Med.* ***2001,*** *7,* 493–496.)

Technically speaking

MALDI-TOF MS is a powerful analytical technique whereby a solid analyte is mixed with a matrix, inserted into a high-vacuum ($\sim 10^{-6}$–10^{-7} Torr) chamber, and exposed to multiple shots from a laser, typically at a UV wavelength of 337 nm. The matrix absorbs the laser energy and assists with analyte desorption and ionization. The ionized analytes then drift down a long flight tube, where they are separated on the basis of their mass-to-charge ratio (m/z) and detected.

For tissue analysis, tissues of interest—which have included human brain gliomas and breast tumor biopsies—are excised, flash-frozen in liquid nitrogen, and kept at –80 °C until ready for analysis (Figure 1). The tissues are then cut into thin sections—12 µm typically suffices to cut through most of the cells and expose the cell contents—and thaw-mounted onto gold-coated MALDI plates. A matrix is then applied either in discrete spots on the tissue surface or evenly coated over the entire tissue, and the plate is inserted into the mass spectrometer.

Discrete spots of matrix allow for a quick examination of the protein signals in a tissue or for profiling protein expression in specific areas of the tissue, such as tumor vs normal breast tissue. An even, homogeneous coating is used for imaging the tissue slice, which is accomplished by moving the laser across the surface of the sample and collecting spectra at each spot. The intensity of a signal corresponding to a protein of interest is

plotted as a function of the location in the tissue, and the resulting image displays the localization of the protein in the tissue. Analysis of the proteins with retention of spatial information is thus obtained directly.

Limitations

For all its advantages, direct tissue analysis by MALDI MS has some drawbacks. Chief among them is that not all mass ranges are represented well, particularly proteins above 50 kDa. This is probably a combined effect of poor desorption and ionization of the larger proteins from the tissue and inefficient detection. In addition, the application of matrix to the tissue can result in analyte delocalization.

Other concerns are that the matrix may not effectively solubilize all proteins of interest and that homogenous crystal coverage over the entire tissue is difficult to achieve. Spectra obtained from different spots on a tissue may thus reflect differences in solubilization and crystallization rather than actual differences in protein composition. Averaging the signal over several spots on a homogeneous tissue, and comparing the resulting spectra from sequential tissue slices to ensure similar protein profiles, minimizes the effect of matrix application on the results. Although the use of MALDI-TOF as a discovery tool is a strength of the technique, unambiguous identification of the proteins of interest must be achieved by other means, typically by tryptic digestion of a protein extract and further MALDI MS and/or LC-MS/MS analysis. Progress in the area of on-tissue tryptic digestion should alleviate the need for additional homogenization and extraction steps and should allow for more rapid identification of the proteins of interest.

Finally, complex data processing is required to fully analyze the spectra. Each tissue section can generate hundreds of protein signals, and the differences between protein expressions of control and cancerous tissue may be subtle. Careful and accurate baseline correction, normalization, peak identification, and clustering programs are necessary to generate meaningful conclusions from the raw data.

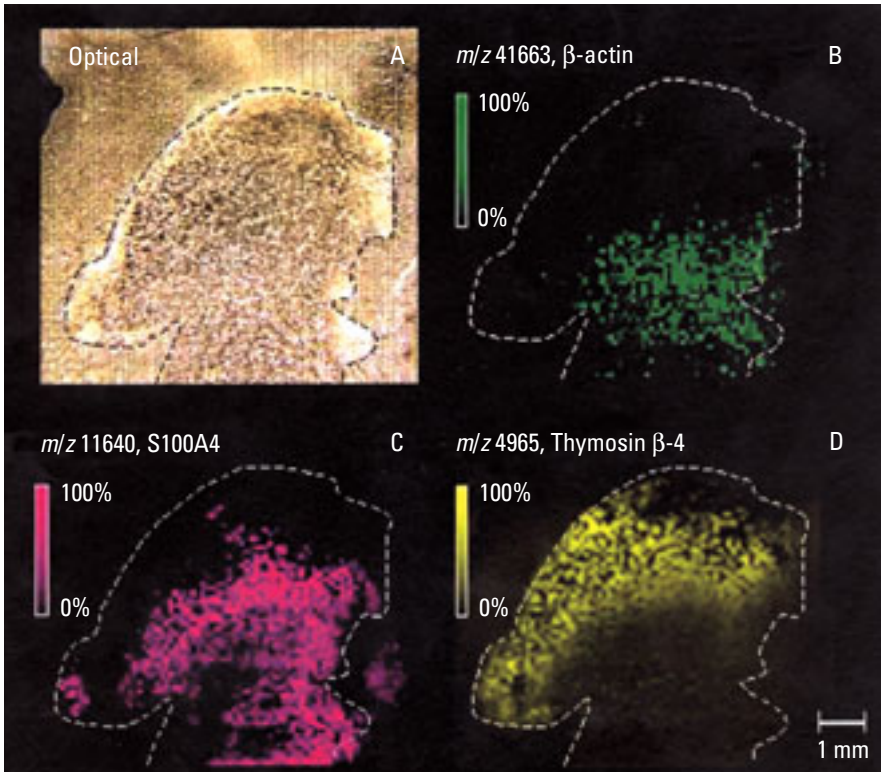

Figure 2. In situ expression. Multiple images of a human glioblastoma section (A) localize three different proteins in the tumor. Subsequent tissue extraction and tryptic digestion followed by LC-MS confirmed their identities as β-actin (B), calvasculin (C), and thymosin β-4 (D). (Reprinted with permission from Stoeckli, M.; et al. *Nat. Med.* **2001,** *7*, 493–496.)

Comparatively speaking

Direct tissue analysis and imaging by MS complement other techniques, including immunochemistry and fluorescent or radioactive labeling, which are currently used for the spatial localization of analytes. In immunochemistry, labeled antibodies that bind to one analyte of interest are exposed to a sample. The bound analytes are then monitored by detection of the labeled antibodies. Immunochemical techniques are sensitive and selective, but they are single-analyte methods, require the target analyte to be known, and require the existence of specific antibodies to the analyte. The specificity of the antibody–analyte interaction also affects the results because structural analogues of the target analyte might bind to the antibody as well. In contrast, the MS method is a multianalyte method that allows for the discovery of unknown proteins. And because each protein is monitored individually, no specific reactions are required.

Similarly, techniques that rely on fluorescence or radioactivity for detection require the target analyte to be known. Many fluorescent stains specifically interact with biological molecules, including DNA, antibodies, and proteins. To localize a particular analyte, the correct stain must be chosen and the resulting fluorescence monitored. In the analysis of pharmaceuticals, for example, the localization of a drug in a target tissue can be monitored by the detection of fluorescence or radioactivity. But again, the drug must be labeled to be observed, and only the tag is monitored. Thus, if a drug is metabolized and the metabolite retains the tag, it is impossible to differentiate the drug signals from the metabolite signals.

Labeling a compound also may change its chemical properties and biochemical reactivities. This effect might be minimal with radioisotope tagging, but large moieties containing one or more aromatic rings often are required to monitor a non- or low-fluorescing compound by fluorescence spectroscopy. MS alleviates these problems by directly evaluating the molecular weight of the compound of interest. As a multianalyte technique, it can monitor a drug together with its metabolites.

Applications

Current experiments using direct tissue analysis with MS can be roughly divided into three groups: protein profiling, protein imaging, and drug imaging. Protein profiling involves analyzing the protein expression in discrete areas on a tissue for comparison with a tissue in a different state (e.g., normal vs cancerous, untreated vs treated). For example, protein profiles obtained from mouse colon tumor revealed at least six distinct signals that were absent or barely present compared with adjacent normal tissue. Conversely, the normal colon tissue contained at least six distinct signals that were absent in the tumor tissue. Thus, 12 possible biomarkers were discovered in this relatively simple and rapid experiment.

Protein imaging is useful for understanding protein distribution in a tissue. Multiple images can be obtained in one experiment by simply selecting the mass ranges of interest to be monitored and then displaying the resulting ion-density maps. Figure 2 shows an example for a human glioblastoma (A). The three images show the localizations of three different proteins in the tumor. Subsequent tissue extraction and tryptic digestion followed by LC-MS confirmed their identities as β-actin (B), calvasculin (C), and thymosin β-4 (D).

Drug imaging is similar to protein imaging. The goal is not only to identify where drugs are localized in the target tissue, but also to correlate the presence of the drug with changes in protein expression.

The emerging technology of protein profiling and imaging by MALDI MS has already demonstrated its usefulness as a discovery tool. The full extent of its promise as a clinical tool for cancer researchers and others is only beginning to be explored.

Further reading

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