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Nanostructure-Initiator Mass Spectrometry Metabolite Analysis and Imaging

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Metabolites are downstream end products of gene and protein activity that closely correlate with the phenotype of a biological organism.[1–7] Therefore, by observing specific metabolic changes, one can gain insight into perturbations underlying disease.[8] Consequently, increasing attention has been dedicated to analyzing metabolites using MS in the context of clinical diagnostics, understanding disease mechanisms, and identifying new therapeutic targets.[5,9,10] The ability to analyze metabolites directly from biofluids and tissues continues to challenge current MS technology, largely because of the limits imposed by the complexity of these samples, which contain thousands to tens of thousands of metabolites.[11] A new technology being developed to address this challenge is Nanostructure-Initiator MS (NIMS), a desorption/ionization approach that does not require the application of matrix and thereby facilitates small-molecule (i.e., metabolite) identification.[12]

Surface-based mass analysis has seen a resurgence in the past decade, with new MS technologies focused on increasing sensitivity, minimizing background, and reducing sample preparation.[4,6,7,13,14] MALDI is one of the primary MS platforms currently used for the analysis of biological samples.[2,15,16] However, the application of a MALDI matrix can add significant background at <1000 Da that complicates analysis of the low-mass range (i.e., metabolites).[17] In addition, the size of the resulting matrix crystals limits the spatial resolution that can be achieved in tissue imaging.[14] Because of these limitations, several matrix-free desorption/ionization approaches have been applied to the analysis of biofluids and tissues.[17] Secondary ion MS (SIMS) was one of the first matrix-free desorption/ionization approaches used to analyze metabolites from biological samples.[18] SIMS uses a high-energy primary ion beam to desorb and generate secondary ions from a surface. The primary advantage of SIMS is its high spatial resolution (as small as 50 nm), a powerful characteristic for tissue imaging with MS.[19] However, SIMS has yet to be readily applied to the analysis of biofluids and tissues because of its limited sensitivity at >500 Da and analyte fragmentation generated by the high-energy primary ion beam.[14] Desorption electrospray ionization (DESI) is a matrix-free technique for analyzing biological samples that uses a charged solvent spray to desorb ions from a surface.[20] Advantages of DESI are that no special surface is required and the analysis is performed at ambient pressure with full access to the sample during acquisition.[20,21] The main limitation of DESI is spatial resolution because “focusing” the charged solvent spray is difficult.[22] However, a recent development termed laser ablation ESI (LAESI) is a promising approach to circumvent this limitation.[23] Desorption/ionization on silicon (DIOS), the precursor to NIMS, is another matrix-free laser-induced desorption/ionization approach.[24,25] DIOS utilizes a porous silicon substrate to absorb the laser energy and vaporize and ionize analytes on the surface without extensive fragmentation.[26] Because it does not use a matrix, it has the advantages of low chemical noise and high sensitivity in the low-mass range when used to analzye metabolites in

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biofluids. NIMS has subsequently replaced DIOS because the NIMS initiator makes the surface more compatible with imaging and experimental variation.[26,27]

FUNDAMENTAL FEATURES OF NIMS

NIMS was developed from the original DIOS technology but uses a liquid initiator to facilitate desorption. As a result, the NIMS surface is stable in ambient air, has improved reproducibility, enables direct biofluid analysis and tissue imaging, and allows for a significantly expanded mass range.[12] NIMS initiators are fundamentally different from MALDI matrices in that the initiators do not absorb UV energy, most do not ionize, and the analytes are not cocrystallized with the initiator.[28] During NIMS desorption/ionization, the porous silicon absorbs laser energy that results in rapid surface heating, vaporization of the trapped initiator, and desorption/ionization of the adsorbed analyte without fragmentation (Figure 1).[12]

NIMS silicon-based surfaces are not yet commercially available but are relatively straightforward to prepare with a procedure that involves three main steps: cleaning, etching, and initiator application.[28] Pre-etch cleaning is important for optimal sensitivity and minimal background noise.[28] NIMS performance is also dependent on etching time, with optimal performance at etching times on the order of 30 min.[28] NIMS has flexibility in the choice of initiator materials.[12,28] Typically, initiators are chosen such that they do not ionize and therefore do not introduce chemical noise into the spectrum. As a result, NIMS facilitates analysis of metabolites in the low-mass range. Additionally, the choice of initiator can substantially affect the ion profile observed in the mass spectrum of a biological sample.[12] Initiator-treated NIMS surfaces can be further modified by coating the substrate with NaCl or $AgNO_3$ to promote cationization of metabolites that are characteristically difficult to detect with traditional MS approaches, such as carbohydrates and steroids.[29] This flexibility to analyze the same biological sample with different NIMS initiators and surface modifications, and in-turn produce distinct ion profiles, is a particularly powerful approach to extract the maximum information from chemically complex samples.

The analysis of biofluids and tissues with NIMS requires essentially no sample preparation.[12,30] This is important for biological samples because sample preparation decreases throughput, potentially reduces analyte concentration, and introduces the possibility for sample degradation, modification, and/or contamination. Conversely, analysis of biofluids with GC/MS, LC/MS, or MALDI MS involves sample preparation and/or modification.[5,31,32] Specifically, GC/MS analysis of metabolites commonly requires molecular derivatization, which is not compatible with all classes of metabolites. Depending on the type of sample being analyzed (e.g., biofluid, cell lysate, metabolite extraction), LC/MS analysis of metabolites can require a sample clean-up step followed by a separation that typically takes ~1 h per sample. Finally, MALDI performance may be reduced in the presence of salts, so biofluids or tissue extracts usually require a desalting step before matrix application.[33,34] Because NIMS does not require derivatization or sample clean-up/desalting, analysis time is reduced to minutes per sample.

The method of sample application on the NIMS substrate affects both the performance and information contained in the mass spectrum.[28] Because NIMS depends on absorption of laser energy by the porous silicon substrate, applying excess analyte effectively reduces the laser intensity at the silicon surface and thereby reduces the overall performance of NIMS.[28] Excellent results from direct analysis of metabolites in biofluids have been achieved using a Z-touch sample application method (Figure 2),30 in which the sample is repeatedly applied and removed from the surface, with the excess ultimately drawn off to leave residual analyte for NIMS analysis.28 In situ extraction is another NIMS sample application method that

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utilizes the hydrophobic nature of the initiator-coated surface.[30] The sample is spotted onto the surface in one solvent (e.g., aqueous) and allowed to dry, and then an extraction is performed in situ from the dried sample spot using one or more different solvents (e.g., solvents with increasing organic content). Finally, the extracted material is reapplied to the NIMS surface as a new sample spot containing only the metabolites that are soluble in the extraction solvent. In this application, the NIMS surface effectively serves as a non-polar stationary phase to separate complex biological mixtures. Given the diverse chemical properties of metabolites, in situ extraction enhances metabolite analysis by facilitating selective extraction from the NIMS surface with different solvents.

Finally, during spectrum acquisition, NIMS requires lower laser fluence than what is typically required by MALDI. Excellent NIMS performance has been achieved recently with laser firing frequencies up to 400 Hz by limiting the total number of consecutive shots to 50, an experimental parameter that improves the throughput of tissue imaging with NIMS. Exposing a single spot (i.e., one-shot width) of the NIMS surface to significantly >50 consecutive laser shots at firing frequencies >100 Hz can degrade performance. This is likely due to excessive surface heating that generates a significant amount of background ions and suppresses analyte signal.

NIMS ANALYSIS OF COMPLEX BIOFLUIDS

Blood, urine, and saliva are examples of complex biofluids that are ideal samples for diagnostics because of the noninvasive manner in which they can be obtained and the rich biochemical information that they provide.[1–3,5] The first examples of using NIMS for direct analysis of biofluids were performed with blood and urine.[12] Submicroliter volumes were applied directly to the NIMS surface for 30 s, and then excess solution was removed with a stream of nitrogen gas. Significant chemical complexity was observed in both blood and urine, particularly in the low-mass (i.e., metabolite) range. Interestingly, markedly different mass spectra were obtained from the biological samples by using two different NIMS initiator materials, bis(heptadecafluoro-1,1,2,2-tetrahydrodecyl)tetramethyl-disiloxane and 1,3-bis(hydroxybutyl)tetramethyldisiloxane.[12]

Xenobiotic compounds can be readily detected in biofluids using NIMS. Using the Z-touch sample application method, ketamine (*m/z* 238.09) was detected in mouse urine and mouse blood 5, 30, and 120 min after intravenous administration.[30] Norketamine (*m/z* 224.08), a major metabolite of ketamine, was also observed.[30] In addition, NIMS sensitivity was shown to be linear with analyte concentration in serum spiked with drugs.[30] Because of the complexity of biofluids, some xenobiotics can be difficult to detect when present at low concentrations. To overcome this, in situ extraction with methanol was performed directly on the NIMS surface with saliva from a smoker and urine spiked with diazepam and raclopride (50–100 ng mL^{-1}).[30] After in situ extraction with 20% methanol, nicotine (*m/z* 163.11) and a major nicotine metabolite, cotinine (*m/z* 177.01), were detected at much higher intensity than without in situ extraction. In spiked urine, diazepam (*m/z* 285.07) and raclopride (*m/z* 347.09) were only detected after in situ extraction with 80% methanol.

Most of the current examples of using NIMS for the analysis of biofluids have been in positive-ion mode. However, analysis of biofluids with negative-ion mode is possible with NIMS when a compatible pre-etch cleaning method or initiator material is used.[35,36] When the highly acidic "Piranha solution" (sulfuric acid/hydrogen peroxide) was replaced with methanol as the pre-etch cleaning solvent, fatty acids were readily detected from a complex biofluid with NIMS.[35] The enhanced negative-mode sensitivity is presumably caused by more efficient anion formation from the less acidic NIMS surface resulting from the methanol pre-etch wash. Alternatively, the basic initiator 3-

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aminopropyldimethylethoxysilane allowed for the detection of several nucleotides from yeast-cell extract with very little background in negative-ion mode.36

A unique application of NIMS for the analysis of biofluids, termed Nimzyme, provides a measure of enzymatic activity in a biological sample (Figure 3).[37] In Nimzyme, single or multiple enzyme substrates are noncovalently immobilized on the porous silicon surface using a fluorous tag that favorably interacts with a perfluorinated initiator material that fills the pores. These immobilized substrates are then incubated with enzyme or biofluid before being washed. Because the fluorous-tagged substrate is insoluble in aqueous buffer, the substrate is retained at the surface during incubation and washing. After washing, the NIMS surface is analyzed by MS to measure the extent of substrate conversion to product based on an observed change in mass. The sensitivity of the Nimzyme assay was shown to be comparable to that of fluorescence-based assays, but with much lower noise.[37] Also, the Nimzyme assay works across pH and temperature ranges, thereby expanding the capabilities of the assay beyond standard biological conditions, which could be particularly useful in industrial applications.[37] Examples of Nimzyme biofluid analysis include a fluorous-tagged lactose substrate (*m/z* 1074.30) immobilized on a NIMS surface and incubated with *Escherichia coli* lysate or a microbial community lysate collected from Yellowstone National Park.[37] In each case, β-galactosidase activity was detected by a mass loss (product *m/z* 911.24) that corresponds to lactose hydrolysis.[37] These examples show the use of Nimzyme with a single enzyme substrate, but multiple fluorous-tagged substrates could be immobilized on the NIMS surface in an array format to significantly enhance the assay throughput (Figure 3).

NIMS IMAGING

Physiological and pathological conditions are characterized not only by the identities and concentrations of metabolites present, but also by the location of metabolites within a tissue.[38] Therefore, analytical tools that can provide both molecular identification and spatial information are essential to understanding tissue physiology and related disease conditions. Immunohistochemistry and fluorescence are conventional methods for identifying and localizing target molecules within a biological tissue.[39,40] However, the number of targets that can be detected simultaneously with these approaches is limited when compared to the number of ions that can be detected in a mass spectrum.[15] Also, with sufficient mass accuracy, MS can achieve higher levels of molecular specificity relative to immunohistochemistry and fluorescence.[15]

NIMS is a powerful platform for MS-based tissue imaging (Figure 4) that addresses several of the aforementioned issues related to SIMS and MALDI. Most importantly, NIMS requires no sample preparation and allows direct analysis of tissue immediately after applying it to the NIMS surface. Also, NIMS has an extended usable mass range when compared to SIMS, and fragmentation is not typically observed.12 Additionally, even with advancements in MALDI matrix application,41 potential limitations in spatial resolution introduced by a matrix exist, such as diffusion of metabolites within the tissue during matrix application and crystal size. These potential spatial resolution limitations are avoided with NIMS.[12] The absence of a matrix and enhanced sensitivity of NIMS not only makes it amenable to metabolite analysis of tissues, but also allows one to focus the laser used for desorption/ionization to a much smaller diameter (15–20 μm) than what is typically used in MALDI imaging (50–300 μm).[15] Taken together, these attributes of NIMS result in significantly improved spatial resolution when compared to MALDI imaging.30

The first example of NIMS tissue imaging was performed with 12 μm-thick mouse embryo slices (Figure 4).[12] Because of the thickness of those original tissue sections, an initial

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ablation step using high laser energy (~4 J cm^{-2}) was necessary. The initial ablation step removed much of the tissue slice and was not used for spectrum acquisition. Following high-energy ablation, a thin area of tissue remains and can be used to acquire a spectrum at lower laser energy (~0.01 J cm^{-2}).[12] Using this approach, anatomical features of the mouse embryo such as heart tissue and vertebra were clearly distinguishable by their ion profiles. Also, this same example demonstrated that different NIMS initiators could be used to generate markedly different ion profiles across the same tissue.[12] More recent NIMS tissue imaging experiments have shown that the initial high-energy laser ablation step can be eliminated by using tissue slices ≤4 μm-thick.[30,42] Here, ablation/desorption and mass spectrum acquisition are accomplished in a single step with moderate laser energies (~0.1 J cm^{-2}), thereby significantly increasing throughput and data integrity. By using 2–4 μm-thick rat brain slices, the antipsychotic drug clozapine (*m/z* 327.13) was shown to be primarily localized in the hippocampus 1 h after administration of a 3 mg kg^{-1} dose through the tail vein (Figure 4).[30] This result demonstrates the sensitivity of NIMS tissue imaging because the dose administered in this study is much lower than doses previously reported for MALDI imaging. Additionally, in these prior MALDI imaging studies, the drug was administered directly into the brain[43] rather than via the tail vein.

Cation-enhanced NIMS can also be applied to NIMS imaging of metabolites (Figure 5). In two recent examples of cation-enhanced NIMS imaging, Na^+ enhanced NIMS was used to image sucrose in a flower stem and Ag^+ enhanced NIMS was used to image cholesterol in a mouse brain slice.[29] In the case of the flower stem, NIMS showed high localization of sucrose in the vascular cambium and absence of sucrose in the xylem, which is consistent with plant metabolism of sucrose.[29] Imaging of mouse brain tissue showed localization of cholesterol in the corpus callosum and the medullary layer of the cerebellum.[29]

One of the unique features of NIMS is its sensitivity, with detection of analytes well into the yoctomole range (Figure 1).[12] Increased spatial resolution requires focusing the laser to a smaller diameter, which in turn desorbs less sample from the surface and requires high sensitivity for analyte detection. A striking example of an application that requires exquisite sensitivity is the detection of analytes from a single cell using NIMS (Figure 1).[12] With NIMS, significantly higher ion complexity was observed from a single cancer cell than that which was observed from 400–500 cancer cells with MALDI or nano-ESI.[12] This is a particularly important example because the analysis of a pool of hundreds or thousands of cells represents an “average” ion profile of many cells in many different states, which significantly complicates biochemical interpretation when trying to study specific pathways. The analysis of a single cell with NIMS eliminates problems of asynchronous metabolic profiles and, in turn, offers a significant advance for metabolomics analyses.[44–46] This is the MS equivalent to the promise of single-cell genomics.[47,48]

CONCLUSIONS

Surface-based MS platforms continue to develop for the analysis of complex samples such as biofluids and biological tissues. NIMS addresses some of the issues that exist with other surface-based approaches. Specifically, NIMS does not require a matrix, produces minimal analyte fragmentation, provides enhanced sensitivity, and requires no sample preparation. Additionally, NIMS offers the flexibility of different initiators, various surface modifications, choice of positive- or negative-ion mode, and in situ extraction methods. These characteristics of NIMS have been used to analyze metabolic profiles and detect specific endogenous and xenobiotic metabolites in biofluids and tissues.

Even with the flexibility and enhancements provided by NIMS, further improvements are needed for the analysis of biological samples. For example, because of the chemical

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complexity that exists in biofluids and tissues, ion suppression remains problematic. Improved ionization efficiencies may be achieved with NIMS through improved initiators or other surface modifications/additives. Furthermore, coupling NIMS to high mass accuracy and/or high-resolution instruments such as a FT mass spectrometer or a quadrupole time-of-flight (Q-TOF) mass spectrometer may increase specificity and thereby improve the deconvolution of complex biological samples. Significant improvements in NIMS imaging, particularly improvements in spatial resolution, may be accomplished through instrument modifications or potentially, by modification of the NIMS substrate. With the current capabilities and continued enhancements, NIMS has the potential to advance the ability to analyze complex biofluids and biological tissues, especially when applied to metabolite analysis.

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Gary J. Patti is a postdoctoral research associate in the laboratory of Gary Siuzdak at the Scripps Research Institute. His research focuses on the application of NIMS and LC/MS-based metabolomics in the area of translational medicine to understand disease pathology and identify novel therapeutic targets. He specializes in the metabolomics analysis of clinical samples such as plasma, cerebral spinal fluid, CNS tissue, and bacterial cultures.

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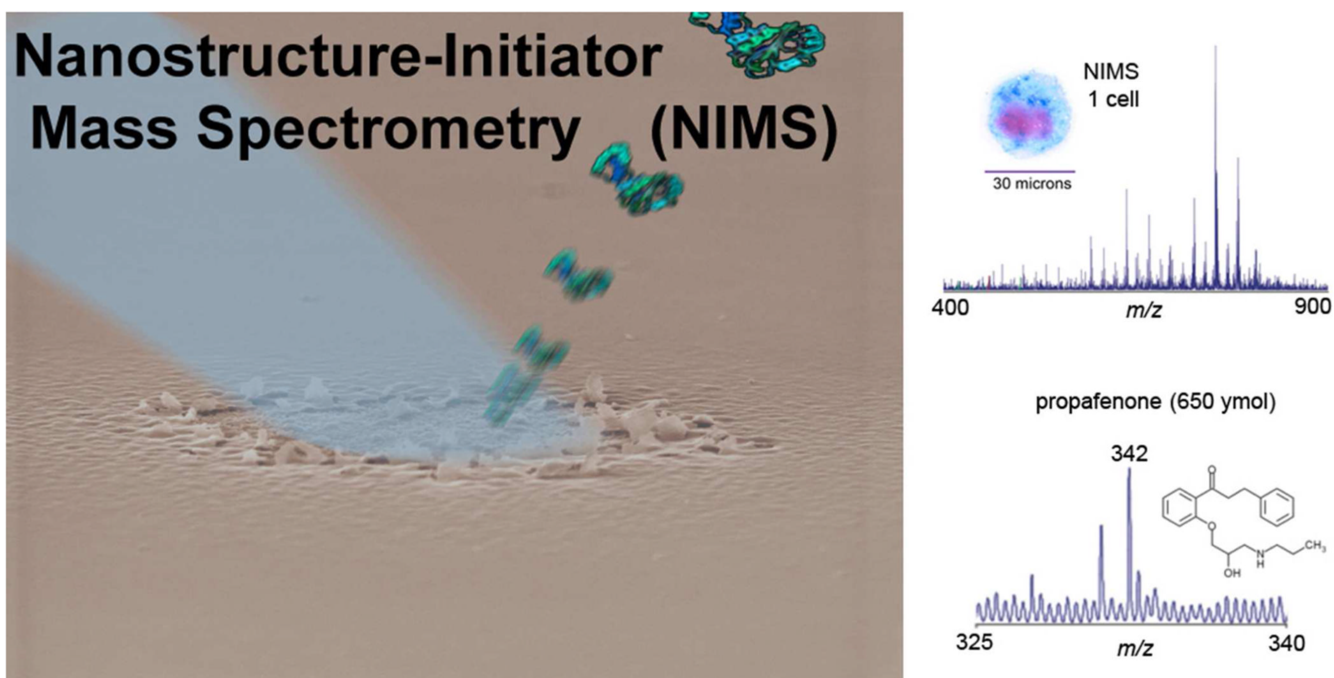

Figure 1.
An illustration of NIMS laser desorption/ionization superimposed on a scanning electron micrograph of the NIMS surface after irradiation with a single laser shot (left). The porous silicon surface absorbs laser energy, which causes rapid heating, vaporization of the initiator, and sample desorption/ionization. Because of the absence of a matrix, NIMS has minimal chemical noise in the low-mass range, thereby facilitating detection of metabolites in the yoctomole (10^{-24}) range. In addition, spatial resolution is limited only by laser spot size rather than matrix crystal size. Both the high spatial resolution and high sensitivity of NIMS have been demonstrated with the analysis of a single cell (right, top) and detection of 650 ymol of the anti-arrhythmic drug propafenone (right, bottom).

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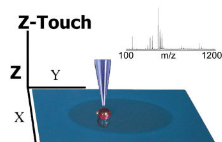

Figure 2.
The Z-Touch sample application method on a NIMS surface. The sample is repeatedly applied and removed from the surface to capture molecules on the surface. The method is effective because of the high sensitivity and the hydrophobic nature of the NIMS surface. Also, the hydrophobic surface facilitates Z-Touch sample application by producing a high contact angle with aqueous biological samples. Removal of the excess sample before drying improves the S/N performance of NIMS.

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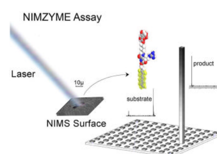

Figure 3.
NIMS analysis of enzymatic activity (Nimzyme). A fluorous-tagged enzyme substrate is noncovalently immobilized on the NIMS surface through hydrophobic interaction with a perfluorinated initiator. The surface is incubated with an enzyme or biofluid, then washed with aqueous solution, leaving the insoluble fluorous-tagged substrate. Analysis by MS indicates the extent of substrate conversion to product by a corresponding change in mass. Nimzyme can be performed in high-throughput by spotting different substrates, enzymes, or biofluids as an array.

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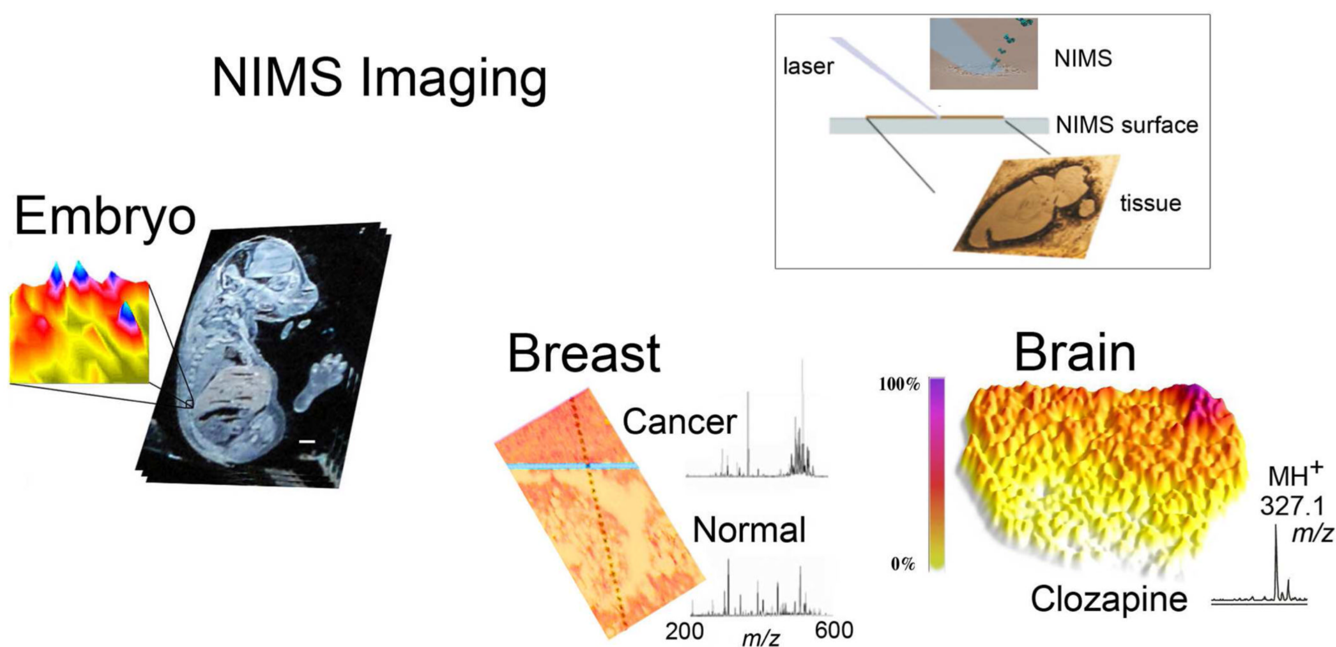

Figure 4.

NIMS as a platform for mass-based tissue imaging. NIMS imaging is performed directly from a 2–4 μm tissue slice placed on the surface, without modification, matrix application, or sample preparation, thereby reducing background noise and improving data fidelity. In addition, with NIMS, spatial resolution is proportional to the laser focus diameter. NIMS tissue imaging has been performed with a broad range of samples. For example, imaging of a mouse embryo demonstrates that NIMS is capable of resolving anatomical features by changes in the ion profile (bottom, left). Breast tissue imaging with NIMS shows clear differences in the low-mass range between cancer and normal tissue (bottom, middle). NIMS imaging of a mouse brain indicates that the drug clozapine (*m/z* 327.1) localizes in the hippocampus 1 h after administration (bottom, right).

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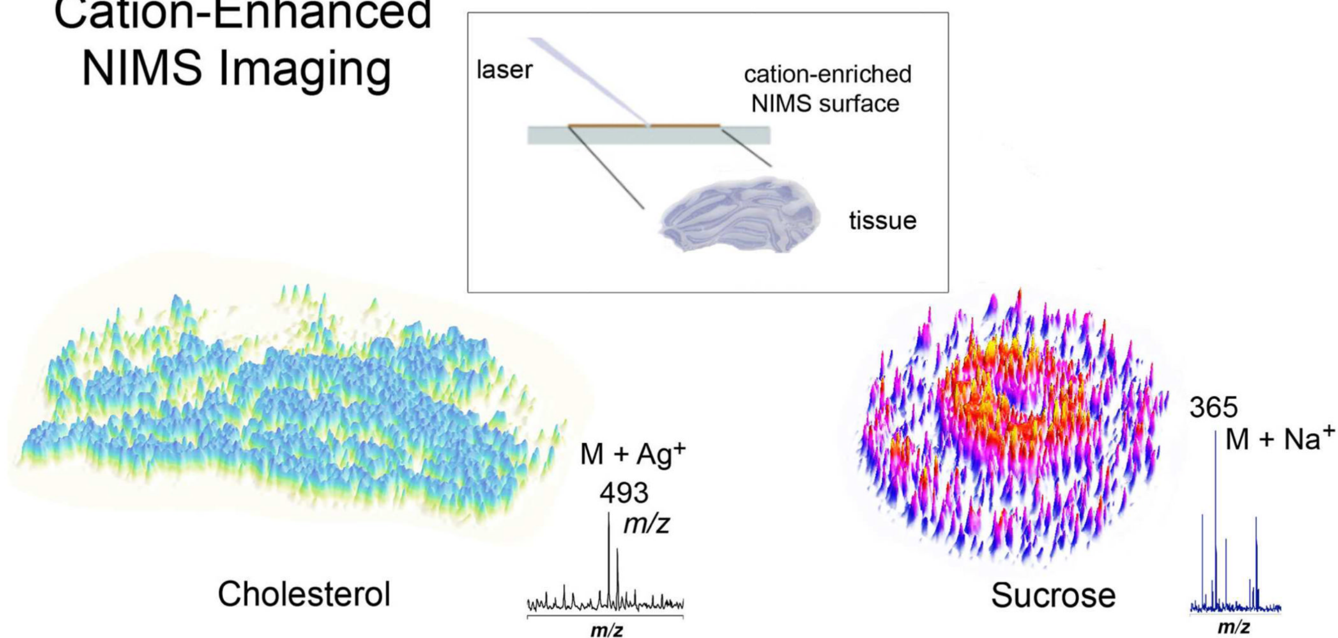

Figure 5.
Cation-enhanced NIMS tissue imaging. Mass-based images of analytes that are traditionally difficult to detect, such as steroids and carbohydrates, can be generated with cation-enhanced NIMS. For example, $AgNO_3$ was deposited onto a NIMS surface to detect cholesterol in a mouse brain (bottom, left). NaCl was deposited onto a NIMS surface to detect sucrose in a flower stem (bottom, right).