High Throughput Screening of Enzyme Activity with Mass Spectrometry Imaging

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Abstract: Mass spectrometry imaging (MSI) has found a diversity of applications ranging from localizing metabolites and proteins in tissues to investigating microbial interactions, and as a result is perhaps the fastest growing subfield of mass spectrometry. Advances in surface mass spectrometry technologies are equally applicable to the analysis of arrayed samples. One promising field in which this capacity has been leveraged is the high-throughput analysis of enzyme activity, an important step in the development of a wide range of biotechnologies. This review article describes several emerging approaches that seek to improve the quality and scope of this application of MSI.

Graphical abstract

Introduction

Understanding enzymatic catalysis is at the heart of biochemistry and essential for biotechnologies from biocatalysis to drug development[1–4]. Functional gene annotation of the overwhelming variety of enzymes identified in recent genomic efforts, combined with the diversity of possible substrates, inhibitors, and reaction conditions entails the exploration of a daunting experimental space. Classically, large-scale enzyme characterization efforts have employed surrogate substrates that change spectroscopic properties upon enzyme action. While high throughput (subsecond/sample), these approaches are applicable only to a narrow range of biochemical transformations, can be difficult to develop, have high false discovery rates, and are typically unable to distinguish multiple competing reactions. HPLC and LC-MS techniques have proven to be important compliments to spectrophotometric screens for many biological applications because of their analytical specificity and accuracy[5]. However, they are much lower throughput (minutes/sample) and are therefore typically

employed for smaller scale studies or hit-confirmation. Here we will focus on the application of Mass Spectrometry Imaging (MSI) towards high-throughput enzyme activity assays.

MSI is perhaps the fastest growing subfield of mass spectrometry (MS), allowing for the mass analysis of thousands of distinct locations on a surface. While the primary application of MSI has been the localization of biomolecules within tissues[6], the technology likewise enables the comparison of thousands of spatially defined samples (for a discussion on sample deposition techniques, see Box 1). One emergent application of this capability is the high-throughput characterization of enzyme activity.

A major challenge for any application of MSI is the limited number of analytes that can be detected simultaneously compared to the much more comprehensive – but low-throughput – analysis achieved by integrating chromatography with MS (e.g. GC-MS or LC-MS), which reduces the number of molecules competing for desorption and ionization at a given time. A variety of approaches have been developed to improve analyte detection in direct infusion methods. For example, the Agilent RapidFire platform automates a solid phase extraction step prior to analysis, in effect accomplishing HT-ESI-MS [7]. Recently, a similar but spatially defined extraction step has been developed for MSI[8]. Additionally, cleanup of samples on a surface can be achieved through selective analyte immobilization and washing off contaminants.

The convergence of sample cleanup technologies with MSI has the potential to provide the sensitivity and throughput needed for enzyme characterization and other applications. This review focuses on a number of these MSI-based enzyme activity profiling technologies, a topic which has not been reviewed since Ken Greis covered it in his comprehensive 2007 review[9]. For other applications of MSI, the reader is referred to a number of excellent reviews on tissue imaging[6,10] and imaging microbial interactions[11,12].

Mass spectrometry and imaging technologies

Generation of gas phase ions is an essential step for mass spectrometry, and in MSI this entails ionization and desorption of molecules from a surface. It is desirable to minimize fragmentation for many applications, including enzyme activity determination, and hence we will focus on prominent ‘soft’ ionization technologies. These can be divided into liquid- and solid-phase desorption/ionization approaches. Electrospray Ionization (ESI) is the foundational technology for most liquid-phase imaging approaches. Desorption ESI (DESI) uses the stream of charged solvent from an electrospray source to solubilize and desorb molecules from the MS surface[13]. Sample preparation is minimal, and the solvent composition can be varied to enrich for specific molecules. In NanoDESI, a nanoliter drop of solvent is scanned over the surface prior to ESI; solvent is delivered and aspirated off by two closely aligned capillaries near the surface [14]. Laser ablation ESI (LAESI) combines IR laser ablation followed by ESI ionization, enabling MSI of a wide range of hydrated samples. These liquid-phase analytical techniques have not yet been applied to high-throughput enzyme screening, however we anticipate that they will be as the technologies mature. One could envision, for instance, interfacing NanoDESI directly with one of the emerging ultrahigh-throughput microfluidic enzyme assays[4,15].

Technologies for solid phase analytics can be roughly divided into two categories: Matrix-Assisted Laser Desorption/Ionization (MALDI)-MS[16] and Surface-Assisted Laser Desorption/Ionization (SALDI)-MS[17] and both are fairly mature technologies regarding enzyme activity analysis. In MALDI-MS, analytes are co-crystallized with a matrix that absorbs laser light and transfers the energy to the analyte. In many cases, molecules <500 Da can be obscured by abundant matrix cluster ions and the requisite crystal formation limits resolution [6]. These drawbacks

of MALDI have motivated efforts to develop matrix-free Surface Assisted Laser Desorption Ionization (SALDI) approaches.

A variety of SALDI techniques have been developed, many of which have recently been reviewed [18]. The majority of these are based some form of nanostructured silicon, including Desorption/Ionization on Silicon (DIOS), NAPA and Nanostructure-Initiator Mass Spectrometry (NIMS). Both DIOS and NAPA are based on the direct laser Desorption/Ionization of analytes directly from these planar substrates[19,20]. NIMS is a unique approach to laser desorption based on a liquid coated nanostructured surface[21]. The ionizing laser heats the surface, which results in the explosive vaporization of the trapped liquid coat as well as the adsorbed analyte molecules.

Mass spectrometry for the spatial localization of enzyme activity

Recent developments in MSI are being used to provide additional information on the turn-over of proteins, metabolites within tissues and cells based on analysis of stable isotope incorporation. This is typically done with ion beam imaging [22,23], but has recently been extended to NIMS, enabling intact metabolite analysis[24]. MSI is also particularly well positioned to directly visualize the spatial distribution enzyme activity in tissues, bacterial communities and biomass. Enzyme activity imaging has traditionally been achieved via *in-situ* colorimetric assays, and activity-based probes in conjunction with fluorescence microscopy or MRI [25–27]. When MSI is employed, it is frequently to take advantage of high-sensitivity mass tags linked to activity-based probes, rather than imaging of enzyme substrates and products directly[28,29]. In a notable exception, Grobe *et al.* soaked tissue sections in a solution of angiotensin, followed by MALDI-MS imaging to detect the proteolysis product[30*,31]. We expect that new desorption approaches as well as some of the analyte enrichment techniques mentioned throughout this review present great promise for this application of MSI, in particular enzyme activity on low-MW molecules.

MSI technologies applied to high-throughput enzyme activity screening

As the most established surface MS method, MALDI has been applied to the detection of enzyme activity repeatedly. By and large, the reports of using MALDI have been of activity with high-MW substrates such as peptides and lipids (to prevent the analytes from being eclipsed by abundant matrix cluster ions), performed in buffer with purified enzymes (to avoid ion suppression). A low-MW enzymatic transformations that has been shown to be amenable to MALDI is catalyzed by acetylcholinesterase: both its substrate and product are stable cations and give ample signal even in the presence of matrix. Further enzymatic activities that have been monitored by MALDI are listed in Table 1. Noise from matrix ions may be diminished by selectively monitoring precursor-daughter ion pairs using MS/MS[32,33,34*], employing a high-MW matrix when analyzing low-MW molecules[35], or using the superior mass-resolution provided by MALDI-FTICR[36,37].

In many situations it is desirable to perform high-throughput enzyme activity assays in complex sample matrices. For example, inhibitor screening under endogenous conditions may give more biologically relevant results. In situations where protein purification is prohibitive, such as directed evolution campaigns, enzyme activity needs to be detectable in cell lysates. A few tactics to simplify these samples prior to analysis by MALDI are the use of microtitre-format SPE cardridges (e.g. Millipore ZipTips®), liquid-liquid extraction, and/or centrifugation[37,38]. Polyvinylidene fluoride membranes have been shown to be capable of selectively binding glycans from a relatively simple enzymatic reaction – a capacity that can likely be expanded to other classes of analytes – and are directly amenable to MALDI-MS[39]. In an alternative approach dubbed MALDI-assisted enzyme screening (MALDI-MES)[40], proteins in a complex sample matrix are covalently immobilized to sepharose beads. The modified beads are then transferred to a solution of the enzyme substrate in a simple buffer or water, and filtered out before analysis of the substrate and product by MALDI-MS.

Perhaps the most promising strategy for high-throughput targeted enzyme activity analysis in complex mixtures by MSI is the immobilization of analytes to the MS surface. This allows for a washing step before MS, reducing sample complexity, with the trade-offs being a potential for bias and effort in substrate preparation. Two variants of this approach are presented in Figure 1: The enzyme substrate can either be pre-immobilized on the surface, and the enzymatic reaction performed on the interface, or the reaction can be performed in solution with subsequent selective capture of the substrates and products onto the surface. One critical consideration in this approach is that the surface immobilization of the substrate needs to be sufficiently reversible during MS analysis to give adequate signal. Various suitable immobilization strategies will be discussed here, and examples of their applications are summarized in Table 1.

Self-Assembled Monolayers for matrix-assisted laser Desorption/Ionization (SAMDI) MS is a prominent implementation of the immobilize-wash-MSI scheme, spearheaded by the Mrksich group[41,42]. In this technique, the enzyme substrate is covalently attached to a self-assembled monolayer (SAM). The SAM, in turn, is attached to the surface through Au-S bonds, which are strong enough to permit thorough washing, yet weak enough to be analyzed by MALDI-MS (see Fig. 2a). Most implementations of the technique executed the enzymatic reaction on-chip with pre-immobilized substrates, but an approach capturing analytes from solution-phase reactions has been described as well[43]. In a superb example of what can be achieved with SAMDI-MS, the technique was applied to screen a library of 10,000 molecules against anthrax lethal factor, identifying a micromolar inhibitor of this the protease [44]. SAMDI is ultimately a MALDI-based technique, and hence it is particularly well suited for high-MW enzyme substrates like peptides and oligonucleotides. One noteworthy exception lies in a report by Ban *et al.* studying glycosyltransferase activity on SAM-immobilized mono-, di- and trisaccharides[45*].

Noncovalent hydrophobic interactions have also been employed as an immobilization strategy in conjunction with MALDI. Hydrophobic surfaces have been constructed using alkanethiolate SAMs on gold, as well as directly on glass microarrays, allowing for simultaneous MSI and fluorescence readout [46*] (see Fig. 2b).

The facile detection of low-MW analytes with DIOS-MS enabled the Siuzdak lab to develop enzyme assays based on this analytical platform. Substrate immobilization was incorporated via fluorophilic interactions[47] as well as covalent linkers that cleave upon laser irradiation[48] (see Fig. 2c). The integration of a liquid 'initiator' phase in the nanostructured silicon used for DIOS has resulted in a high-sensitivity desorption/ionization approach dubbed nanostructure-initiator mass spectrometry (NIMS). Since the initiator molecule used is perfluorinated[49], this platform inherently allows for a wash step that retains perfluorinated analytes (see Fig. 2d). This was put into practice by Northen *et al.*[50], who were able to detect glycosylhydrolase activity in lysate prepared from a complex microbial community. This technology was titled "Nimzyme". Since then, this assay has been extended to other classes of enzymes, as well as alternative capture chemistries by designing tagging reagents that contain, besides a perfluoroalkyl chain, a sugar-reactive hydroxylamine[51**] or a sulfhydryl-reactive maleimide[52]. Nimzyme has been implemented using on-chip as well as solution-phase enzyme reactions, where the perfluorinated substrates have been shown to form micelles[53]. Recently, Heins *et al.* applied NIMS to the high-throughput functional characterization of glycosylhydrolases[54**]. 10,000 different sample conditions were tested for activity on four mass-tagged substrates[55] simultaneously.

The realization that porous aluminum oxide may have SALDI activity similar to porous silicon led to the development of aluminum-coated glass that can be analyzed by MS[56]. Compared to DIOS and NIMS, this technique avoids the need to use corrosive hydrofluoric acid to etch the silicon surface. Covalent and fluorophilic immobilization of glycans has been achieved, as well as the ability to monitor glycosylhydrolase activity[57]. However, the technologies necessary to deposit a thin layer of aluminum onto glass slides are currently not widely

available, and the presence of aluminum oxide clusters in the lower mass ranges (<500 Da) may restrict the applications of aluminum coated glass to those already covered by existing MALDI-based techniques.

It should be noted that many of the approaches mentioned here are not truly label-free: The substrate that comes in contact with the enzyme is frequently modified with e.g., a perfluorinated tag, or attached to a self-assembled monolayer. While these substrates are a considerable improvement over fluorogenic surrogates, they are not perfect mimics of the enzyme's native substrates. Thus it will be critical to develop more innocuous affinity labels, as well as a greater variety of label-free approaches. Deng *et al.* [51**] recently reported a label-free approach based on oxime chemistry to label oligosaccharides following solution-phase enzymatic reactions, while Patrie *et al.* [58**] used antibodies to selectively capture a peptide of interest before subjecting it to SAMDI-MS. These approaches avoid the potential bias of surrogate substrates and allow for the analysis of complex and heterogeneous substrates.

Quantification of enzyme kinetics and inhibition

The aforementioned assays can provide critical qualitative as well as quantitative measures of enzyme performance including kinetics, specificity and inhibition. The high sample throughput of surface MS makes it an attractive technique to determine the kinetic constants of enzymes by means of acquiring detailed reaction time courses. When no washing step is employed (e.g. in traditional MALDI), pixel-to-pixel variation in ionization suppression and contamination necessitates the use of internal standards (usually an isotopomer of the reaction product spiked in at a known concentration after quenching)[5,51**,59,60]. In the case of a sufficiently simple mixture or successful washing, the substrate-to-product peak ratios can be sufficient to accurately model kinetic constants[45*,58**]. A notable approach described by Nichols *et al.* employs a droplet-based microfluidics device interfaced with a DIOS chip[61]. The progress of an arginase-catalyzed reaction could be determined near-continuously by letting a droplet of the reaction mixture travel along the length of a microfluidic channel while depositing analytes onto the DIOS chip, followed by MSI.

Conclusion

Throughout the last decade, great strides have been made towards MS-based high-throughput enzyme activity assays as an alternative to colorimetric assays. MSI-based methods have the potential to be robust, selective and high-throughput, and will likely become an important tool for enzyme activity screens. Overall, currently SAMDI and NIMS/Nimzyme are the most thoroughly developed techniques, though we anticipate that DESI, NanoDESI, and LAESI will all find important applications in this area. SAMDI and NIMS each have mass range limitations compared to the ESI approaches, but their mass ranges are complimentary: NIMS is more suitable for analyzing enzymes acting on low-MW molecules, while SAMDI excels in the high-MW range. In addition, a plethora of affinity and immobilization chemistries exist for MALDI and SALDI that have yet to be applied to enzyme activity assays[62]. MSI is also remarkably well positioned to visualize the spatial distribution of enzyme activity in tissue sections, and we expect that the *in-situ* sample cleanup techniques discussed could contribute greatly to such studies. As the throughput of MSI-based enzyme screening continues to grow, processing the large quantities of data generated will soon become a significant bottleneck, and hence streamlined data analysis platforms such as Open-MSI[63*] will be indispensable.

ACKNOWLEDGEMENTS

The DOE Joint BioEnergy Institute is supported by the US Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231.

Box 1: Liquid handling for MSI-based enzyme activity screening

Sample deposition onto the mass spectrometry surface is an essential step in the analysis of arrayed samples – such as those in an enzyme activity screening experiment – using MSI. It is desirable to maximize spot density and minimize sample volume to maximize throughput by increasing the number of samples per plate and minimizing the time spent swapping plates. Various technologies have been applied to tackle this requirement. Perhaps the simplest and most widely available is standard liquid-handling robots with 96- or 384-well heads[64**]. These instruments can usually only reliably transfer volumes > 1µL and can typically only spot 384 spots/plate.

Pin- and inkjet-based microarray fabrication technologies are widely accessible and have been employed to transfer small volumes onto a surface for MS analysis [21,57,65]. They produce spots within an acceptable volume range with exquisite special accuracy. The dispensing mechanism (usually custom-tailored steel pins) comes in direct contact with the liquid, so thorough washing is necessary between samples. Depending on the application, this can be a limitation; especially when transferring a large library of samples each with a characteristic enzyme activity profile.

Acoustic deposition is a contact-free liquid transfer approach that has been an efficient transfer technology in our hands [66*]. This technique is capable of transferring nL-scale droplets by transmitting bursts of sound waves into liquids in microtitre plate wells, resulting in droplet ejection from the well onto the MS surface. The technique requires no pin washing and can easily achieve a high spot density (~400 spots per cm², 10k/surface) for printing aqueous/alcohol solutions. Changing solvent systems has a strong effect on printing accuracy, which may be a limitation in some applications. Given that laser diameters can be focused to 1 µm resolution, in principle millions of samples could be printed and analyzed on a single MS surface.

Table 1: Examples of MSI technologies applied towards assaying enzyme activity

	MALDI			SALDI (Matrix-Free)		
Typical mass range	500 – 300,000 (matrix cluster ions obscure the low mass range)			< 4,000		
Surface chemistry	Traditional MALDI-MS [16]	SAMDI-MS [41]	Hybrid MALDI-MS [65]	DIOS-MS [20]	NIMS/Nimzyme [21]	Aluminum-oxide coated glass [56]
Enzymes activities monitored	Lipase [60,67], Decarboxylase [67], Peptide kinase[34*,68–70], Peptide hydroxylase [68], Acetylcholinesterase [32,36] Pyranose oxidase[71] Beta-lactamase[37,38], Glycosyltransferase [39], Pectin hydrolase [39]	Peptide kinase [42], Peptide arginine methyltransferase [43], Protease[44,58**,72], Deoxyribozyme[73], Polynucleotide kinase[73], histone deactylase [64**,74,75*], Glycosyltransferase [45*,76]	Glycosyltransferase [65], Glycosylhydrolase [46*,65,77]	Acetylcholinesterase [59,78–80] Phenylalanine hydroxylase [80], Glycosyltransferase [80], Arginase [61], Peptide kinase [47]	Glycosylhydrolase [50,51**,53,55,66*,81], Glycosyltransferases [50], Peptide kinase [82], Chloramphenicol acetyltransferase [83]	Glycosylhydrolase [57]
On-surface enzymatic reaction followed by washing (top path in Fig. 1)	N/A	Demonstrated [42,44,45*,64**,72–74,76]	Demonstrated [46*,65,77]	Not Demonstrated	Demonstrated [50,82]	Demonstrated [57]
In-solution enzyme reaction followed by capture on surface and washing (bottom path in Fig. 1)	N/A	Demonstrated [43,45*,58**,75*]	Not Demonstrated	Demonstrated[48]	Demonstrated [51**,53,55,66*,81,83]	Demonstrated [57]
Capture chemistries employed	N/A	Cysteine-Maleimide [43,64**,74,75*], His6-Ni ²⁺ [58**], Antigen-Antibody [58**], Biotin-Streptavidin [73], Alkyne-Azide [45*]	Hydrophobic [46*,65,77]	Fluorous [47,84] Biotin-Streptavidin [48], Alkyne-Azide[48], Cyseine-Iodoacetamide [84]	Fluorous (inherent in NIMS surface)[50], Hydroxylamine-Aldehyde [51**], Cysteine-Maleimide [52]	Fluorous [57]
Complex sample matrices investigated	Supernatants of: Whole bacterial cells in buffer [38], bacterial cell lysate [33], milk [37]	Mammalian cell lysate [72,74,75*] Blood plasma [58**], Cell-free expression system [45*]	Environmental isolate supernatant [77], Saliva [77]	Artificial mixtures of peptides [48] or small molecules [47]	Bacterial cell lysate [50,83], Microbial community lysate [50]	Enzymatic activity demonstrated only with purified protein in buffer.

This table enumerates the circumstances under which 6 different surface MS-based high-throughput enzyme activity assays have been shown to work. Both proof-of-concept studies and true high-throughput screens are included. For an in-depth description of the techniques, please refer to text and **Fig. 2**.

Figure 1

Generalized workflow of high-throughput targeted enzyme activity analysis through surface immobilization, washing and mass spectrometry imaging. In one implementation, the enzyme substrate (or a panel of candidate substrates) is pre-immobilized on the MS surface and treated with samples to be tested for enzymatic activity (top path). Alternatively, the enzymatic reaction can be performed in solution, and the substrate and/or product selectively captured from the mixture onto the surface (bottom path). In either situation, the surface is subsequently washed, which eliminates signal suppression by cell debris, salts, and other contaminants possibly present in the sample. For MALDI-based approaches this is followed by matrix application, while for SALDI-based technologies this step can be omitted. Finally, the surface is analyzed by MSI and the signals quantified to attain enzyme activity measurements.

Figure 2

Surface immobilization chemistries that have been applied towards enzyme activity screening by MSI. Analyte immobilization allows for cleanup of the sample prior to MSI by washing. Hence, the interaction between MS surface and the analyte needs to be able to withstand washing, yet labile during analysis by surface MS. a) In SAMDI[41], immobilization is achieved through self-assembled monolayers that, thanks to the weak S–Au bond, can be analyzed by MALDI-MS following the application of matrix. b) In the approach described by Belouqui *et al.*[46*], analytes are immobilized through non-covalent interactions with a hydrophobic glass microarray slide, and visualized by MALDI-MS. c) In the method of Meng *et al.*[48], analytes are covalently captured onto a nanostructured silicon surface through a linker that undergoes a retro-Diels-Alder reaction when subjected to DIOS-MS. d) In Nimzyme[50], perfluorinated analytes interact non-covalently with a perfluorinated initiator liquid trapped in a porous silicon surface. During analysis by NIMS, the initiator liquid is vaporized along with the trapped analyte. DIOS and NIMS are SALDI methods and as such do not require addition of matrix prior to MSI.

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Mouse kidney tissue sections were soaked in a solution of angiotensin and after a brief incubation were imaged using MALDI-MS. Distinct spatial distributions of different angiotensin proteolysis products could be visualized. Addition of inhibitors allowed the authors to deconvolute which protease is active in which area of the tissue section.

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MS/MS (performed here on an AP-MALDI-Orbitrap instrument) can be used to quantify low-MW molecules that would be obscured by matrix ions in a conventional MALDI-TOF instrument. This principle is applied here to the high-throughput screening of inhibitors of kanamycin phosphotransferase while simultaneously monitoring inhibition of a native human peptide kinase, through Multiple Reaction Monitoring. The use of Atmospheric Pressure MALDI presumably improves throughput by eliminating instrument pump-down time.

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