# Applications and Continued Evolution of Glycan Imaging Mass Spectrometry

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## Abstract

Glycosylation is an important post-translational modifier of proteins and lipid conjugates critical for the stability and function of these macromolecules. Particularly important are N-linked glycans attached to asparagine residues in proteins. N-glycans have well defined roles in protein folding, cellular trafficking and signal transduction, and alterations to them are implicated in a variety of diseases. However, the non-template driven biosynthesis of these N-glycans leads to significant structural diversity, making it challenging to identify the most biologically and clinically relevant species using conventional analyses. Advances in mass spectrometry instrumentation and data acquisition, as well as in enzymatic and chemical sample preparation strategies, have positioned mass spectrometry approaches as powerful analytical tools for the characterization of glycosylation in health and disease. Imaging mass spectrometry expands upon these strategies by capturing the spatial component of a glycan's distribution in-situ, lending additional insight into the organization and function of these molecules. Herein we review the ongoing evolution of glycan imaging mass spectrometry beginning with widely adopted tissue imaging approaches and expanding to other matrices and sample types with potential research and clinical implications. Adaptations of these techniques, along with their applications to various states of disease, are discussed. Collectively, glycan imaging mass spectrometry analyses broaden our understanding of the biological and clinical relevance of N-glycosylation to human disease.

## Keywords

Imaging mass spectrometry; N-glycan; MALDI; Glycosylation; Mass spectrometry

## I Introduction

### A Glycobiology of N-glycans and mass spectrometry

This chapter is provided in recognition of the many seminal studies and advances from David M. Lubman to the field of mass spectrometry, glycosylation and biomarker research (Mengmeng Wang et al. 2019; Zhu et al. 2019; Patwa et al. 2010). This review will summarize the advantages, limitations and applications of N-glycan imaging mass

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spectrometry approaches applied to tissues and biofluids from clinical and animal models of disease (Richard R. Drake et al. 2015; Richard R. Drake 2015; R. R. Drake et al. 2017; Angel et al. 2017; Richard R. Drake, West, et al. 2018; Scott and Drake 2019; C. R. K. Blaschke et al. 2021). Glycosylation is the enzymatic addition of carbohydrate residues (or glycans) to a biomolecule, most commonly to a protein or lipid. These sugars can either be attached as monomers or can be linked together to form long, complex chains in a non-template driven synthetic manner. Individual sugar moieties used to produce glycans are taken up directly by the cell or synthesized via numerous metabolic pathways and include, but are not limited to, glucose (Glu), mannose (Man), galactose (Gal), fucose (Fuc), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and N-acetylneuraminic acid (Neu5Ac) (Cummings 2009) (Figure 1A). Together these sugar chains form a dense carbohydrate scaffold on the cell's outer membrane known as the glycocalyx (Reitsma et al. 2007). This surface is comprised of multiple subclasses of glycosylated entities, namely N- and O- linked glycoproteins, glycosaminoglycans (GAGs), proteoglycans and glycolipids (Martinez-Seara Monne et al. 2013). The glycocalyx governs interactions with the local microenvironment by acting as a barrier, filter, substrate and active chemical or enzymatic agent. Glycosylated proteins and lipids in this surface are implicated in cell-cell communication, signal transduction, secretion, interactions with the immune system, cellular migration and adherence, proliferation and regulation amongst other processes (Alphonsus and Rodseth 2014; H. Liu et al. 2020). Critically, the glycocalyx is sensitive to the temporal physiological state of the cell and reflects internal cellular health (Kuo et al. 2018). Thus, it has been well studied that the structure and composition of the glycocalyx changes in response to a variety of diseases (Yilmaz et al. 2019). N-linked glycans, attached to asparagine residues as part of N-X-S/T motif sites on the glycoprotein backbone, are able to play diverse cellular roles due to their structural heterogeneity. Biosynthesized in the endoplasmic reticulum (ER) as a dolichol-linked oligosaccharide substrate, N-glycans are transferred to nascent protein cotranslationally and subsequently processed by a series of glycosidases and glycosyltransferases in the ER and Golgi as part of the secretory pathway (Stanley P 2017). Three main structural classes of N-glycans result from this processing, and are termed oligo- or high mannose, hybrid and complex. Examples of these three N-glycan classes, as well as common N-glycan structural motifs referred to in this review, are shown in Figure 1B,C. The wide variety of possible N-glycoforms interface with an even wider network of molecular interactors to govern cellular processes such as protein folding, immune regulation, cellular mobility and cell signaling.

N-glycosylation is known to be significantly altered in cancer, both as a response to pathophysiology and as a direct governor of oncogenesis, tumor maintenance, immune evasion and metastatic escape (Mereiter et al. 2019). To this end a variety of methodologies have been developed to analyze N-glycosylation (Mulloy et al. 2017) (Figure 2). Lectins, carbohydrate binding proteins that recognize specific structural epitopes of N-glycans and other glycoconjugates, have been used historically for glycomic analyses, and have since been optimized and adapted into a wide variety of technologies, including microarray and chip formats (Cummings et al. 2017; Patwa et al. 2010). For analysis of tissues and surface-attached targets, lectins are typically used to detect N-glycans through immunohistochemical (IHC), immunofluorescent (IF) or colorimetric assays. Lectins are generally specific for

binding glycan structural motifs rather than individual glycan species, and have somewhat variable affinities, limiting their use for precise glycan structural determinations. A wide variety of mass spectrometry approaches have been developed to address the structural characterization of glycans in biological samples (Mengmeng Wang et al. 2019; Ruhaak et al. 2018). Analysis of N-glycans by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) has emerged as a powerful approach in the last decade. An advantageous feature of LC-MS/MS is the ability to separate N-glycan isomers using online high- or ultra-performance performance liquid chromatography (HPLC/UPLC), facilitated by porous graphitized carbon (PGC) or similar columns (Veillon et al. 2017). When coupled with sequential exoglycosidase digestions or ion mobility-equipped mass spectrometers, more precise structural information can be obtained (Guile et al. 1996; Lane et al. 2019). While effective, in the context of potential diagnostic or clinical applications, these strategies require lengthy purification, chemical derivatization and separation steps which preclude the analysis of large sample cohorts in a feasible timeframe. Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of spotted, released N-glycans is another established approach (Ruhaak et al. 2018). While this reduces data acquisition time, most MALDI-TOF MS analyses still require lengthy purification and derivatization steps prior to analysis (Morelle et al. 2009). Imaging mass spectrometry for N-glycans addresses the shortcomings of these previously mentioned technologies as well as introduces advantageous features of its own (Richard R. Drake, West, et al. 2018). Even more complex investigations into precise N-glycan structural linkages can be completed in timeframes acceptable for high-throughput or even clinical applications.

## B  Imaging mass spectrometry

Imaging mass spectrometry (IMS) provides the ability to spatially localize analytes detected by MS, and it was first developed for the analysis of metal alloys and semiconductors in industrial applications (Pacholski and Winograd 1999; Galle, Hamburger, and Castaing 1970). Matrix-assisted laser/desorption ionization (MALDI) IMS on biological tissues was first reported in 1997 (Caprioli, Farmer, and Gile 1997). The Caprioli group demonstrated that by rastering the laser beam across the sample surface in a gridded fashion, pseudo-colored heat maps could be generated to show the in situ distributions and abundances of ions of interest where each "pixel" represented a mass spectrum at each tissue location. Imaging experiments utilizing a MALDI source rely on two primary factors: the application of an energy-absorbing organic matrix to the tissue sample and the use of a nitrogen or neodymium-doped yttrium aluminum garnet (Nd:YAG) laser for ion generation from a single coordinate within the tissue, called a data pixel (Robinson, Steven, and Bunch 2018; Holle et al. 2006). These characteristics made MALDI an ideal technology for the initial development of N-glycan IMS, as N-glycan ions have been traditionally difficult to ionize due to their large size and complex structural properties (Powers et al. 2013; Richard R. Drake, Powers, et al. 2018; Powers et al. 2014).

Imaging mass spectrometry has distinct advantages as an imaging modality. Imaging by light microscopy limits the number of identifiable analytes in a single experiment, whereas IMS platforms may detect hundreds of analytes at the same time (Tan et al. 2020). Unlike immunohistochemical or immunofluorescent staining, IMS experiments do

not require recognition of an epitope sequence, allowing for in depth and multiplexed profiling of analytes that are not easily recognizable by antibodies, e.g., metabolites, lipids, post-translational modifications. The high-resolution mass analysis afforded by current mass spectrometers allows for reporting of analytes with very similar masses and high confidence in the specificity of molecular assignments, a feature especially useful in metabolomics and lipidomic investigations (Bowman et al. 2020). New advancements in laser, computational and instrumental technologies have facilitated high spatial resolution ( 10 μm) combined with high speed data acquisition (Prentice and Caprioli 2016). A broad range of analytes are detectable by label free imaging of proteins, lipids, nucleic acids, carbohydrates, metabolites and pharmaceuticals ranging from less than 100 to more than 100,000 Da (Mainini et al. 2013). Additionally, the sensitivity of imaging mass spectrometry platforms allows for the detection of molecules of interest at femtomolar levels directly from their biological milieu (Leopold et al. 2018). Most critically, the ability to analyze disease-mediated molecular and morphological features from patient tissue specimens is core to the insights gained by many IMS experiments. Linking histopathologically-defined tissue structures to the expression of specific biomolecules potentiates the discovery of disease markers and therapeutic targets. At its most reductive function, IMS acts as a "molecular microscope" to reveal the biological and chemical mechanisms underpinning pathophysiology, resulting in better understanding of the origins, maintenance and progression of disease.

## II    IMS for N-glycans in tissues

The basic N-glycan MALDI IMS schematic is shown in Figure 3. A key component of the N-glycan IMS workflow is the use of a solvent sprayer to apply a thin molecular layer of peptide N-glycosidase F (PNGase F) enzyme onto tissue or cells to release only N-linked glycans from their carrier proteins. Diffusion of the released glycans is minimal allowing spatial co-localization to tissue regions and specific cell types. The first IMS workflows were developed around MALDI platforms, thus, the focus of this review is primarily on MALDI-IMS strategies, although alternative technologies will be briefly covered. Glycan IMS enzymes, chemical matrix, tissue preparation and data analysis strategies are also summarized.

### A    Sample Preparation

**2.1.1    Tissue sources and sample types—**Current MALDI-IMS workflows are able to accommodate both fresh-frozen (FF) and formalin-fixed paraffin-embedded (FFPE) tissue sources from clinical pathology labs, tissue biorepositories, and animal models, providing in essence a nearly unlimited supply of specimens for analysis (Richard R. Drake, Powers, et al. 2018) (Figure 4A). The automated and systematic solvent exchanges required to produce an FFPE block seem to improve and facilitate reproducible detection of N-glycans in FFPE tissue, likely by extensive removal of competing metabolites and lipids. Most N-glycans do not have free amino groups, and thus are not crosslinked in formalin. For optimal results, FF tissue also requires extensive washing to remove metabolites and lipids for detection of N-glycan signal. FFPE tissue slices (3–7 μm; 5 μm standard) are cut from pathology blocks or cores from tissue biopsies and affixed to glass slides (indium tin oxide (ITO)-coated for analyses in TOF instruments). Sections are dewaxed and rehydrated in a graded series of

ethanol and water washes prior to heat induced epitope retrieval in a citraconic anhydride-based buffer, required to break bonds created by formalin fixing (Figure 4B,C). For FF tissues, desiccated cryosections (5–12 μm; 10 μm standard) are rinsed in organic solvents to remove lipids, salts and other metabolites, thus increasing downstream N-glycan signal. FF tissues do not require antigen retrieval prior to enzymatic N-glycan release, but heat denaturation improves signal. In addition to whole-tissue IMS, both FFPE and fresh-frozen tissue samples are routinely homogenized for N-glycan extraction and purification (Balog et al. 2012). Doing so enables the analysis of multiple tissues on the same MALDI target plate and can aid in fragmentation and additional structure confirmation tests that support tissue findings. Liquid-phase extraction of N-glycans from tissue homogenate is routinely used orthogonal to whole-tissue MALDI-IMS for precise structural identification via ESI-LC-MS/MS (Holst et al. 2017).

**2.1.2    PNGase F de-glycosylation and Matrix application—**N-glycans are released from their glycoprotein carriers via on-tissue incubation with peptide N-glycosidase F (PNGase F), which cleaves between asparagine and the first GlcNAc residue on the chitobiose core of the N-glycan (Tarentino and Plummer 1994) (Figure 4D). For N-glycan IMS, the retention of analyte spatial information is accomplished through a "dry" molecular coating of PNGase F that limits diffusion of released N-glycans across the tissue. Current protocols using an automated sprayer system to apply this coating are robust and highly reproducible (Richard R. Drake, Powers, et al. 2018). Tissue slides sprayed with PNGase F are incubated in a pre-warmed (37°C) chamber at    80% relative humidity for a minimum of two hours to remove the N-glycan structures (Figure 4E). Orthogonal structural analyses can be done using tissue homogenates following deglycosylation with PNGase F followed by N-glycan purification and derivatization steps prior to matrix incorporation and spotting for MALDI-TOF MS (Jensen et al. 2012) or ESI-LC-MS/MS (Holst et al. 2017; Briggs, Condina, Klingler-Hoffmann, et al. 2019).

Desorption and ionization of analytes into a MALDI mass spectrometer for detection is dependent on the application of a crystalline chemical matrix, which absorbs laser energy and transfers it to the substrate below resulting in a transition to the vapor phase and the ionization of analytes (Karas, Bachmann, and Hillenkamp 1985; Karas and Hillenkamp 1988; Jaskolla and Karas 2011) (Figure 4F). The most common organic acids used for N-glycan IMS are α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) (R. R. Drake et al. 2017). Other matrix molecules like 1,5-diaminonaphthalene (DAN) and 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP) have also been used (Hossain and Limbach 2012; Nishikaze 2017). Because matrix crystal size and consistency of matrix application are critical determinants of analyte signal intensity and reproducibility, most current approaches use an automated sprayer for N-glycan imaging experiments. Ideally, the CHCA-coated tissue slides are analyzed within a few days after preparation but can be stored in a desiccator for several weeks (Figure 4G).

## B    Instrumentation and ion sources

N-glycan ions span a wide mass range, with the simplest sugar chains detected at ~700 *m/z* and multiply branched, fucosylated and sialylated structures detected upwards of 4,000 *m/z*,

which approaches the limit of detection for most current MALDI-IMS configurations. There are glycan species of unknown compositions that exceed 4000 m/z, and routine detection of these species represents an area for further study. Further complicating factors for N-glycan MALDI-IMS analysis are variable formation of sodium adducts associated with sialic acids, isotopic overlap between structures, the presence of sulfate groups on some species and interference from the chemical matrix. N-glycans are typically detected in positive mode, however certain special applications are better suited to negative mode analysis (Harvey 2020). To meet these needs, two broad types of MALDI mass spectrometers, each with their own advantages and disadvantages, are typically used to detect released N-glycan ions from tissue, those being Fourier transform ion cyclotron resonance (FT-ICR) and time of flight (TOF) instruments. Recent instrument configurations incorporating a second MALDI laser (termed MALDI-2) and ion mobility separations coupled with MALDI-Q-TOF have recently been used for N-glycan IMS. These are each briefly summarized as follows.

**2.2.1   Time of flight MS—**Time of flight mass spectrometry, although typically less sensitive, offers access to a broad m/z range of molecules from metabolites to DNA and higher throughput than FT-ICR instruments, and are thus present in many mass spectrometry laboratories. The basic principle of TOF is that ions separate based on the time it takes them to travel through a flight tube of known length and reach the detector (Greaves and Roboz 2013). Although TOF mass spectrometers have generally less resolving power, the rapid time from the laser-ejection of N-glycan ions to detection increases the number of pixels which can be analyzed in a given time frame (up to 50X faster than FTICR-MS for some instruments), resulting in the practical feasibility of much higher spatial resolution analyses (< 5 μm) (Ogrinc Poto nik et al. 2015; Kamata et al. 2020). Also, because of their capacity for higher throughput, TOF mass spectrometers are routinely used for assessment of N-glycans from tissue homogenate via spotting on multi-sample target plates. Another advantage of TOF instruments is that there is no theoretical bound to their upper m/z detection limits. As m/z in these instruments is ultimately a function of flight time, increasing the length of the drift tube allows for detection of analytes with larger masses (J. Lee and Reilly 2011).

**2.2.2   FTICR-MS—**Ion-Cyclotron Resonance (ICR) detectors excite N-glycan ions using alternating radiofrequency pulses inside a powerful magnetic field (typically ranging from 7T to 21T), resulting in differential cyclotron resonance frequency of the ion packets within the detector proportional to their masses (Karabacak et al. 2010; Smith et al. 2018; Marshall, Hendrickson, and Jackson 1998; Scigelova et al. 2011). This amalgam of resonances is detected by opposed detection plates inside the ICR cell as a single time domain current which is then deconvoluted into its component frequencies by Fourier transformation, for which this class of mass spectrometers bears its name. FT-ICR instruments achieve the highest resolving power (R) in the space (R > 1,600,000) (Bowman et al. 2020) proportional to the strength of the instrument's magnetic field. However, this resolving power drops off for high-mass analytes (> ~2,000 *m/z*) without specialized source adjustments (Prentice et al. 2018). Much of the initial N-glycan IMS workflows were developed using a MALDI-FTICR instrument (Drake et. al., 2017).

**2.2.3  Ion mobility MS**—One of the challenges facing N-glycan MALDI-IMS analysis is the separation of isomeric analytes. The non-linear, non-template driven biosynthesis of N-glycans results in a breadth of structural diversity. Within this heterogeneity are a variety of MS-detected masses that comprise multiple compositionally-identical analytes which differ only with respect to the anomeric linkage of specific residues (Devakumar et al. 2008). Terminal versus bisecting GlcNAc moieties, the specific attachments of sialic acids and core versus outer arm fucosylation, amongst other characteristics, are typically indistinguishable using conventional IMS analyses. Non-imaging mass spectrometry experiments typically resolve glycan isomers using sequential exoglycosidase digestions, potentially coupled with or orthogonal to HPLC/UPLC column-based strategies prior to analysis of electrosprayionized analytes via LC-MS/MS (Mauko et al. 2012; Tao et al. 2014; Yin et al. 2016). Laser ionization of N-glycan ions directly from tissue has not traditionally afforded incorporation of isomeric separation techniques. The recent introduction of MALDI MS instruments with ion mobility capabilities like drift tube ion mobility (DTIMS), traveling wave ion mobility (TWIMS), trapped ion mobility (TIMS) and high-field asymmetric ion mobility (FAIMS), amongst others, helps to deconvolute this complexity (May et al. 2015; Fernandez-Lima 2016; Spraggins et al. 2019). In trapped ion mobility separation instruments, N-glycan isomers within a single $m/z$ ion packet migrate along with an inert drift gas against an oppositely opposed electric field, with their final equilibrium migratory distance dependent on their collisional cross sectional (CCS) areas (Kirk et al. 2019). Gradual reduction of the counter-acting electric field thus elutes these ions in a structural geometry-dependent manner from the mobility cell into the mass analyzer for detection. This technology has already demonstrated utility in parsing out N-glycan isomers (Pu et al. 2016). It should be noted that adding a 4th dimension of data (ion mobility) to IMS experiments exponentially increases the data footprint acquired during a single run. The data handling challenges associated with this new field of analysis are complex and require the development of advanced software solutions before the routine implementation of ion mobility into the imaging space (Sans, Feider, and Eberlin 2018; Rivera et al. 2020).

**2.2.4  MALDI-2 MS**—One difficulty common to all MALDI platforms is the detection of analytes of very low abundance or very high molecular weight. This complicates the analysis of mammalian N-glycans, which are synthesized to well past 10,000 $m/z$ via the addition of polylactosamine extensions (North et al. 2010). Critical factors contributing to this shortcoming are ionization efficiency and analyte decomposition in the MALDI-source (C. C. Wang et al. 2016). When compared to alternative ionization schema such as ESI, laser-induced ionization of analytes in a MALDI instrument is relatively inefficient (1 out of every ~$10^5$ molecules ionized). This is especially true for low-proton affinity substrates like carbohydrates, which ionize at an efficiency of $10^{-7} – 10^{-8}$ in MALDI experiments (Lai and Wang 2017; Page et al. 2007). Recently, novel instrumentation approaches have been developed to overcome this obstacle. Inefficient primary MALDI-ionization is rectified by an additional, subsequent ionization of the analyte plume by a secondary laser oriented perpendicular to the axis of ion ejection in laser positronization-coupled MALDI-IMS (MALDI-2) platforms (Barré et al. 2019). Thus, low abundance structures in the analyte cloud are more likely to be ionized and detected by the mass spectrometer. This new

technology has already shown applicability for N-glycan analyses, where a MALDI-2 instrument operating in negative mode demonstrated an order of magnitude more sensitive detection of N-glycans from brain tissue samples than did a positive mode traditional MALDI platform (Heijs et al. 2020). Another possibility afforded by MALDI-2 outfitted mass spectrometers is tissue analysis at subcellular resolution. Coupling a secondary positronization laser with a transition-geometry MALDI mass spectrometer, which positions the primary laser behind the tissue sample, allows pixel sizes on the order of 600 nm and thus the analysis of single cells (Niehaus et al. 2019). Secondary post-ionization is critical for generating acceptable signal at these low rasters, as the volume of sample ejected into the instrument decreases proportionally with reduced pixel size (Zavalin et al. 2012).

**2.2.5    Structural analysis of N-glycans by CID fragmentation—**Mass spectrometry approaches typically identify N-linked glycans by accurate mass alone, which allows for the precise identification of a particular N-glycan's carbohydrate composition. Lost in these analyses however is precise information on the linkages which define N-glycan structural features. A traditional approach to deconvoluting N-glycan structural heterogeneity is fragmentation by tandem mass spectrometry (Harvey 2005). The sequential dissolution of glycosidic linkages from the complete N-glycan is typically facilitated by collision induced dissociation (CID) fragmentation. Other tandem mass spectrometry strategies such as higher-energy collisional dissociation (HCD) and electron-transfer dissociation (ETD) are more commonly employed for glycopeptide identification (Ford et al. 2015). Although ESI-based LC-MS/MS traditionally yields more rich fragmentation information, MALDI-MS/MS systems employing on-line CID collision cells now routinely provide fragmentation sufficient for structural identification in imaging experiments (Prentice, Chumbley, and Caprioli 2015; Yang et al. 2007; McDowell et al. 2020) (Figure 5A). Briefly, tandem mass spectrometry by CID involves selective isolation of precursor masses of interest via a multi-pole ion guide followed by introduction of an inert collision gas. Acceleration of precursor ions through application of an electric potential results in gas-phase fragmentation upon collisions of N-glycan ions with the neutral gas molecules (Mitchell Wells and McLuckey 2005) (Figure 5B). Fragment ions are then introduced into the mass analyzer for detection. Sequentially increasing the collision voltage yields a proportional increase in N-glycan fragments. CID fragmentation typically generates both B/Y and C/Z ions and at high energies may also yield A/X cross-ring fragments of individual sugar monomers (Harvey 2000). By observing the sequential loss of sugar monomers from the complete N-glycan, an accurate understanding of the specific linkages composing the structure of interest may be inferred (Figure 5C). These structural inferences are made more concrete by multiplexing CID-based experiments with orthogonal analyses on ion-mobility instruments as previously discussed (Harvey and Struwe 2018).

**2.2.6    Alternative Ionization Strategies—**Although MALDI clearly sets itself apart as the ionization strategy of choice for the analysis of N-glycans, additional classes of imaging mass spectrometers, namely desorption electrospray ionization (DESI) and secondary ion mass spectrometry (SIMS) instruments, have potential applicability for N-glycan analyses.

Introduced to the imaging field in 2004, desorption electrospray ionization allows for the analysis of tissue samples under ambient conditions (Takáts et al. 2004). Electrospray-generated charged solvent droplets are directed at the tissue in a rastered fashion similar to that of MALDI experiments (Eberlin 2014). Contact of these droplets with the sample surface extracts molecules and transfers them into the gas phase for introduction into the mass spectrometer (Takáts, Wiseman, and Cooks 2005). Because these sources operate under atmospheric pressure, they can be paired with a wide variety of simpler, less costly mass analyzers making this technology more accessible to both academic and clinical laboratories (Cooks et al. 2011). Another clear advantage of these systems is that desorption by electrospray ionization imparts less energy to the analytes than does a MALDI laser, DESI-IMS may be advantageous for the analysis of oligosaccharides with more labile residues which are typically decomposed in-source during MALDI imaging experiments. Hybrid instruments employing both MALDI and ESI sources (termed MALDESI) are capable of generating multiply charged species, aiding in the detection of low $m/z$ analytes which are not as well resolved in FTICR instruments (Sampson et al. 2006; Nazari et al. 2016). To these ends, multiple groups have demonstrated analyses of carbohydrates, gangliosides and other biomolecules using DESI imaging strategies (Bereman, Williams, and Muddiman 2007; Škrášková et al. 2016; Pace and Muddiman 2020).

Developed prior to MALDI-MS for surface sampling of semiconductors and other inorganic films with industrial applications in mind, secondary ion mass spectrometry (SIMS) employs an ion beam of gaseous clusters which sputters a fixed sample, once again in a rastered manner (Benninghoven 1994). High-energy collisions between these gas molecules and sample molecules results in the ejection of analytes from a sample as secondary ions for analysis by time of flight mass spectrometers. The use of an ion-beam for ionization rather than a laser or microdroplet significantly reduces the footprint of individual pixels and thus allows for sub-micron resolution (< 100 nm) in imaging experiments (Anderton and Gamble 2016; Winograd 2015). Imaging of a wide variety of biomolecules, including glycans from carbohydrate-modified surfaces, by TOF-SIMS has been repeatedly demonstrated (Yoon and Lee 2018; Bolles et al. 2010). Although the complexity of released N-glycans has up-to-now limited their analysis from tissue samples by SIMS, recent chemical derivatization strategies may potentiate N-glycan ionization (Kaya et al. 2018).

### C    Data processing for N-glycan IMS

Data processing of the spectral datasets generated from imaging mass spectrometry experiments are challenging to analyze due to their complexity and large data footprint size (Alexandrov 2012). These challenges require significant computational resources to overcome. For example, a typical high spatial resolution MALDI-IMS experiment consists of hundreds of thousands of individual pixels, where each pixel represents a spectrum comprising hundreds of thousands of small $m/z$ bins. These spectra require thresholding and normalization amongst other processing steps and finally spatial mapping to create a composite dataset of pseudo-colored images for every possible $m/z$ of interest. Adding to this complexity, most IMS instruments operate vendor-specific software for initial data processing, making cross-platform comparisons and data sharing traditionally difficult (Chambers et al. 2012).

There is a recent emergence of vendor-neutral IMS processing software options. Open-source packages like MSiReader, Cardinal, and msIQuant utilize MATLAB, R and C++ programming, respectively, to analyze imzML data, the common imaging mass spectrometry file format (Bokhart et al. 2018; Bemis et al. 2015; Källback et al. 2016; Römpp et al. 2011). Even vendor specific software like Bruker Daltonic's SCiLS Lab and Waters' High Definition Imaging can accommodate this shared format which allows for the integration of multiple imaging mass spectrometry datasets from different platforms. The complex analyses performed by these software packages are critical for extracting the pathological relevancy of a particular N-glycan's spatial distribution. Overlaying high-resolution, annotated images of histological staining with N-glycan IMS data reveals co-localization of analytes of interest with distinct tissue morphologies (Aichler and Walch 2015). These programs also allow for statistical considerations such as segmentation, classification and multivariate analyses (Alexandrov et al. 2010; McCombie et al. 2005). New to the space are applications for the multiplexed integration of orthogonal imaging modalities including immunohistochemical and immunofluorescence microscopy, autofluorescence scans and magnetic resonance imaging with N-glycan IMS data to link glycosylation differences to biomarkers of interest or specific pathological features (Porta Siegel et al. 2018; Levenson, Borowsky, and Angelo 2015; Clift, Drake, et al. 2020; Angel et al. 2017).

Unlike peptide, lipid and small molecule imaging which is more established, the relative novelty of N-glycan IMS meant that limited software resources were initially available for peak assignment (Woodin, Maxon, and Desaire 2013). Currently, most laboratories manually assign N-glycan structures to mass spectra via the use of an in-house N-glycan database generated using software such as GlycoWorkbench or GlycoMod or rely on shared online databases like GlyConnect or GlyTouCan (Damerell et al. 2015; Cooper, Gasteiger, and Packer 2001; Alocci et al. 2019; Tiemeyer et al. 2017). Software for automated peak picking of N-glycans from IMS-generated spectra is still in its infancy, but new applications such as Bruker Daltonic's Metaboscape have shown promise for alignment of mass spectra peaks to an extensive database of known N-glycan masses with matched structures.

## III    Applications of N-glycan IMS

The utility of N-glycan IMS has been most thoroughly demonstrated in the field of cancer, as summarized in Table 1, facilitated by access to vast biorepository archives of FFPE tissues. N-glycan IMS has also been applied to non-cancer disease tissues like osteoarthritis and heart valve stenosis (Briggs et al. 2016; Angel et al. 2017; Clift, Drake, et al. 2020). Summaries of N-glycan IMS studies of liver, breast, pancreatic, colorectal, lung, ovarian and renal cancer tissues are discussed in this section. General findings from these studies often compliment serum or plasma N-glycomic studies from the same cancers, although it should be acknowledged that the tumor and circulatory N-glycomes are independently regulated (Kirwan et al. 2015; Gudelj et al. 2018; Dotz et al. 2019). An example panel of N-glycan images for a pancreatic cancer tissue is shown in Figure 6. These investigations have revealed distinct N-glycosylation patterns across tissue subtypes, N-glycan structural motifs associated with particular cancers as well as N-glycan ions whose detection specifically delineates healthy versus diseased tissue or discriminates between cancer stages.

## A Liver cancer

Perhaps no other disease has been more frequently associated with N-glycosylation changes than cancers of the liver, specifically hepatocellular carcinoma (HCC) (Mehta, Herrera, and Block 2015; Blomme et al. 2009; Comunale et al. 2006). Interest in liver cancer glycosylation stems from initial studies of alpha-fetoprotein (AFP), which determined core fucosylation as a critical indicator of hepatic dysfunction in HCC (Aoyagi et al. 1988; Zhao et al. 2020). Core fucosylated N-glycans, amongst other structural motifs, have since been associated with a number of circulating glycoproteins from patients with liver cancer (Norton et al. 2008; Comunale et al. 2009; 2010; Mengjun Wang et al. 2009; Comunale et al. 2011; DelaCourt et al. 2021). To this end multiple N-glycan IMS analyses have been directed towards HCC tissue cohorts in hopes of linking in situ expression of N-glycan species directly with those found in circulation. In 2018 West et al. surveyed the N-glycome of a large HCC cohort along with cirrhotic and non-transformed tissue samples (West et al. 2018). Highly branched N-glycans with one or more fucose modification were expressed abundantly in HCC samples while in cirrhotic and adjacent normal tissues their expression was detected minimally or not at all. Critically, this analysis was able to associate expression of the fucosylated tetra-antennary N-glycans Hex7dHex1HexNAc6 and Hex7dHex2HexNAc6 with significantly decreased survival times, demonstrating the functional relevance of N-glycan IMS findings to patient outcomes. A later orthogonal analysis confirmed that the majority of the detected N-glycans with at least one fucose residue in the HCC TMA, especially those associated with poor survival, are core-fucosylated (West et al. 2020). N-glycan imaging of hepatoblastoma (HepG2) cells in 2019 detected afucosylated, bi- and tri-antennary structures as well as the high mannose species Hex9HexNAc2, suggesting disparate glycoprofiles between liver cancers with different origins (Angel et al. 2019). Antibody panel-based N-glycan imaging, described in a later section, was initially developed with screening for liver malignancies in mind (Black, Liang, et al. 2019). The introductory demonstration of the technology featured α-1-antitrypsin (A1AT) and immunoglobulin G (IgG), but expanded panels have since included common serum proteins with well documented N-glycosylation changes in hepatocellular carcinoma. N-glycan signatures between healthy and cirrhotic human serum samples assessed via this platform were distinct, suggesting that future panel designs may facilitate the early detection of HCC transformation in the clinic by N-glycan IMS.

## B Breast cancer

The role of N-glycosylation in the development, maintenance and progression of breast cancer has been extensively studied, but there has been limited translation of results to clinical or diagnostic applications (Scott and Drake 2019). The emergence of imaging mass spectrometry for the investigation of N-glycosylation in breast cancer directly from clinical tissues, however, has shifted this dynamic. In 2018, in situ analyses of breast cancer tissue microarrays (TMAs) and whole tissue sections by MALDI-IMS revealed increases in polylactosamine containing N-glycans specific to the aggressive human epidermal growth factor receptor 2 (HER2) and triple negative (TN) subtypes (Scott, Casadonte, et al. 2019). Polylactosamine extensions are implicated in cellular adhesion, potentially giving mechanistic insight into the metastatic capacity of these subtypes (Srinivasan et al. 2009). This finding has been corroborated by an orthogonal study utilizing MALDI-IMS which

implicated tetra-antennary N-glycans containing just a single N-acetyllactosamine branch with poor survival outcomes in breast cancer patients (Herrera et al. 2019). Interestingly, imaging mass spectrometry has revealed patterns of N-glycosylation which are specific to regions of necrosis in breast cancer tissues (Scott, Norris-Caneda, et al. 2019). N-glycans localizing to this area of cellular remnants were uniquely sialylated and lacked fucose residues when compared to the high mannose and multiply branched structures of the tumor tissue. Similar patterns have been observed in other cancer types, suggesting a possible role for N-glycosylation in the pathways leading to cellular death (Seyrek, Richter, and Lavrik 2019).

In 2019, N-glycan IMS of a mouse stage IV breast cancer cell line (4T1) detected increased expression of the high mannose Hex9HexNAc2 N-glycan (Angel et al. 2019). The same high mannose N-glycans seen in breast tumors and the aforementioned cultured cells have also been detected in the sera of breast cancer patients using MALDI-MS (de Leoz et al. 2011). In this context too, the Hex9HexNAc2 structure is especially elevated, which posits the incomplete trimming of pre-golgi N-glycan intermediates as a feature of breast cancer. Other MALDI-based studies of N-glycosylation in breast cancer serum have detected N-glycan signatures which delineates recurrent versus non-recurrent cancer, stage 1 breast cancer from healthy controls and signatures specific to the lymph node invasive subtype (J. W. Lee et al. 2020; S. B. Lee et al. 2020). Taken together, these studies on the complex glycosylation landscape of breast cancer by MALDI imaging mass spectrometry are potentially exploitable for clinical surveillance, early detection or delineation of subtypes.

## C   Pancreatic cancer

The role of N-glycosylation in pancreatic ductal adenocarcinoma (PDAC) and associated neoplasia has been previously described (Munkley 2019). Early experiments using N-glycan MALDI-IMS demonstrated biantennary N-glycan structures with and without core fucose residues specific to areas of fibrosis in pancreatic cancer (Powers et al. 2014). Additionally, this study identified undecorated structures (those without fucose, sialic acid or bisecting GlcNAc residues) as characteristic of areas of tumor necrosis. A further exploration of PDAC N-glycosylation by the same group in 2015 corroborated the finding of biantennary N-glycans as features of fibrotic tissue regions in pancreatic cancer (Powers et al. 2015).

Building off these prior studies, an extensive imaging mass spectrometry analysis of the N-glycome of healthy and PDAC pancreatic tissue was reported in 2020 (McDowell et al. 2020). Normal human pancreas samples in this study were defined by an abundance of high mannose N-glycans localized to exocrine acinar tissue while inter- and intralobular ductal tissue regions featured core-fucosylated bi- and triantennary structures. A unique finding of this study was the detection of sulfated, bi-antennary N-glycans with terminal N-acetylgalactosamine residues which localized specifically to pancreatic islets of Langerhans. PDAC tissues from this cohort exhibited a variety of structural themes including increases in fucosylation, sialylation, branching, bisecting N-acetylglucosamines and polylactosamine extensions, amongst others. In addition to these findings, this study demonstrated the utility of a number of the N-glycan IMS adaptations, discussed further later in this review. Sialic acid stabilization by amidation revealed the distinct localizations of α2,3- versus α2,6-

sialylated N-glycans in PDAC tissues, suggesting expression of disparate sialyltransferases across different tissue subtypes. Chemoenzymatic cleavage of N-glycans from these tissues using Endo F3 revealed that most structures in this PDAC cohort were predominantly, if not exclusively, core-fucosylated. N-glycan IMS is particularly relevant to pancreatic cancer, as the sole FDA-approved biomarker for pancreatic cancer is carbohydrate-antigen 19–9 (CA19–9), a four-sugar carbohydrate motif attached to both O- and N-linked glycans (E. Poruk et al. 2013). To this end, this analysis demonstrated the potential clinical relevancy for studies which multiplex N-glycan IMS with orthogonal imaging modalities. By combining expression data of select IMS-derived N-glycan masses from PDAC TMAs with immunofluorescence-assessed expression data of current and prospective PDAC biomarkers from the same TMAs, models for classification of pancreas tissue samples as normal or cancerous were built which outperformed modeling based on either dataset alone (Staal et al. 2019; Goonetilleke and Siriwardena 2007). These results suggest the future utility of cross-disciplinary, multi-marker panels which exploit N-glycan IMS in the early detection and diagnosis of pancreatic and other cancers.

## D Colorectal cancer

In colorectal cancer (CRC), N-glycosylation plays a well-defined oncological role (De Freitas-Junior and Morgado-Díaz 2016). Studies utilizing MALDI-TOF MS have analyzed N-glycans from colorectal cancer tissue homogenate, various CRC cell lines and patient serum (Balog et al. 2012; Holst, Deuss, et al. 2016; de Vroome et al. 2018). These studies detected a wide variety of N-glycan structural features specific to CRC tumor tissue, including high mannose, core fucosylation, sialylation, increased branching and even sulfation. These distinctions were born out between healthy and diseased samples but also across molecular subtypes and disease stage. Although the imaging functionality of MALDI-MS has only recently been applied to colorectal cancer, distinct spatial localizations of N-glycan species have been elucidated and the detected structural themes agree with the MALDI-TOF-derived findings from orthogonal sample sources above. In validation of the sialic acid stabilization by amidation protocol developed by the Wuhrer group, elevated levels of sialylated N-glycans were shown in colorectal cancer tissue samples (Holst, Heijs, et al. 2016). An additional proof-of-concept study, which demonstrated both N-glycan and peptide IMS from the same tissue sections, detected high mannose N-glycans localized to tumor regions in a representative colorectal cancer sample (Heijs et al. 2016). These initial findings were solidified in 2020 by a comprehensive survey of the N-glycome of stage II CRC by MALDI-IMS, which confirmed expression of sialylated and high mannose structures in cancer cells (Boyaval et al. 2020). This increased expression was shown to be propagated into the surrounding stroma, suggesting the modification N-glycosylation in the tumor microenvironment ahead of the tumor's invasive front in advanced stage colorectal cancer.

## E Lung cancer

The role of glycosylation changes in lung cancer are well known and initial studies suggest the existence of disease-specific N-glycosylation (Lemjabbar-Alaoui et al. 2015). One of the proof-of-concept studies which demonstrated the applicability of MALDI-IMS to N-glycan analyses featured TMAs with lung adenocarcinoma cores matched to normal lung tissue. In

these cores, high mannose N-glycan structures, especially Hex6HexNAc2, were found to be elevated (R. R. Drake et al. 2017). Although not a direct analysis of lung cancer, a recent study employed the use of radiation to recapitulate pre-neoplastic transformation which may lead to lung cancer in rhesus macaques (Carter et al. 2020). Areas of irradiation-induced hyperplasia in these lung samples analyzed by N-glycan IMS exhibited elevations in high mannose structures consistent with the human lung TMA cores from the aforementioned study, as well as fucosylated bi-antennary structures with and without bisecting GlcNAc residues. This elevation in high mannose N-glycans has also been observed in a MALDI-TOF MS analysis of lung adenocarcinoma patient sera across stages, positing that a further IMS evaluation of the lung cancer N-glycome may discover additional N-glycan features which map to serum expression for possible use as biomarkers (Lattová et al. 2020).

### F    Ovarian cancer

Glycosylation changes in ovarian cancer were first observed in the mid 1960's (Garcia-Bunuel and Monis 1964). Of recent interest is the translation of N-glycosylation analytical applications to clinical strategies for ovarian cancer, as N-glycans are known to be heavily involved in the oncogenesis and maintenance of this disease (Guo and Abbott 2015; Briggs, Condina, Klingler-Hoffmann, et al. 2019). Consistent with other types of cancers, a 2016 study detected increased expression of high mannose N-glycans in ovarian cancer tumor tissue as compared to normal tissue from the same samples (Everest-Dass et al. 2016). This same analysis also detected increases in hybrid-type structures specific to areas of intervening stroma in this cohort. All N-glycan structures in these experiments were orthogonally verified by LC-MS/MS. N-glycans extracted and purified from FFPE ovarian cancer tissue samples for MALDI-TOF analysis by the Li group supported the specificity of high mannose N-glycan expression in ovarian tumor tissue (Chen et al. 2017). This analysis also found that a seven-glycan panel consisting of four high mannose and three fucosylated complex structures was able to delineate epithelial ovarian cancer from healthy controls, while a similar yet distinct N-glycan panel could distinguish specific ovarian cancer grades. N-glycan IMS analyses have also been able to differentiate early-versus late-stage ovarian cancer using specific N-glycosylation signatures (Briggs, Condina, Ho, et al. 2019). The high mannose N-glycan Hex9HexNAc2 was detected abundantly in stage III ovarian cancer samples as compared to stage I tissues. Additionally specific to late-stage ovarian cancer were fucosylated, bisecting and sialylated complex species. These glycosylation changes were not only detected in the whole tissue cohort analyzed in this report, but in corresponding TMAs which lends additional statistical power to these findings. Taken together these early N-glycan IMS studies suggest glycosylation changes as a feature of ovarian cancer potentially exploitable for clinical detection and surveillance of this disease.

### G    Renal cancer

The first publication of a standardized N-glycan MALDI-IMS workflow was demonstrated on a normal kidney tissue section which contained both medulla and cortex tissue regions (Powers et al. 2013). Although the technology was in its infancy, the few N-glycan analytes detected were localized to specific tissue subtypes. A subsequent analysis of FFPE murine kidneys demonstrated similar results (Gustafsson et al. 2015). This initial work was

furthered by a more comprehensive 2020 study of clear cell renal cell carcinoma (ccRCC), which spatially profiled N-glycosylation in a cohort of normal kidney and renal cell carcinoma tissues (Richard R. Drake, McDowell, et al. 2020). A defining feature of healthy kidney tissue from this study was broad structural diversity between tissue subtypes, with bisecting, high mannose and fucosylated structures localized to the cortex while bi- and tri-antennary N-glycans with core fucose residues localized to medullary tissue. Interestingly, the interface between cortex and medulla also had a distinct glycosylation signature, defined by multiply-fucosylated structures which lacked bisecting GlcNAc residues. A high spatial resolution analysis from this study detected tetra-antennary N-glycans with and without sialic acid modifications specific to kidney glomeruli. These normal-associated structural features were conspicuously absent from ccRCC tumor tissue, which exhibited increased expression of tetra-antennary N-glycans with polylactosamine extensions of varying length. The fibrillar capsule surrounding a Fuhrman grade 2 ccRCC tumor was characterized by a bi-antennary, monosialylated N-glycan. For comparison, a non-ccRCC polycystic kidney disease (PKD) tissue was also analyzed, which again showed a disparate N-glycome as well as distinct glycosylation pattern differences between cyst fluid and tissue regions. N-glycan IMS findings were mapped directly to transcriptomic profiling by RNA sequencing from a prior study, which revealed altered expression of fucosyltransferases FUT3 and FUT6 which attach fucose residues via an a1,3 linkage to N-glycan termini (von Roemeling et al. 2014; Neely et al. 2016; Schneider, Al-Shareffi, and Haltiwanger 2017). The decreased expression of these glycosyltransferases in ccRCC tissue across all stages matches less abundant outer-arm fucosylation of tumor N-glycan structures observed by IMS. Taken together the findings from this multi-omic study comprise the most comprehensive analysis to-date of the healthy and diseased renal N-glycome.

## IV   Alternate enzymes and chemical modifications

### A   Amidation-amidation to stabilize sialic acids

As previously mentioned, characterization of N-glycan isomers can be challenging using traditional N-glycan IMS workflows. Particularly difficult is the separation of sialylated N-glycan isomers (Nishikaze 2019). Sialic acid monomers are attached to terminal galactose residues on N-glycan branched arms by the β-galactoside sialyltransferases (STGAL) family of enzymes, which catalyze the formation of either α2,3 (ST3GAL1) or α2,6 (ST6GAL1) anomeric linkages (Harduin-Lepers et al. 2001). Adding to this complexity is the labile nature of sialic acid residues, which often decompose in-source during MALDI ionization due to a combination of high vacuum and heat (Harvey 1999). Improved MALDI source configurations have improved overall retention of sialic acids, but not completely (Richard R. Drake, West, et al. 2018). To facilitate analysis by mass spectrometry, multiple chemical derivatization approaches have been developed to stabilize these residues (Xin Liu et al. 2010; Sekiya, Wada, and Tanaka 2005; Powell and Harvey 1996; Reiding et al. 2014; de Haan et al. 2020). Relevant to N-glycan IMS are the strategies that take advantage of the differential chemical properties between α2,3- and α2,6-linked anomers to stabilize each as a distinct moiety. In amidation syntheses developed by the Wuhrer group, α2,3- and α2,6-linked sialic acid residues are modified with amide and dimethyl amine functional groups, respectively, by sequential incubations with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide

(EDC)/1-hydroxybenzotriazol (HOBt) and ammonia (Holst, Heijs, et al. 2016; Pongracz et al. 2021) (Figure 7A). These reactions are adapted to use directly on tissue in situ prior to traditional MALDI-IMS N-glycan workflows. The differential incorporation of these moieties introduces well-defined mass shifts for each linkage which aid in their identification by mass spectrometry. This chemical amidation strategy has been successfully used in situ to characterize sialylation differences between healthy and diseased tissue (McDowell et al. 2020; Boyaval et al. 2020). Of interesting note from the aforementioned studies is the finding that differential α2,3 versus α2,6 sialylation of the same base N-glycan structure drove disparate spatial localization within the same tissue, suggesting the compartmentalization of specific sialyltransferases to distinct tissue subtypes or disease morphologies (Figure 7B–D).

## B    Alternate glycosidases

Although PNGase F is the gold standard for enzymatic deglycosylation of N-linked glycoproteins and N-glycan IMS, there are a many different glycosidases reported for N-glycan analysis applications (M. S. Kim and Leahy 2013). Most of these glycosidases have substrate specificities for other parts of N-linked glycan structure, or specific to individual glycoprotein classes in the immunoglobulin family, Incorporation of these enzymes requires minimal modification of existing IMS workflows and allows for the targeted analysis of specific N-glycosylation features. One note, a key consideration when evaluating new glycosidases for spraying on tissue is to identify any salts or stabilizers that may be present in the formulation that could interfere with laser ionization efficiency.

One non-PNGase F example is endoglycosidase F3, which has already been incorporated into N-glycan MALDI IMS workflows alone or combined with PNGase F (West et al. 2020). Similar to sialylation isomers of N-glycans, fucosylation isomers are especially difficult to delineate. Fucose monomers are linked to the growing N-glycan by the fucosyltransferase (FUT) family of enzymes. Core fucose residues, those bonded to the terminal N-acetylglucosamine linked to the glycoprotein's asparagine, are attached exclusively via an α1,6 linkage by FUT8. Outer arm fucoses are attached to branched N-glycan antennae via α1,2 linkages by FUT1,2 or by α1,3 linkages via FUT3,4,6,7,9–11 (Schneider, Al-Shareffi, and Haltiwanger 2017). Endoglycosidase F3 (Endo F3) hydrolyzes the glycosidic linkage between the N-acetylglucosamine residues of the N-glycan chitobiose core preferentially in the presence of an α1,6-linked core fucose, thus freeing for detection only N-glycan structures that are core fucosylated (Plummer, Phelan, and Tarentino 1996; West et al. 2020). Those N-glycan isomers which are exclusively outer-arm fucosylated are not cleaved and therefore not detected. The process introduces a distinct   349 m.u. mass shift loss from the parent N-glycan structure, aiding in the specific detection of these Endo F3-cleaved glycans. A fucose and GlcNAc residue remain on the glycoprotein, providing a searchable peptide modification for glycoproteomic identification of core-fucosylated glycoproteins (Ma et al. 2018). Recent analyses incorporating Endo F3 into standard N-glycan IMS workflows have demonstrated the specific detection of fucosylation isomers in situ (West et al. 2020). The distinction between core and outer-arm fucosylation is an important one, as the location of fucose residues can be a critical determinant of glycoprotein function (Tu, Lin, and Lin 2013; H. I. Kim et al. 2013). Suggestive of their

disparate biological functions, core versus outer arm fucosylation isomers often display distinct spatial localization in both healthy and diseased tissues.

## C  O-glycans

Although this review is targeted towards N-glycan imaging mass spectrometry, it is worth briefly discussing O-glycan strategies as these carbohydrates make up a large portion of the glycocalyx and are equally relevant to biological and pathological processes in the cell (Varki 2017). Unlike N-glycan analyses, which enjoy a variety of enzymatic and chemical cleavage reagents, O-linked glycan analysis suffers from a lack of reliable enzymes to free these species for detection. This is due in part to the heterogeneity of the modifying sugar directly linked to the glycoprotein's serine/threonine residue, which could be an N-acetylgalactosamine on mucin glycoproteins or an N-acetylglucosamine, galactose, mannose or fucose on non-mucin glycoproteins (Darula and Medzihradszky 2018). The lack of a consensus motif excludes the broad cleavage of O-glycans by just a single enzyme, making the design of streamlined analysis workflows, like those for N-glycan IMS, more challenging.

Strategies to focus on a subset of O-glycans, namely O-GalNAc-based structures, have been more successful (Saldova and Wilkinson 2020). Endo-α-N-acetylgalactosaminidase (O-glycanase) releases core 1 (Gal[β1,3]GalNAc[α]Ser/Thr) and core 3 (GlcNAc[β1,3]GalNAc[α]Ser/Thr) O-glycans from their glycoprotein carriers, but its efficiency is reduced when the O-glycan contains additional saccharide units beyond the core structure. Both reductive and non-reductive hydrolyses of the O-glycosidic linkage by β-elimination represent alternative chemical approaches to liberate O-glycans, although side reactions with these reagents may generate additional ions that obfuscate analytes of interest (Goso, Tsubokawa, and Ishihara 2009). O-glycans released via these strategies are typically detected after chemical derivatization using LC-MS/MS approaches, although detection by MALDI-TOF of purified O-glycans spotted on target plates has been described (Morelle et al. 2009). An alternative approach for O-glycan analysis is use of a novel, bacteria-derived mucin protease, typified by StcE (secreted protease of C1 esterase inhibitor) (Malaker 2019). These mucin proteases, reported in the literature and also commercially available, cleave at O-glycosylated sites. Detection of the resulting mucin glycopeptides by MALDI IMS could be amenable a workflow similar to those for on-tissue tryptic digestion for imaging mass spectrometry of peptides (Malaker et al. 2019). In situ analyses of released O-glycans and mucin O-glycopeptides by IMS is likely possible, but still requires extensive further optimizations of the release methods and glycosidase conditions specific for tissue IMS approaches.

# V  Expanded approaches

## A  Tissue microarrays

Tissue microarrays are single pathology slides to which multiple, small tissue punches, collected from biopsies or surgical resection, are affixed in a manner similar to the preparation of clinical FFPE tissue sections (Jawhar 2009). Histological staining of resected tissues allows clinical pathologists to sample healthy or diseased tissue subtypes of interest

and compile samples from large numbers of patients into the same TMA block for the creation of individual FFPE TMA sections. Often, samples will be taken of both healthy and malignant tissue from the same patient, resulting in matched pairs for analysis. While the spatial information aspect of imaging mass spectrometry is less relevant to these analyses, TMAs provide the opportunity to assay N-glycosylation information from hundreds of patient samples in a high throughput manner (Powers et al. 2014) (Figure 8A). For example, one tissue microarray representing over 100 patient biopsies can be analyzed via N-glycan MALDI-IMS in the same (or less) time as it takes to run a single whole tissue section. Critically, microarray formats enable the cost effective and rapid high throughput screening required for biomarker discovery (Hewitt 2012). An additional advantage of this tissue specimen layout is that the large sample sizes afforded by TMAs provides adequate statistical power for complex analyses of differences in N-glycosylation between healthy and diseased tissue types (Xueli Liu et al. 2004). In multiple studies, N-glycan imaging of tissue microarrays has demonstrated utility in the screening of large patient cohorts for N-glycan expression differences in a variety of cancers (Powers et al. 2015; West et al. 2020; 2018; Richard R. Drake, McDowell, et al. 2020; Conroy et al. 2020; Herrera et al. 2019; Scott, Casadonte, et al. 2019). Like whole-tissue sections, N-glycan IMS data from TMAs may be multiplexed with immunohistochemical, immunofluorescence or autofluorescence microscopy for comparison with biomarkers or other molecules of interest (McDowell et al. 2020).

## B Biofluids

An emerging role for the field of imaging mass spectrometry is that of clinical diagnostics for the specific detection of disease. The utilization of IMS for carbohydrates to assess healthy versus pathological states is potentiated by the fact that all current FDA-approved cancer biomarkers are glycoproteins or are themselves glycan motifs (Adamczyk, Tharmalingam, and Rudd 2012; Kailemia, Park, and Lebrilla 2017). The adaptation of N-glycan imaging strategies to other clinical sample types is an attractive alternative to FFPE or FF tissues. By far the most clinically accessible medium for analysis are patient biofluids, namely sera and plasma, which can be collected via minimally invasive blood draws as part of routine diagnostic screenings. Discussed below are two novel adaptations of N-glycan IMS towards clinical biofluids (Figure 8B).

**5.2.1 Biofluid N-glycan profiling—**Human serum and plasma contains thousands of proteins and metabolites that are reflective of the disease state and overall health of the donor patient. (Anderson and Anderson 2002). These complex mixtures are comprised of a relatively high-abundance (10X order of magnitude) of a small number of glycoproteins which have the potential to obfuscate detection of low-abundance yet potentially clinically relevant analytes, thus making these matrices sometimes difficult to analyze (Qian et al. 2006). N-glycosylation of human serum is traditionally assessed by high- or ultra-performance liquid chromatography (HPLC/UPLC), capillary gel electrophoresis (CGE) or MALDI-TOF of purified N-glycans (Stöckmann et al. 2015; Ruhaak et al. 2010; Vreeker et al. 2018). While these technologies have helped elucidate N-glycan functions in disease, lengthy derivatization, labeling and cleanup steps in these workflows make them incompatible with the rapidity required for clinical diagnoses.

To address the necessity for rapid, specific diagnostics of serum glycosylation, a novel imaging mass spectrometry approach has been developed (C. Blaschke et al. 2020). A single microliter of patient serum is diluted in sodium bicarbonate and spotted on amine-reactive hydrogel-coated glass slides to immobilize serum glycoproteins, followed by desalting and delipidating through a series of organic washes and drying under desiccation. The dried, serum-spotted slides are then processed with the standard N-glycan MALDI-IMS workflow described in section 2.1.1, starting with enzymatic release of N-glycans from serum proteins by PNGase F. Analysis by MALDI-FTICR or MALDI-QTOF instruments has demonstrated the specific detection of over 75 N-glycan structures from human serum using this method. This new imaging platform is also compatible with Endo F3 and sialic acid amidation approaches traditionally applied to tissue samples as described above. MALDI-MS is already routinely used in the clinical laboratory for identification of microbial samples via analyte "barcodes" corresponding to particular pathological signatures (Kostrzewa 2018; Richard R. Drake, Boggs, and Drake 2011; Mellmann et al. 2009). It is foreseeable that this biofluid IMS method or some further iteration of the technology could be used to generate N-glycan barcodes for the identification of various diseases in the clinic.

**5.2.2. Antibody panel-based N-glycan IMS**—In 2019 a more targeted imaging mass spectrometry strategy for the identification of N-glycosylation signatures on common serum glycoproteins was developed (Black, Angel, et al. 2019; Black, Liang, et al. 2019). Antibody Panel-Based N-glycan Imaging (APB) takes advantage of established slide-based antibody array formats (Jones et al. 2020) repurposed for mass spectrometry analysis of N-glycans.

APB N-glycan imaging capitalizes on decades of antibody engineering, using high binding affinity anti-human antibodies to specifically capture individual serum glycoproteins (Black, Angel, et al. 2019; Black, Liang, et al. 2019). Briefly, antibodies targeting specific serum glycoproteins are manually spotted on nitrocellulose slides then washed and blocked with bovine serum albumin (BSA). Incubation with human serum samples results in the capture from the total protein pool of only the glycoprotein antigens specific to the antibody spots on the array. Initial demonstrations of this technology focused on six abundant serum proteins, alpha-1 antitrypsin (A1AT), haptoglobin, hemopexin, immunoglobulin G (IgG), low molecular weight kininogen and transferrin, although any combination of antigens of interest may be used dependent on the availability of an appropriate antibody. Similar to the biofluid imaging strategy described above, slides with captured glycoproteins can subsequently be incorporated into MALDI-IMS workflows at the point of enzymatic deglycosylation (Black, Liang, et al. 2019). This method has demonstrated the specific detection of over 20 individual glycan species with high sensitivity and the capability to detect ~3 fmol of glycan from just 10 ng of captured protein. Initial results from analyses of healthy versus cirrhotic human serum revealed differential glycosylation signatures between healthy and diseased samples. Currently under development is a neural network framework for the analysis of N-glycan IMS data from these panels which seeks to assign specific glycosylation barcodes to disparate disease states. Like whole-serum N-glycan IMS, further development could see this technology integrated into the clinical laboratory.

## C Cell culture

Cultured cells are one of the most frequently-used tools for the study of human disease, as they model the basic biological processes underlying both healthy and diseased states and are inherently manipulatable for experimental design (Gillet, Varma, and Gottesman 2013). Cultured cells have long been used as model systems to probe the biosynthesis, function and regulation of N-glycans and glycosylated proteins (Montreuil 1995). Comprehensive studies of N-glycans in cell culture conducted today typically utilize LC-MS or MALDI-TOF MS approaches (Aich, Lakbub, and Liu 2016; Shajahan et al. 2017; Parry et al. 2006).

In 2019, a novel approach for the analysis of N-glycans directly from cultured cells was developed utilizing imaging mass spectrometry (Angel et al. 2019; Angel, Mehta, and Drake 2021) (Figure 8C). Cells cultured on standard chambered glass slides were fixed and delipidated in organic solvents prior to microscopy imaging to confirm appropriate cellular density. Using conditions optimized specifically for cultured cells, the standard N-glycan IMS processing workflow was applied to the cells on the slide. Protocol modifications include the adjustment of both PNGase F and CHCA spraying parameters and the incorporation of a post-matrix ammonium phosphate spray to limit the formation of signal-suppressing matrix clusters (Chubatyi and McEwen 2015; Ucal and Ozpinar 2018). Analyses of a panel of human and animal cell lines cultured and prepared using this method by MALDI-FTICR MS detected over 70 N-glycoforms. This assay is innovative in a number of ways. Cells for this method can be cultured using standard approaches without requiring further optimization. The inclusion of media blanks for background signal subtraction excludes obfuscation by glycans derived from fetal bovine serum (FBS) (Carr, Huddleston, and Bean 1993; Lin, Franc, and Heck 2018). Incorporation of Coomassie blue staining after IMS data acquisition to infer total protein expression allows for both intra-and inter-run normalization, making this method inherently quantitative (Bradford 1976; Butt and Coorssen 2013). Expanded use of this method allows for the monitoring of N-glycan turnover through stable isotopic labeling of amino acids in cell culture (SILAC) using an isotopic detection of amino sugars with glutamine (IDAWG) approach (Orlando et al. 2009). N$^{15}$ labeling in this way could also be used to measure cellular N-glycosylation response to stress or therapeutics interventions induced with a variety of chemical or pharmaceutical antagonists (Mechref et al. 2013; Xu et al. 2019; Delafield and Li 2020). Consequently, revealing the N-glycan profiles of cultured cell lines allows assessment of how faithfully these models recapitulate glycosylation seen in clinically-derived tissue specimens and thus in human biology. Linking these glycosylation states will help better understand which cell lines are most representative of a given disease state for studying N-glycosylation in both basic science and therapeutic contexts.

## VI Conclusions

The specific adaptation of imaging mass spectrometry as a tool for N-glycan analysis has exponentially expanded glycomics research efforts into organ, tissue and cell-type specific glycosylation studies. Capturing the in situ spatial component of a particular N-glycan's expression has led to insights into the multifaceted role these molecules play in a variety of diseases, with potential for multiple clinical applications. Discussed in this review are

the most common neoplasias studied by N-glycan IMS, summarized in Table 1. The broad applicability of the approach suggests that any type of cancer may be investigated, as well as non-cancer diseases, assuming availability of appropriate clinical specimens. Emphasis on the MALDI IMS application to human disease was discussed, but tissue and biofluids from any biological source could be used with these workflows. For many organisms, the N-glycomes of most organisms, as well as individual organs in humans, remain largely uncharacterized. While the basic structural features of high mannose and bi-antennary glycans are fairly conserved across all species, there are many known species-specific glycans synthesized by a broader class of glycosyltransferases and glycosidases than that found in humans. For example, different sialic acid structural variants are prevalent in non-human mammals (Varki 2008; Irie et al. 1998). Analysis of non-mammalian N-linked glycosylation by N-glycan IMS is a wide-open space for application of this method.

No single MS method is sufficient to fully characterize the structural complexities of N-glycans, However, in regard to providing N-glycan co-localization information with tissue histopathology, N-glycan IMS approaches have proven to be highly effective and informative. The utility of N-glycan IMS to distinguish isomers, novel glycans and larger structures will continue to evolve with improvements in instrumentation, data analysis and incorporation of other approaches. This includes use of other ionization approaches, in particular atmospheric pressure methods like DESI and IR-MALDESI, hybrid instruments and increased use of integrated ion mobility separation (Bereman, Williams, and Muddiman 2007; Pu et al. 2016; Škrášková et al. 2016; Lane et al. 2019; Pace and Muddiman 2020). N-glycan IMS is amenable to multiplexing with immunohistochemical and immunofluorescence imaging, generating multi-modal data for both glycan and protein markers of interest which has demonstrated utility in the characterization of disease states (Levenson, Borowsky, and Angelo 2015; Tan et al. 2020; McDowell et al. 2020; Clift, Mehta, et al. 2020; Yagnik et al. 2021). Linkage of N-glycan IMS with more advanced molecular imaging modalities like mass cytometry/mibiTOF (Keren et al. 2019), and in the other resolution direction, clinical MRI combinations are being pursued (Abdelmoula et al. 2019; Richard R. Drake, Angel, et al. 2020; Basu and Agar 2021).

While initially developed for N-glycans, glycan IMS is adaptable to a wide range of enzymatic and chemical release agents, which expands the repertoire of detectable analytes beyond N-linked structures to O-linked glycans, glycosaminoglycans and glycolipids amongst other species (Morelle et al. 2009; Goto-Inoue, Hayasaka, and Setou 2010; Malaker et al. 2019; Saldova and Wilkinson 2020; Clift, Drake, et al. 2020). Alternative endoglycosidases like Endo F3, Endo H and Endo S recognize distinct epitopes on glycans, cleaving them in can be advantageous for the delineation of specific glycan anomeric linkages, specific glycan classes and glycans specific to certain proteins or cell populations (Plummer, Phelan, and Tarentino 1996; Collin et al. 2001; Freeze and Kranz 2010; West et al. 2020). A recent study incorporating isoamylase, to free glycogen polymers for detection, into the N-glycan IMS workflow has demonstrated a novel use of imaging mass spectrometry to link glucosamine metabolism and storage to the biosynthesis of N-linked glycans (Sun et al. 2021). As more specific enzymes and chemistries are discovered or engineered, an even wider array of carbohydrate analytes will become available for detection by IMS.

Together, glycan imaging mass spectrometry approaches have deepened our understanding of the glycobiome and the complex biology and chemistry which governs the synthesis, function and physiological effects of these carbohydrates. The continued evolution and expanded adaptations of this technology promises further insight into the biochemical underpinnings of health and disease in the context of glycosylation. Ultimately, in the near future glycan imaging mass spectrometry platforms may be integrated into clinical laboratories for disease surveillance and early discovery by the detection of specific glycan molecular signatures.

## Acknowledgements

## VII Abbreviations

| | |
|---|---|
| **A1AT** | α-1-antitrypsin |
| **AFP** | α-fetoprotein |
| **APB** | Antibody panel-based |
| **BSA** | Bovine serum albumin |
| **CA19–9** | Carbohydrate-antigen 19–9 |
| **ccRCC** | Clear cell renal cell carcinoma |
| **CCS** | Collisional cross-sectional area |
| **CGE** | Capillary gel electrophoresis |
| **CHCA** | α-cyano-4-hydroxycinnamic |
| **CID** | Collision-induced dissociation |
| **CRC** | Colorectal cancer |
| **DAN** | 1,5-diaminonaphthalene |
| **DESI** | Desorption electrospray ionization |
| **DHB** | 2,5-dihydroxybenzoic acid |
| **dHex** | Deoxy hexose |
| **DTIMS** | Drift tube ion mobility separation |
| **ECM** | Extracellular matrix |
| **EDC** | 1-ethyl-3(3-dimethylaminopropyl)carbodiimide |

| | |
|---|---|
| **Endo F3** | Endoglycosidase F3 |
| **ER** | Endoplasmic reticulum |
| **ESI** | Electrospray ionization |
| **ETD** | Electron-transfer dissociation |
| **FAIMS** | High-field asymmetric ion mobility separation |
| **FBS** | Fetal bovine serum |
| **FDA** | Food and Drug Administration |
| **FF** | Fresh-frozen |
| **FFPE** | Formalin-fixed paraffin-embedded |
| **FT-ICR** | Fourier transform ion cyclotron resonance |
| **Fuc** | Fucose |
| **FUT** | Fucosyltransferase |
| **GAG** | Glycosaminoglycan |
| **Gal** | Galactose |
| **GalNAc** | N-acetylgalactosamine |
| **GlcNAc** | N-acetylglucosamine |
| **Glu** | Glucose |
| **HCC** | Hepatocellular carcinoma |
| **HCD** | Higher-energy collisional dissociation |
| **HER2** | Human epidermal growth factor receptor 2 |
| **Hex** | Hexose |
| **HexNAc** | N-acetylhexosamine |
| **HOBt** | 1-hydroxybenzotriazole |
| **HPLC** | High-performance liquid chromatography |
| **IDAWG** | Isotopic detection of amino sugars with glutamine |
| **IF** | Immunofluorescence |
| **IgG** | Immunoglobulin G |
| **IHC** | Immunohistochemistry |
| **IR** | Infrared |

| | |
|---|---|
| **ITO** | Indium tin oxide |
| **IMS** | Imaging mass spectrometry |
| **LC-MS** | Liquid chromatography-coupled mass spectrometry |
| **MALDESI** | Matrix-assisted laser desorption electrospray ionization |
| **MALDI** | Matrix assisted laser desorption/ionization |
| **Man** | Mannose |
| **MIBI-TOF** | Multiplexed ion beam imaging by time of flight |
| **MRI** | Magnetic resonance imaging |
| **MS** | Mass spectrometry |
| **Nd:YAG** | Neodymium-doped yttrium aluminum garnet |
| **Neu5Ac** | N-acetylneuraminic acid |
| **PGC** | Porous graphitized carbon |
| **PDAC** | Pancreatic ductal adenocarcinoma |
| **PKD** | Polycystic kidney disease |
| **PNGase F** | Peptide N-glycosidase F |
| **QTOF** | Quadrupole time-of-flight |
| **SILAC** | Stable isotopic labeling of amino acids in cell culture |
| **SIMS** | Secondary ion mass spectrometry |
| **StcE** | Secreted protease of C1 esterase inhibitor |
| **STGAL** | B-galactoside sialyltransferase |
| **THAP** | $2',4',6'$-trihydroxyacetophenone monohydrate |
| **TIMS** | Trapped ion mobility separation |
| **TMA** | Tissue microarray |
| **TN** | Triple negative |
| **TOF** | Time-of-flight |
| **TWIMS** | Traveling wave ion mobility separation |
| **UPLC** | Ultrahigh-performance liquid chromatography |

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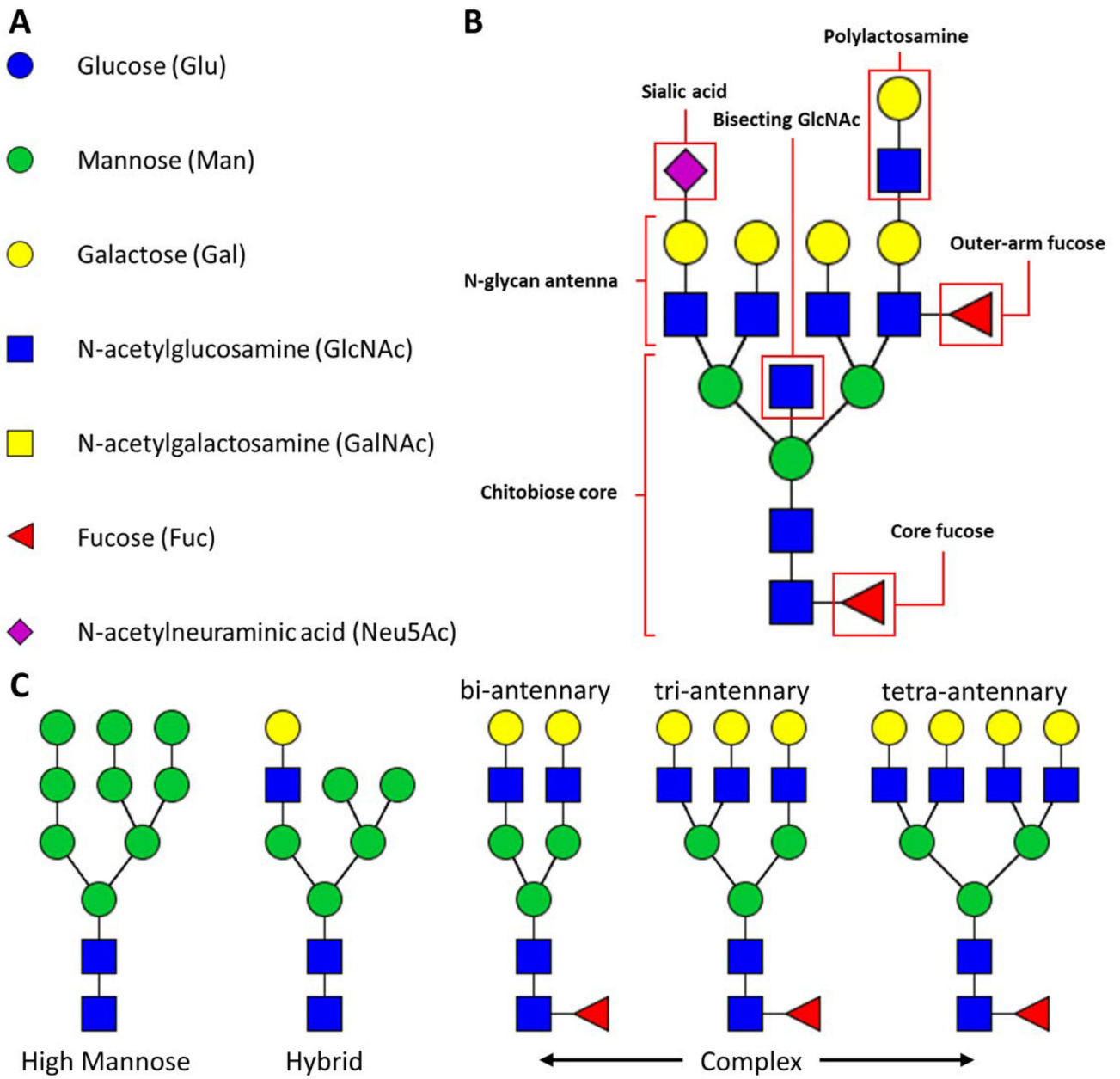
**Figure 1. N-glycan structural annotations.**
A) individual monosaccharide unit symbols and abbreviations. B) N-glycan structural motifs referenced in this review. C) N-glycan structural classes and examples of N-glycan branching.
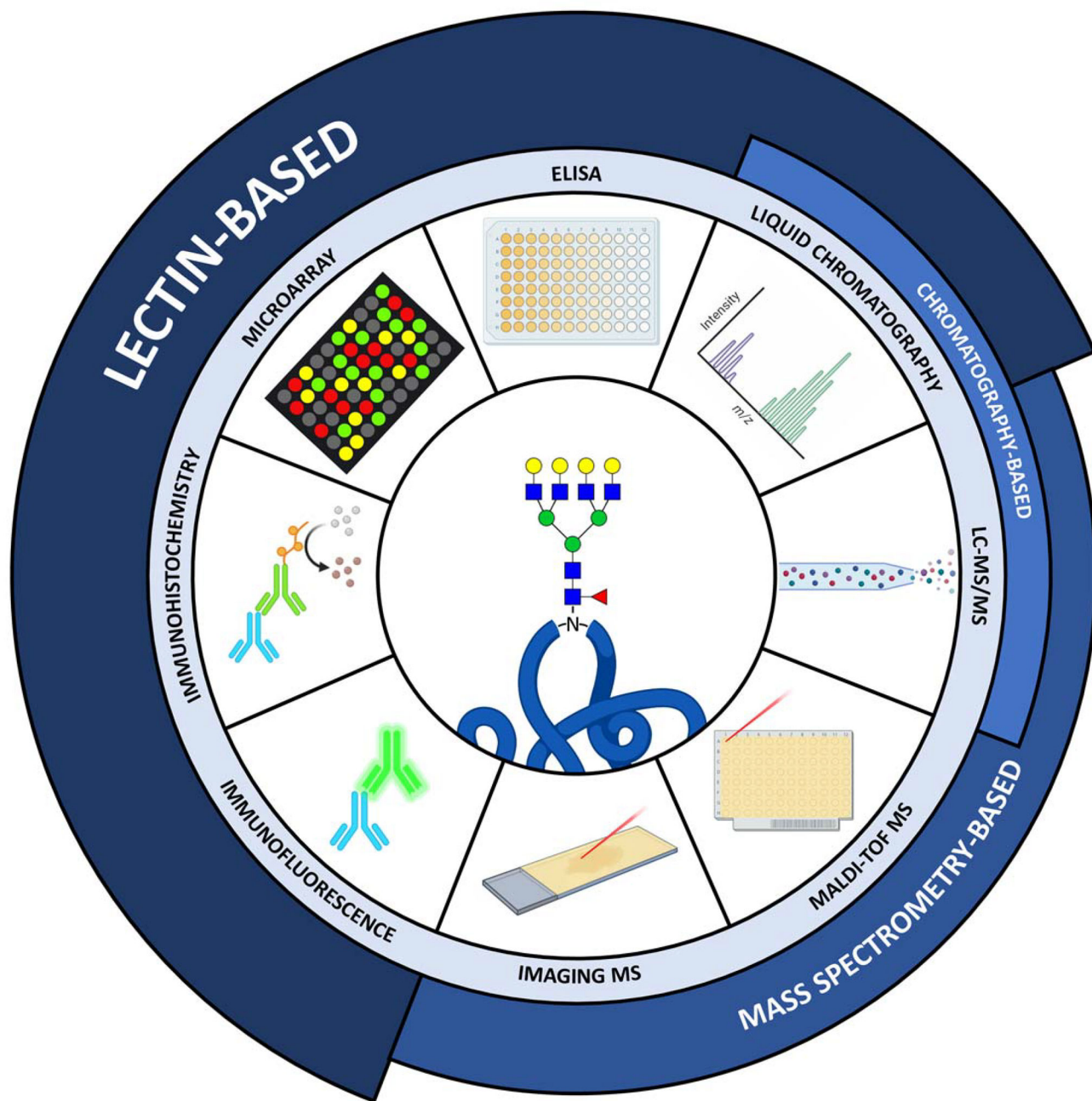
**Figure 2. Techniques to analyze N-glycans.**
Immunofluorescence, immunohistochemistry, lectin microarray, ELISA, liquid chromatography, LC-MS/MS, MALDI-TOF and Imaging MS techniques for analysis of N-glycosylation. Broadly, these analyses are either lectin-based, chromatography-based or mass spectrometry based, although significant overlap exists between these classes (e.g. liquid chromatography-coupled mass spectrometry or lectin affinity chromatography).
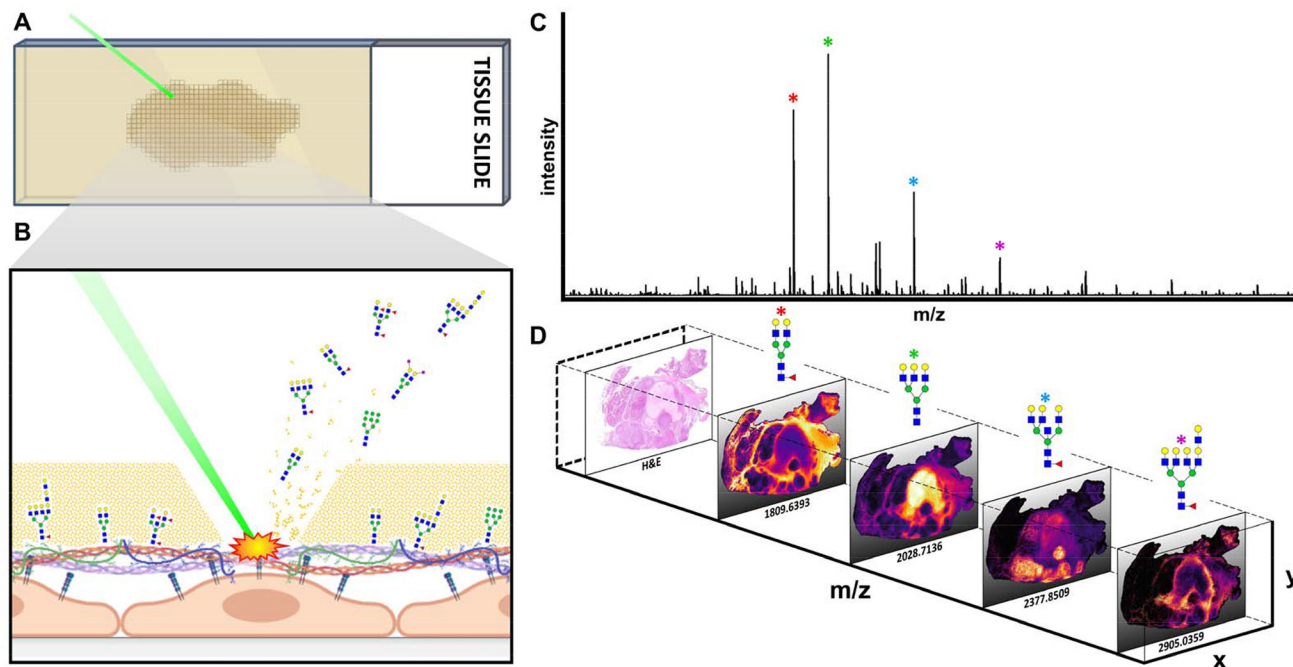
**Figure 3. MALDI imaging mass spectrometry for the analysis of N-glycans.**
A) MALDI laser striking a rastered FFPE tissue section. B) Close up view of MALDI laser ablating the tissue surface and subsequent desorption/ionization and ejection of N-glycan ions into the mass spectrometer for detection. C) Representative mass spectrum generated from a single MALDI laser strike. D) Spatial mapping of individual pixel spectra back to their original X,Y coordinates generates N-dimensional N-glycan data.
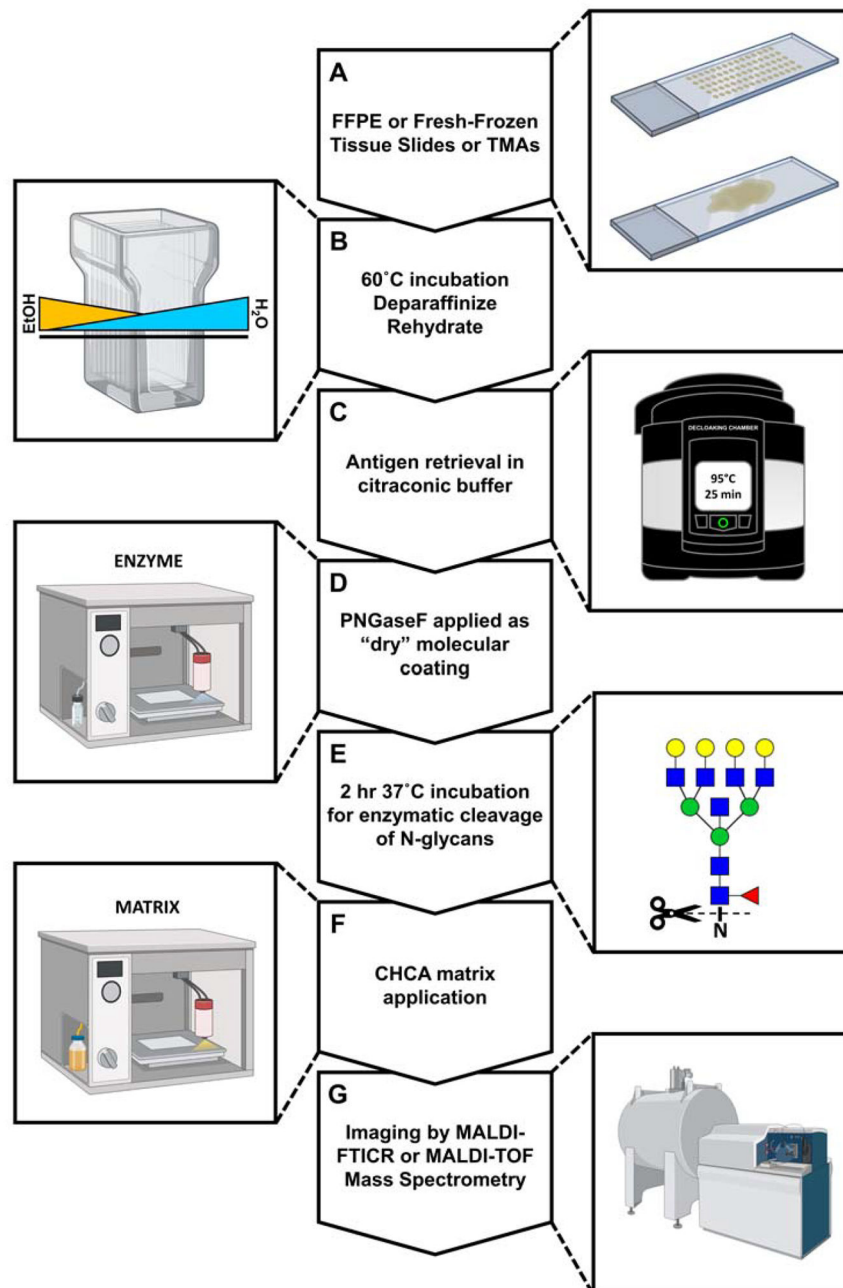
**Figure 4. A standardized workflow for in-situ N-glycosylation analysis by MALDI-IMS.**
A) FFPE or FF tissue slides. B) Dewaxing and rehydration of tissue specimens prepares them for antigen retrieval and removes signal-suppressing lipids and metabolites. C) Heat-induced epitope exposure in low-pH citraconic buffer. D) Use of an automated solvent sprayer to apply a dry molecular coating of peptide N-glycosidase F. E) Enzymatic cleavage of N-glycans from their glycoprotein carriers. F) Application of a crystalline organic acid matrix by an automated solvent sprayer as in D. G) Spatial analysis of released N-glycans by matrix-assisted laser desorption/ionization and FT-ICR MS detection.

**Figure 5. CID fragmentation for N-glycan structural identification.**
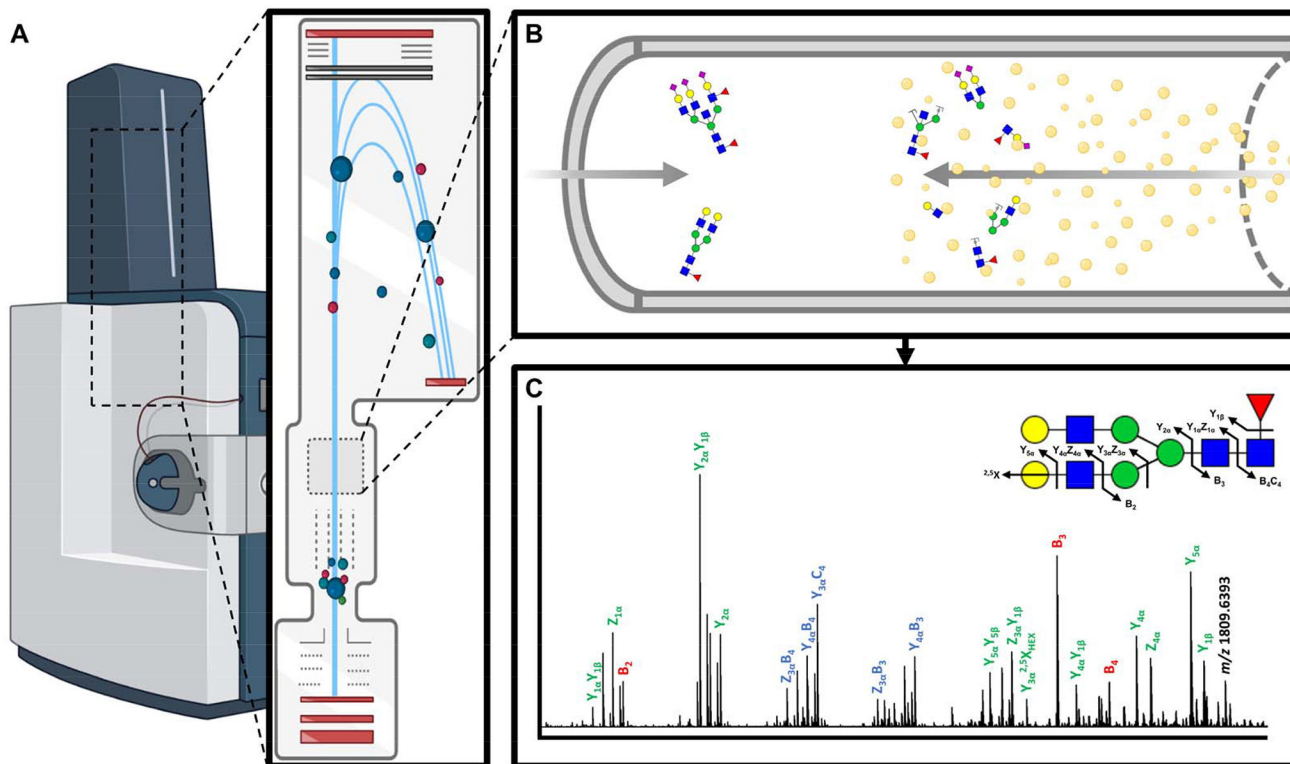A) MALDI-TOF mass spectrometer with on-line CID collision cell. B) Accelerated precursor N-glycan ions collide with an inert gas (e.g. nitrogen) leading to gas-phase fragmentation. C) Analysis of the N-glycan fragment ions allows for reconstruction of the complete N-glycan structure. CID generates B/Y, C/Z and A/X fragment ions.

**Figure 6. N-glycan imaging mass spectrometry of a late stage pancreatic ductal adenocarcinoma tissue.**

A) H&E staining and histopathological annotation of a late stage PDAC tissue with adenocarcinoma, necrosis, tumor margin and adjacent normal subtypes. B) Overlay image of four representative N-glycan masses specific to each tissue subtype (adenocarcinoma, orange, Hex7dHex1HexNAc7, *m/z* 2743.9831; necrosis, cyan, Hex5HexNAc4, *m/z* 1663.5814; tumor margin, white, Hex8dHex1HexNAc7, *m/z* 2905.0359; adjacent normal, pink, Hex6dHex1HexNAc5, *m/z* 2174.7715). C) Segmentation analysis based on 85 unique N-glycans detected. D) Individual N-glycan heatmaps for tissue-subtype specific N-glycans *m/z* 1663.5814, *m/z* 2174.7715, *m/z* 2743.9831 and *m/z* 2905.0359.
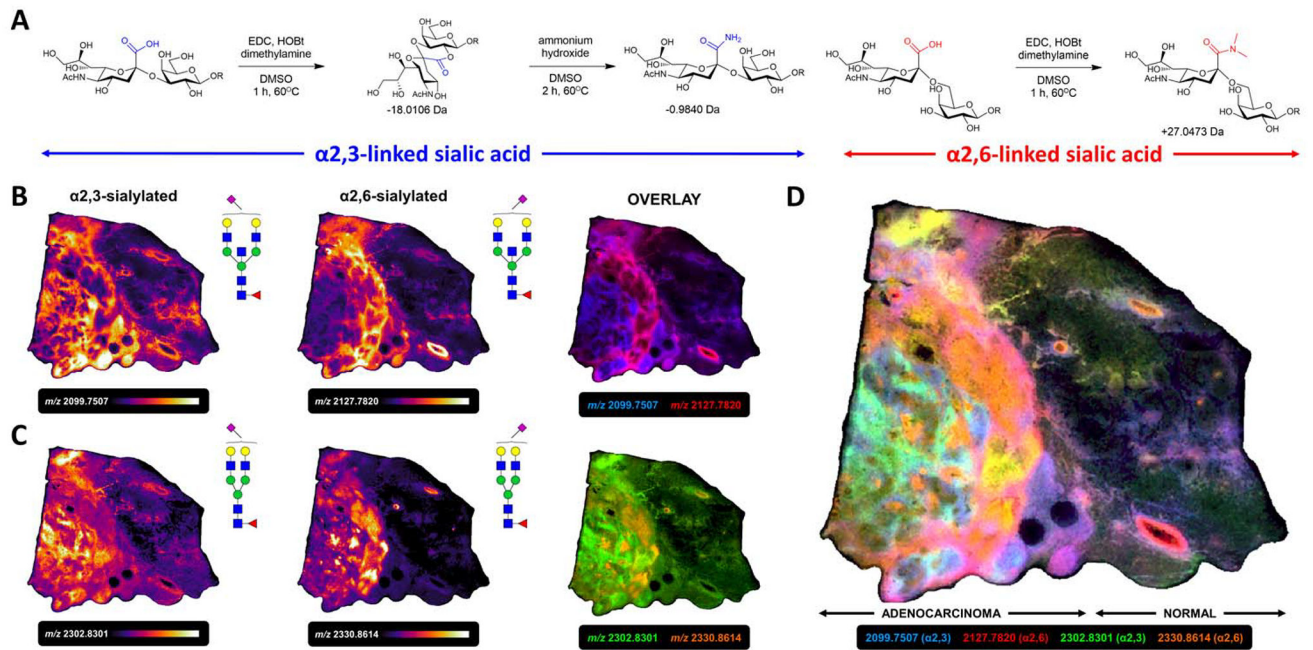
**Figure 7. Sialic acid stabilization and linkage differentiation by on-tissue amidation chemistry.**
A) Derivatization of α2,3- and α2,6-linked sialic acid anomers with amide (−0.9840 m.u.) and dimethyl amine (+27.0473 m.u.) functional groups. B) α2,3 (*m/z* 2099.7507) and α2,6 (*m/z* 2127.7820) linkage isomers of Hex5dHex1HexNAc5Neu5Ac1 drove distinct spatial localization. C) α2,3 (*m/z* 2302.8301) and α2,6 (*m/z* 2330.8614) linkage isomers of Hex5dHex1HexNAc4Neu5Ac1. D) Overlay of 4 α2,3- and α2,6-sialylated N-glycans suggests compartmentalization of specific sialyltransferases.
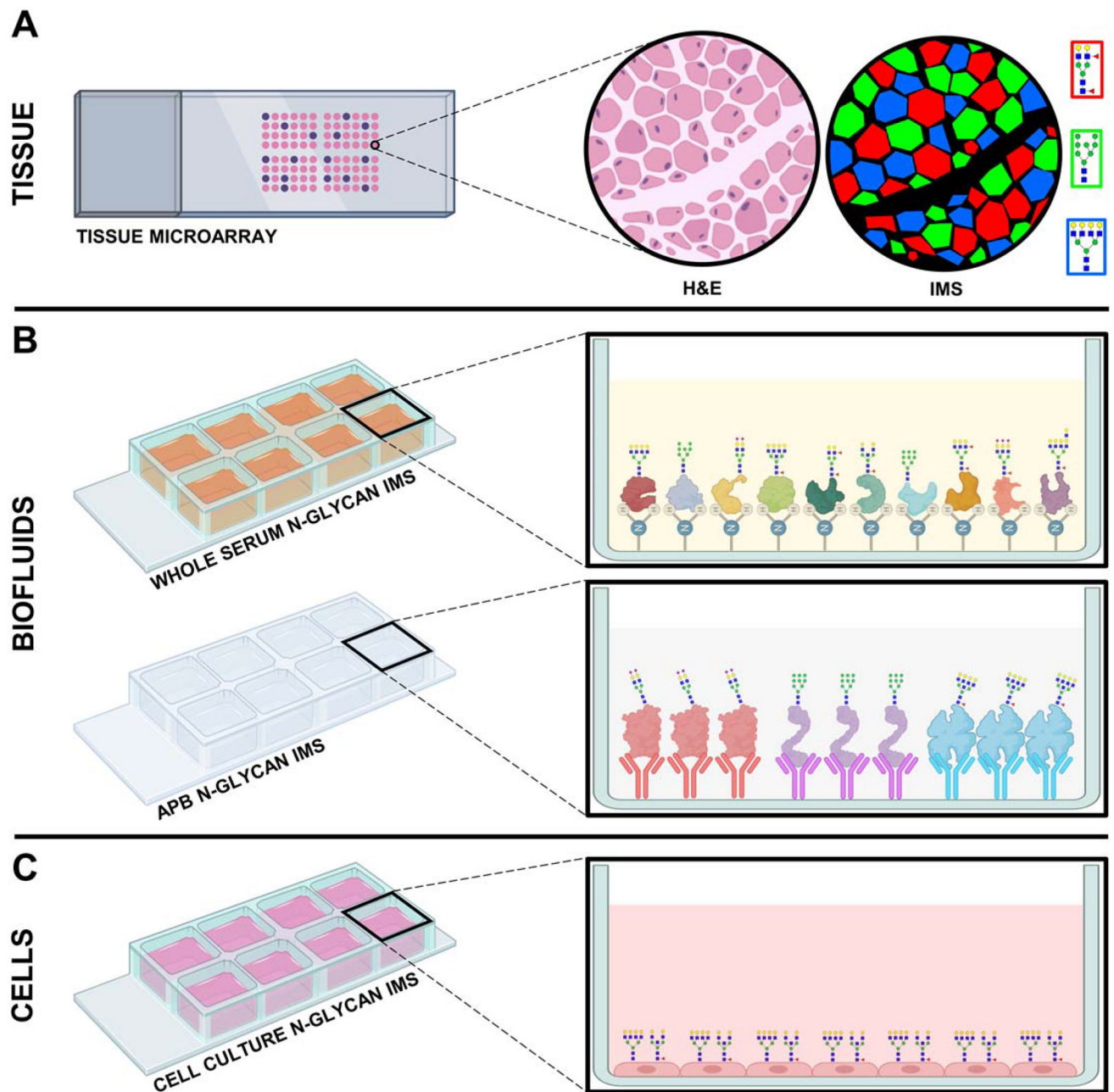
**Figure 8. Expanded N-glycan IMS approaches for alternative clinical and laboratory sample types.**

A) N-glycan imaging mass spectrometry of tissue microarray cores correlates histopathologically-annotated tissue features with N-glycan analytes in a high-throughput fashion. B) N-glycan imaging of biofluids. Whole-serum N-glycan analysis examines amine reactive slide-captured serum total protein, while APB N-glycan imaging employs targeted N-glycan analysis of specific glycoproteins through high-affinity capture by slide-bound antibodies. C) N-glycan imaging mass spectrometry of cultured cells fixed to a glass microscope slide.

**Table 1.**

Applications of N-glycan IMS

| Disease | Year | Group | Cohort | Disease-Associated N-glycans |
|---------|------|-------|--------|------------------------------|
| *Cancer* | | | | |
| *Breast* | 2019 | Scott et al. | Whole tissue, TMA | Polylactosamines ↑ (HER2 and TN-associated) |
| | 2019 | Herrera et al. | Whole tissue, TMA | Tetra-antennary with LacNAc ↑ (poor survival-associated) |
| | 2019 | Scott et al. | Whole tissue | Sialylation ↑ Fucosylation ↓ (necrosis-associated) |
| | 2019 | Angel et al. | 4T1 cells | Hex9HexNAc2 ↑ |
| *Colorectal* | 2016 | Holst et al. | Whole tissue | Sialylation ↑ |
| | 2016 | Heijs et al. | Whole tissue | High mannose ↑ |
| | 2020 | Boyaval et al. | Whole tissue | High mannose ↑ |
| | | | | Sialylation ↑ |
| *Lung* | 2017 | Drake et al. | TMA | High mannose ↑ |
| | | | | Hex6HexNAc2 ↑ |
| | 2020 | Carter et al. | Whole tissue | High mannose ↑ (hyperplasia-associated) |
| | | | | Fucosylated biantennary ↑ (hyperplasia-associated) |
| *Liver* | 2014 | Powers et al. | TMA | Hex7HexNAc6 ↑ |
| | | | | Hex8HexNAc2 ↑ |
| | 2015 | Powers et al. | Whole tissue, TMA | Hex7HexNAc6 ↑ |
| | | | | Core fucosylation ↑ |
| | 2018 | West et al. | Whole tissue, TMA | Hex7HexNAc6 ↑ |
| | | | | Branching ↑ |
| | | | | Fucosylation ↑ (poor survival-associated) |
| | 2019 | Angel et al. | HepG2/C3A cells | Afucosylated bi-/tri-antennary ↑ |
| | | | | Hex9HexNAc2 ↑ |
| | 2020 | West et al. | Whole tissue, TMA | Core fucosylation ↑ (poor survival-associated) |
| | 2020 | Black et al. | Serum | Hex3dHex1HexNAc4 ↑ (cirrhosis-associated) |
| | | | | Hex5HexNAc4NeuAc2 ↑ (cirrhosis-associated) |
| *Ovarian* | 2016 | Everest-Dass et al. | Whole tissue | High mannose ↑ |
| | 2019 | Briggs et al. | Whole tissue, TMA | Hex9HexNAc2 ↑ (stage III-associated) |
| | | | | Fucosylation ↑ (stage III-associated) |
| | | | | Bisecting GlcNAc ↑ (stage III associated) |
| | | | | Sialylation ↑ (stage III-associated) |
| *Pancreatic* | 2014 | Powers et al. | Whole tissue | Biantennary ± fucose ↑ (fibrosis-associated) |
| | | | | Undecorated ↑ (necrosis-associated) |
| | 2015 | Powers et al. | Whole tissue | Biantennary ↑ (fibrosis-associated) |
| | 2020 | McDowell et al. | Whole tissue, TMA | Fucosylation ↑ |
| | | | | Branching ↑ |
| | | | | Bisecting GlcNAc ↑ |
| | | | | Sialylation ↑ |
| | | | | Polylactosamines ↑ (tumor margin-associated) |
| | | | | Undecorated ↑ (necrosis-associated) |

| Disease | Year | Group | Cohort | Disease-Associated N-glycans |
|---------|------|-------|--------|------------------------------|
| | | | | High mannose ↓ |
| | | | | Sulfated, terminal GalNAc biantennary ↑ (islet-associated) |
| *Renal* | 2019 | Drake et al. | Whole tissue, TMA | Bisecting GlcNAc ↑ (proximal tubule-associated) |
| | | | | Hex7dHex1HexNAc6 ± sialic acid ↑ (glomeruli-associated) |
| | | | | Polylactosamine ↑ |
| | | | | Branching ↑ |
| | | | | Outer-arm fucosylation ↓ |
| | | | | Hex5dHex1HexNAc4NeuAc1 ↑ (fibrillar capsule-associated) |
| | | | | Fucosylation ↓ (PKD cyst fluid-associated) |
| ***Non-Cancer*** | | | | |
| *Osteoarthritis* | 2016 | Briggs et al. | Whole tissue | Hex5HexNAc4NeuAc2 ↑ (bone marrow lesion-associated) |

↑ increased expression relative to normal healthy tissue

↓ decreased expression relative to normal healthy tissue

( ) association with a specific disease or tissue subtype