Published in final edited form as:

Adv Cancer Res. 2017 ; 134: 85–116. doi:10.1016/bs.acr.2016.11.009.

MALDI mass spectrometry imaging of N-linked glycans in cancer tissues

Richard R. Drake^{1,*}, Thomas W. Powers¹, E. Ellen Jones¹, Evelyn Bruner², Anand S. Mehta¹, and Peggi M. Angel¹

¹Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA

²Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC, USA

Abstract

Glycosylated proteins account for a majority of the post-translation modifications of cell surface, secreted and circulating proteins. Within the tumor microenvironment, the presence of immune cells, extracellular matrix proteins, cell surface receptors and interactions between stroma and tumor cells are all processes mediated by glycan binding and recognition reactions. Changes in glycosylation during tumorigenesis are well documented to occur, and affect all of these associated adhesion and regulatory functions. A MALDI imaging mass spectrometry (MALDI-IMS) workflow for profiling N-linked glycan distributions in fresh/frozen tissues and formalin-fixed paraffin-embedded (FFPE) tissues has recently been developed. The key to the approach is the application of a molecular coating of peptide-N-glycosidase to tissues, an enzyme that cleaves asparagine-linked glycans from their protein carrier. The released N-linked glycans can then be analyzed by MALDI-IMS directly on tissue. Generally 40 or more individual glycan structures are routinely detected, and when combined with histopathology localizations, tumor specific glycans are readily grouped relative to non-tumor regions and other structural features. This technique is a recent development and new approach in glycobiology and mass spectrometry imaging research methodology, thus potential uses such as tumor-specific glycan biomarker panels and other applications are discussed.

Keywords

Glycobiology; glycomics; N-linked glycosylation; glycoproteins; fucosylation; formalin-fixed paraffin embedded tissue; tumor antigens glycoprotein; biomarkers; MALDI imaging mass spectrometry

1. Introduction

MALDI imaging mass spectrometry (MALDI-IMS) profiling of N-linked glycan distributions in fresh/frozen tissues, formalin-fixed paraffin-embedded (FFPE) tissue blocks

*To whom correspondence should be addressed: Richard R. Drake, 173 Ashley Avenue, Charleston, SC, 29425; (tel) 843 792 4505; draker@musc.edu.

and tissue microarrays (TMA) is a recent development and new approach in glycobiology research methodology. Reported initially in frozen kidney tissues [Powers et al., 2013] and quickly followed by application to different FFPE cancer tissues [Powers et al., 2014], this methodology is particularly relevant for cancer tissues, as most known cancer biomarkers are glycoproteins or carbohydrate antigens. The basic approach is to spray a molecular coating of the enzyme that releases N-linked carbohydrates from carrier proteins, peptide N-glycanase (PNGaseF), on-tissues, followed by matrix application and MALDI-IMS. Beyond the initial publications, eight other reports from multiple laboratories have been published for FFPE cancer tissues [Powers et al., 2015; Drake et al., 2015; Holst et al., 2016; Heijs et al., 2016; Everest-Dass et al., 2016] and other non-cancer FFPE tissue types [Briggs et al., 2016; Toghi Eshghi et al., 2014; Gustaffson et al., 2015]. Compared to other biomolecules targeted by MALDI-IMS like lipids, metabolites and proteins/peptides, IMS of N-glycans has some unique analytical aspects. One major consideration for N-glycan imaging is that FFPE tissues are the best starting material, which is a particular advantage due to the vast repositories of clinical FFPE tissues, primarily from cancer patients, archived worldwide. Similarly, tissue microarrays derived from multiple FFPE tissues can also be efficiently used [Powers et al., 2014; Powers et al., 2015]. The use of the enzyme, PNGase F to release the N-glycans means that the derived IMS signal is highly specific to only released N-glycan patterns from tissue. Many N-glycan structures are known and can be confirmed by collision-induced dissociation (CID) directly from tissues or following extraction off of tissue [Powers et al., 2015; Holst et al., 2016]. For most cancer samples analyzed thus far, the numbers of glycans detected per tissue sample are manageable, generally 40–60 per sample. This in turn facilitates the generation of different glycan panels associated with specific histopathology features and tissue sub-regions useful for cancer biomarker assessment. These localized regions of interest can be further targeted for identification of the carrier glycoproteins.

The focus of this chapter is to provide an overview of the methods and challenges associated with the emerging area of N-glycan tissue imaging by mass spectrometry as applied to cancer. A background of the significance of glycosylation in cancer development and progression is provided, as well as a summary of other methods used to evaluate glycan expression in cancer tissues. Using a colorectal adenocarcinoma FFPE tissue that has been evaluated for N-glycan and peptide MALDI-IMS, examples of the type of glycan data obtained from this tissue are provided to illustrate the strengths of the approach, as well as highlight the challenges and limitations of the method. Application of the method to tissue microarrays has been covered in previous studies [Powers et al., 2014; Powers et al., 2015], but data from an example custom tissue microarray that is being used for continued method development is provided. Future applications of the approach are numerous, and are discussed in context with other large data “omics” analyses and clinical diagnostics.

2. Glycosylation and Cancer

2.1. Function and types of glycosylation

The functions and regulation of glycoproteins carrying N-linked and O-linked sugar chains have been extensively reviewed [Moremen, Tiemeyer, & Nairn, 2012; Varki 2016; Kudelka,

Ju, Heimbürg-Molinaro, & Cummings, 2015]. It is estimated that over 50% of human proteins are glycosylated, making it one of the most common and complex post-translational modifications. There are over three hundred metabolic enzymes, glycosyltransferases and glycosidases involved in glycan biosynthesis and processing [Zoldoš, Novokmet, Be eheli, & Lauc, 2013]. Glycoproteins account for ~80% of the proteins located at the cell surface and in the extracellular environment, and serve as binding ligands for cell adhesion, extracellular matrix molecules, signaling receptors, immune cells, lectins and pathogens [Varki 2016]. Glycans present on newly synthesized glycoproteins also aid in basic protein folding, intracellular transport and secretion processes.

There are ten monosaccharide units from which mammalian glycans are constructed, but additional diversity can be achieved by further modification of those monosaccharides, like sulfation [Moremen, Tiemeyer, & Nairn, 2012]. Structurally, hexose (Hex) monosaccharides consist of glucose (Glu), galactose (Gal) and mannose (Man) residues. N-acetylhexosamine (HexNAc) monosaccharides consist of both N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc). The remaining monosaccharides include fucose (dHex, Fuc), N-acetylneuraminic acid (NeuAc), glucuronic acid, iduronic acid and xylose. The glycans will be further discussed in this chapter and their representative symbols are listed in Figure 1A.

2.2. N-linked glycan biosynthesis

The generation of the many possible N-glycan structures that are attached to glycoproteins is the result of a series of sequential glycan addition and subtraction reactions mediated by specific glycosidases and glycosyltransferases [reviewed in Rini, Esko, & Varki, 2009; Stanley, Schachter, & Taniguchi, 2009]. Briefly, the addition of N-glycans to proteins occurs co-translationally in the endoplasmic reticulum (ER), whereby a Glc3Man9GlcNAc2-P-P-Dolichol donor substrate is transferred to an asparagine residue on the recipient protein by an oligosaccharyltransferase complex. The N-glycan consensus sequence is N-X-S/T, where X is any amino acid other than proline. After transfer, the glycan is trimmed sequentially in the ER and cis-Golgi by glucosidases and mannosidases to Man5GlcNAc2, which serves as the precursor to complex and hybrid glycan structures (see Figure 1). Glycans that are not processed or incompletely processed by the mannosidases are termed high mannose glycans, containing 5–9 mannose residues (Man5-9GlcNAc2). (Figure 1A). To generate complex glycans, Man5GlcNAc2 is acted upon by N-acetylglucosaminyltransferases I and II to generate an initial biantennary complex glycan. Triantennary and tetraantennary complex glycans are generated from the activity of N-acetylglucosaminyltransferases IV and V to add additional branches. N-acetylglucosaminyltransferase III is an additional enzyme that transfers a bisecting GlcNAc residue onto the complex glycan (Figure 1A). In the trans-Golgi, further maturation may take place to provide additional glycan diversity. Among the most common additions are the transfer of β -linked galactose residues to the non-reducing end, the addition of α 1,6-linked fucose residues to the GlcNAc directly bound to the asparagine (termed core fucosylation), and the addition of sialic acids and fucose residues on the branched chains. Different examples of these N-glycan structures are shown in Figures 1–7.

2.3. N-linked glycans and cancer

Alterations and changes in cell surface glycosylation during tumorigenesis are well documented and have been extensively reviewed [Christiansen, Chik, Lee, Anugraham, Abrahams, & Packer, 2014; Kudelka, Ju, Heimbarg-Molinaro, & Cummings, 2015; Pinho & Reis, 2015; Taniguchi & Kizuka 2015;]. This is underscored by the fact that the majority of current FDA-approved tumor markers are glycoproteins or glycan antigens, including PSA, and also CA19-9 [Adamczyk, Tharmalingam, & Rudd, 2012; Ruhaak, Miyamoto, & Lebrilla, 2012]. Broadly for cancers, increased expression of N-acetylglucosaminyl transferase V (GnT-V) transcripts in tumors leads to an increase in β 1–6 GlcNAc branching in N-linked structures necessary for larger tri- and tetraantennary structures associated with the metastatic phenotype of multiple cancer types [Miwa, Song, Alvarez, Cummings, & Stanley, 2012; Pinho & Reis, 2015; Schultz, Swindall, & Bellis, 2012; Taniguchi & Kizuka 2015]. Structurally, increased branching of glycans in cancers is typified by increased detection of sialyl Lewis X and sialyl Lewis A antigens, as well increases in polylactosamine modifications. These structures in turn are recognized by selectins and other carbohydrate lectins expressed on different tissues involved with immune cell binding, and in the case of extravasation, binding to cells in distant organ/tissue sites. Conversely, the presence of bisecting-GlcNAc residues from the activity of N-acetylglucosaminyl transferase III (GnT-III) alter the structural conformation of the glycan chains and tend to limit branching [Miwa, Song, Alvarez, Cummings, & Stanley, 2012; Pinho & Reis, 2015; Taniguchi & Kizuka 2015] and therefore tumor progression. Within the tumor microenvironment, the presence of immune cells, extracellular matrix proteins, cell surface receptors and interactions between stroma and tumor cells are all processes mediated by glycan binding and recognition reactions. For example, increased levels of α 2,6 sialylation on integrins has been implicated in metastasis [Schultz, Swindall, & Bellis, 2012; Uemura et al., 2012].

3. Methodology for N-linked Glycan Detection by MALDI imaging

3.1. Tissue sources

Most imaging mass spectrometry techniques that are attempting to detect protein, lipid, and metabolite targets rely on the availability of fresh frozen tissues. The original N-glycan MALDI-IMS study described the technique for use in frozen tissues [Powers et al., 2013]. In subsequent studies, it was observed that use of FFPE tissues processed by standard histochemistry workflows, i.e., xylene washes to remove paraffin and antigen retrieval to reverse cross-links, yielded more N-glycan signal intensities as well as more target analytes [Powers et al. 2014]. The reasons for this difference are not completely understood, but one major factor is the significant processing of FFPE tissues. Formalin readily reacts with many biological molecules including lipids, proteins, RNA and DNA. The process of the reaction not only forms crosslinks between tissue content, but also modifies almost all end groups in biological molecules such as primary, and secondary amines, amides, hydroxyls, and sulfhydryls [Dapson 2007]. In FFPE tissue, such molecules therefore become undetectable through massive crosslinking. After undergoing formalin fixation, FFPE tissues are processed for paraffin embedding. This procedure involves heat-mediated solvent exchanges of ethanol, xylenes, and molten paraffin through the tissue, further washing out metabolites, lipids and molecules that may not have been crosslinked [Scalia, et al. 2016]. In particular

for N-glycans, which generally do not have any free amino groups and are not cross-linked, the extensive processing most likely serves to expose the N-linked glycan moieties on the remaining glycoproteins, allowing PNGaseF digestion to be more efficient. The methods certainly can be applied to frozen tissues, and hypothesize that in these tissues the N-glycans are less exposed to enzyme, and that cleaved glycans may still be bound to binding partners and/or shielded from ionization. The remainder of the chapter will focus on use of FFPE tissues as the starting material.

3.2. Glycan visualization in tissues: lectins and anti-carbohydrate antibodies

The most common methods used to evaluate N-glycans and other carbohydrates in tissues are the use of specific anti-carbohydrate antibodies and lectins. These reagents can be used for standard histochemistry types of analysis with FFPE tissues. Antibodies to anti-carbohydrate antigens are frequently derived as tumor antigens associated with known blood group antigen designations [Heimburg-Molinaro, Lum, Vijay, Jain, Almogren, & Rittenhouse-Olson, 2011; Kudelka, Ju, Heimburg-Molinaro, & Cummings, 2015; Ravn & Dabelsteen, 2000]. A well-known example carbohydrate antigen is that of CA19-9, and its measurement is used clinically in the prognosis for pancreatic and other gastrointestinal cancers. CA19-9 antibody recognizes a four sugar sialyl-Lewis-A carbohydrate motif comprised of GlcNAc-Gal, α 1–4 Fuc and α 2,3 sialic acid. Another carbohydrate antibody that has been described associated with prostate cancer detection is the F77 antigen, which recognizes blood group H antigen related Lewis Y glycan structures with α 1,2-Fuc [Nonaka et al., 2014]. Most carbohydrate targeted antibodies recognize a sugar structural epitopes of 1–4 sugars. While they can be effective for tissue immunohistochemistry staining, they may be less useful for defining the type of glycan class or carrier; i.e., the detected glycan could be present on glycolipids, O-linked glycoproteins or N-linked glycoproteins, or possibly all three, depending on the tissue.

Carbohydrate-binding proteins termed lectins, obtained from plants, bacteria, fungi, and animals, have been used for many decades to evaluate structural features of glycoproteins in many biomedical applications [Hirabayashi 2004; Lis & Sharon, 1998]. Like the carbohydrate-antigen antibodies, lectins recognize specific structural motifs of 2–4 sugar linkages. A frequent limitation to their use, especially for tissue histochemistry applications, are their poor affinity binding constants, generally in the low micromolar range but can be millimolar [Dam, Roy, Das, Oscarson, Brewer, 2000; Lis & Sharon, 1998]. Because they frequently possess multivalent binding capabilities, they are frequently used for affinity capture purposes and chromatography separations [Dam, Roy, Das, Oscarson, Brewer, 2000; Drake et al., 2006; Lis & Sharon, 1998]. For histochemistry purposes in tissues, they can be used to stain for known carbohydrate motifs using workflows similar to standard immunohistochemistry procedures in FFPE tissues. They cannot be used to distinguish whether the glycan motif that is bound by a given lectin is present on a glycolipid, O-linked glycoprotein or N-linked glycoprotein. The use of imaging MS for N-glycans addresses this issue of identifying specific individual N-glycans and their localization within a tissue, but does not replace the use of lectins or anti-carbohydrate antibodies. There are many potential synergistic uses of all three detection modalities in tissue that warrant further exploration.

3.3. Histochemistry stains

There are many histological stains that target functional groups on carbohydrates through binding or chemical reactions to produce carbohydrate specific staining [Myers, Fredenburgh, & Grizzle, 2008; Reid, Owen, Kruk, & Maitland, 1990]. Alcian Blue (AB) and Periodic Acid Schiff (PAS) are two of the most commonly used carbohydrate stains. Alcian Blue is a cationic, water soluble dye that has a blue color due to copper bound in the molecule [Scott, Quintarelli, & Dellovo, 1964]. AB binding to specific functional groups of carbohydrates is controlled by the pH of the solution. In acidic solution (pH 2.5), Alcian Blue binds to negatively charged sulfate and carboxylate groups of chondroitin sulfate, dermatan sulfate, heparin sulfate, hyaluronic acid as well as sialo and sulfo-mucins [Mowry 1956; Myers, Fredenburgh, & Grizzle, 2008; Scott, Quintarelli, & Dellovo, 1964]. At pH of 1.0 or below, sulfated mucins are stained [Spicer 1960]. Specificity of the stain is shown using controls that have been exposed to acidified methanolic solution or digested with hyaluronidase [Myers, Fredenburgh, & Grizzle, 2008; Spicer 1960]. Alcian blue is frequently used in pathologic evaluation of epithelial and connective tissue tumors [Kindblom & Angervall, 1975; Myers, Fredenburgh, & Grizzle, 2008]. An example of this stain for a colon cancer FFPE tissue, in comparison to an adjacent slice hematoxylin and eosin stain, is shown in Figure 1C and D.

The periodic acid Schiff (PAS) stain is used to target hyaluronins and acid mucopolysaccharides (proteoglycans and glycosaminoglycans). Unlike AB, PAS is a chemical reaction dependent on structural features of the monosaccharide unit. Periodic acid oxidizes 1,2 glycol groups to aldehydes, followed by a colorimetric reaction of the aldehydes with the Schiff reagent to produce a dark pink color [McManus & Cason, 1950]. PAS may be used at different concentrations to stain target carbohydrate components. Mild PAS (0.01% PAS for 10 minutes at 4°C) selectively oxidizes nonsubstituted OH groups of sialic acid [Richter & Makovitzky, 2006], while a 0.5% solution of PAS stains glycogen and mucins deep purple [Gomori, 1946].

Alcian blue and PAS are often combined to show pathological changes in distribution of mucin glycoproteins [Myers, Fredenburgh, & Grizzle, 2008]. Most mucin glycoproteins are heavily O-glycosylated, but do frequently contain one or two N-linked glycan chains. Alcian blue is used to stain sialo and sulfo components of carbohydrates blue (used at pH 2.5), while the PAS stain targets the neutral components dark pink. An example for using Alcian blue with PAS was reported in colorectal tissues [Jain, Mondal, Sinha, Mukhopadhyay, & Chakraborty, 2014]. Although not necessarily specific to N-glycans, this type of histochemical stain is useful in assessing the overall carbohydrate abundance in a given tissue, and can be used to correlate with N-glycan image distribution within that tissue.

3.4. Peptide N-glycosidase F (PNGaseF)

A key aspect of N-glycan imaging is the selectivity of the peptide N-glycosidase F enzyme to cleave the N-linked glycans attached to asparagines in the protein carrier (Figure 1B). The asparagine is deamidated to an aspartic acid, and this change within the N-X-S/T motif can be used to identify peptides that had been glycosylated. To facilitate use of this enzyme for the scale required for tissue imaging studies, the entire PNGaseF gene (P21163.2) from the

genome of *Flavobacterium meningosepticum* was cloned, expressed and purified in the Mehta laboratory as described [Powers et al., 2013]. A histidine epitope tag was included to facilitate purification, and it is stored with minimal buffer components and no other additives. Many commercially available preparations of PNGaseF have low amounts of enzyme and contain additives like glycerol or detergents, which can lead to ion suppression during ionization. The recombinant enzyme has proven to be stable and highly reactive when 20 µg are sprayed robotically onto tissue and incubated over a 2–4 hour period at 37°C in a humidified chamber [Powers et al, 2013; Powers et al. 2014].

Prior to application of PNGase F, waxed FFPE tissue sections are heat treated at 60°C for one hour. This step is essential for denaturing the protein content and substantially increases N-glycan signal. Tissue is then dewaxed using typical xylene washing steps, followed by high temperature antigen retrieval in a citraconic anhydride buffer. It is important to note that placing sections on positively charged slides or poly-l-lysine coated slides limits sample loss occurring through the antigen retrieval process. Other standard antigen retrieval buffers like Tris can also be used. Parameters often need to be optimized per tissue type (e.g., breast cancer vs prostate cancer). Continued optimization of each of these parameters is still ongoing.

3.5. Matrix and instrumentation choices for N-glycans

Classically, the matrix dihydroxybenzoic acid (DHB) has been used as for MALDI analysis of carbohydrates [Harvey 2015]. For MALDI IMS of N-glycans, however, both DHB and alpha-cyano-4-hydroxycinnamic acid (CHCA) have been used with success [Powers et al., 2013; Powers et al, 2014; Powers et al. 2015; Holst et al., 2016]. When deciding which matrix, a consideration is the type of instrument that will be used for the imaging experiment. Most MALDI time of flight (TOF) instruments have higher pressure sources that tend to produce intense matrix clusters, so that matrices are selected to limit interference with signals of interest. DHB has been the choice when using TOF instruments for many years. MALDI-IMS of N-glycans has been done on a Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometers [Powers et al., 2013; Powers et al., 2014; Powers et al., 2015; Heijs et al., 2016]. This instrument has an intermediate pressure source, with limited high mass matrix cluster peaks. Here, CHCA appears to result in more intense N-glycan signal from tissue.

When applying matrix, thickness of the tissue section must be considered as the ratio of matrix to tissue influences efficient production of signal [Yang and Caprioli, 2011]. Thick applications of matrix can also result in increased production of matrix clusters, obscuring target analytes. On the other hand, not enough matrix results in poor ionization and loss of signal. Our group has routinely used FFPE tissue sections 5 to 7 µm thick, and apply matrix at 0.002154 mg/mm². For FFPE tissue sections of 3 µm thickness, 0.001725 mg/mm² works well. Currently, matrices for MALDI IMS are applied by robotic sprayers which increase throughput and provide robust, consistent results [Angel & Caprioli, 2013; Schuereberg & Deininger, 2010]. The example N-glycan images shown in this chapter were all processed using a robotic sprayer to spray the PNGaseF and apply CHCA matrix. In unpublished evaluations of other matrix molecules for N-glycan imaging of tissues, no matrix has yet

outperformed CHCA or DHB. A formal analysis of different matrix molecules is still warranted, as well as evaluations of glycan data obtained in negative ion mode.

Primary instruments that have been used for N-glycan imaging are MALDI-TOFs and MALDI-FT-ICRs. Each has certain advantages. TOF instrument provide high throughput but lower mass resolution capabilities. A situation that frequently occurs on-tissue is that the second isotope from one peak may be near-isobaric to another. For N-glycans, this is very common in tumor tissues for complex glycans that may have two fucoses (292 m.u.) versus a single sialic acid (291 m.u.). An example is Isotope 2 of Hex5dHex1HexNAc4NeuAc1 + 1Na at m/z 2101.7426 versus Isotope 1 of Hex5dHex3HexNAc4 + 1Na at m/z 2101.7551, suggesting a needed resolving power of ~168,000 (assuming equal height at 50% peak height). MALDI-FT-ICRs can easily detect this difference with imaging data typically acquired at estimated resolving powers of 85,000–160,000. FT-ICRs are generally lower throughput but the advantage is the higher mass resolution and increased sensitivity when compared to MALDI- TOF instruments.

MALDI sources frequently generate structural fragments due to in source decay. In a MALDI spot comparison of carbohydrates, this can be leveraged as pseudo MS3 for structural elucidation [Mechref, Novotny, & Krishnan, 2003]. In imaging studies, this becomes a problem as it is impossible to determine an intact structure produced by the biology versus the fragmented structure induced by instrumentation. Sialic acids are a particularly challenging problem as they readily cleave under even low source energy. Vendors for MALDI-FT-ICR instruments have been aware of this problem for analysis of carbohydrates and have built in a cooling nitrogen gas stream into the source, limiting sialic acid cleavage by in source decay [O'Connor & Costello, 2001; O'Connor, Mirgorodskaya, & Costello, 2002].

Although high mass accuracy is useful in distinguishing between near-isobaric species, this cannot determine glycoforms. Ion mobility mass spectrometry incorporates post source gas phase separation to separate glycoforms based on collision cross section. Ion mobility has been used extensively for carbohydrate characterization [Fenn & McLean, 2011; Gray et al., 2016] in MALDI spot or in liquid chromatography analyses. A few reports of on-tissue imaging analyses show that on fresh frozen tissue, ion mobility can be used to separate and determine structural characteristics of carbohydrates apart from other biological molecules [McLean, Fenn., & Enders, 2010]. In preliminary studies, an ion mobility imaging approach has been applied to PNGaseF digested FFPE tissues (data not shown). The approach easily distinguishes distinct glycoforms in tumor and non-tumor regions, as well as potential separation of glycan isomers.

3.6. Structural confirmation

Studies on structural information for N-glycans are performed parallel with imaging experiments, as a combination of on-tissue and off-tissue experiments. The on-tissue MSI experiments provide structural information by tandem mass spectrometry, enzymatic approaches, or chemical derivatization in a way that preserves localization of the N-glycan species. For tumor tissues, this aids in confirming that a specific N-glycoform has non-tumor or tumor specific localization. Tandem mass spectrometry is performed either on the same

tissue section as was used for the imaging experiment, or on a serial tissue section [Holst et al., 2016; Powers et al., 2015]. Serial tissue sections used for tandem mass spectrometry are prepared the same way as for an imaging experiment. Most commonly, the same mass spectrometry source used in the imaging experiment is employed as this eliminates issues of differential ionization. For MALDI MSI, on-tissue tandem mass spectrometry experiments are generally directed at specific N-glycan species that have been shown to be altered in N-glycan imaging profiles from large cohorts. Tertiary and larger N-glycan structure may be difficult to fragment on-tissue by MALDI MS/MS. The use of differential exoglycosidases in combination with PNGase F has been used to uncover and define large structures that are not as accessible on-tissue by MALDI-MS/MS methods [Powers et al., 2013]. On-tissue chemical derivatization, used to increase detection sensitivity and stabilize moieties such as sialic acid, is applied to tissue sections serial to the tissue section used for the imaging experiment. The use of chemicals in on-tissue derivatization is challenging since chemicals that result in ion suppression must be removed in a way that does not delocalize the N-glycan species. Recently, an ethyl esterification method for high throughput profiling of sialic acid containing N-glycans [Reiding, Blank, Kuijper, Deelder, & Wuhrer, 2014] was modified for application to tissue sections [Holst et al., 2016]. Application to leiomyosarcoma and colon carcinoma tissue sections showed localization of α 2,3 and α 2,6 linkages shifts in sialic acid content and stabilized the potentially labile sialic acid moieties [Holst et al., 2016].

Off-tissue approaches to defining N-glycan structures first release N-glycans from tissue sections, and collect released N-glycans from the tissue as a supernatant. Derivatization methods are then applied to improve fragmentation and detection, followed by chromatography to separate and quantify N-glycoforms [Briggs et al., 2016; Gustafsson et al., 2015; Powers et al., 2013]. An advantage to this approach is that virtually any chemical derivatization approach may be used since the sample will be cleaned up by the following chromatography approaches. A disadvantage is that the data no longer has region specific information. This disadvantage may be partly overcome by microdissection of tissue sections performed prior to releasing the N-glycans [Briggs et al., 2016]. Nano LC-MS/MS strategies paired with IMS allow high sensitive detection and fragmentation of molecules up to intact proteins from low microliter extractions of molecules from tissue sections [Schey, Anderson, & Rose, 2013], but this type of strategy has not yet been applied to the imaged N-glycome. LC-MS/MS experiments could also be performed on larger amounts of the same tissue that was used for tissue imaging, as has been done for identification of proteins from melanoma or gastric cancers [Angel & Caprioli, 2013; Balluff et al., 2011; Hardesty, Kelley, Mi, Low, & Caprioli, 2011]. The use of this strategy would allow extensive cataloguing of N-glycome structural information but without region and cell specific information. Both top down and bottom up approaches should be considered for structural characterization of N-glycans parallel with imaging experiments.

4. N-glycan distribution linked with histopathology

4.1. Major structural classes

In the analysis of the different types of N-glycans that are detected in the tissues, a structural biosynthetic and/or catabolic theme has been observed that is linked to their histopathological localizations. For reference throughout this chapter, we have selected to highlight a FFPE human colon adenocarcinoma tissue that has many distinct features, including regions of adenocarcinoma, mucinous tumor, adjacent normal colon crypts and different non-tumor stroma regions. Imaging of this tissue for N-glycans by FTICR-MALDI, and different histological stains of it, will be presented throughout as examples of the type of information that can be obtained with the approach. In this sub-section, examples of five main structural classes will be shown in the same colon tissues. The representative distributions of these five N-glycan groups are highlighted in Figure 2, and discussed individually in the next sections.

4.2. High mannose N-glycans

N-glycans with high mannose chains represent an early stage in biosynthetic processing, as described in Figure 3. If glycans are detected in tissues with primarily only mannose ($n = 1-9$) and two N-acetylglucosamines, these would be considered to be intracellular intermediates residing in the ER or Golgi membranes in normal tissues. As these structures are degraded by mannosidases to only three mannose residues and then further processed to complex N-glycans, these structures would not be expected to be on the cell surface. However, in tumor tissues this is frequently not the case, as high mannose containing glycans and other biosynthetic intermediates (also called pauci-mannose glycans) are found on secreted glycoproteins and on the cell surface [Loke, Kolarich, Packer, & Thaysen-Andersen, 2016; Nyalwidhe, et al., 2013]. Specific mannose recognizing C-type lectin receptors are also known to bind to them. These mannose structures are also immunogenic, and specific auto-antibodies to high mannose structures can be detected in sera from cancer and infectious disease patients [Wang 2012; Wang et al. 2013]. Whether high mannose glycans are secreted or transported to the cell surface to represent a programmed response in tumors, an immune response to the tumor, or are reflective of aberrant transport regulation in cancer cells remains to be determined.

In the published MALDI imaging studies of N-glycans detected in tumor tissues, the high mannose structures are routinely detected in abundance [Everest-Dass et al., 2016; Heijs et al., 2016; Holst et al., 2016; Powers et al., 2014; Powers et al., 2015]. An example of this is shown in Figure 3, where the number of mannose residues is decreasing from top left to bottom right, reflecting mannosidase processing. The tumor specific localization of these structures from high mannose (Man4-9) and paucimannose (Man2-3, +/-Fuc) classes is readily apparent. For most tissue types, the paucimannose and Man6-Man9 structures are most abundant, while the Man4 is usually the lowest in abundance. In this tissue example, distribution of the Man5 glycan is both tumor-localized and located in non-tumor regions. Overall, we have found the distribution of Man5 to be primarily in tumor regions, with a presence in non-tumor locations to be highly variable. As a class, the high mannose and

paucimannose structures represent easily detectable tumor-localized glycans using MALDI imaging approaches.

4.3 Non-tumor stroma and normal tissue glycans

Complex N-glycans are derived from the Man3GlcNAc2 precursor, built sequentially by the activity of individual glycosyltransferases that add multiple N-acetylglucosamine, galactose, fucose and sialic acid sugars. Branching of the biantennary chains to include tri- or tetraantennary structures, if present, also occurs during this process (discussed further in section 4.4). To illustrate this biosynthetic pattern and the role of co-localization within stroma features, images of the colorectal tissue for biantennary structures with (Figure 4) or without (Figure 5) a core fucose modification are shown. The structures shown in these two Figures represent the most commonly detected N-glycans in tissue MS imaging studies [Holst et al., 2016; Powers et al., 2014], typified by Hex5HexNAc4Fuc1 ($m/z = 1809$; Figure 4) and Hex5HexNAc4 ($m/z = 1663$; Figure 5). The singly sialylated versions of these structures ($m/z = 2122$ and 1976 , detected as doubly sodiated) are also abundant, and share the same overall tissue distributions. In many FFPE tumor tissues, these classes of glycans tend to provide distinct distribution patterns from each other in the non-tumor stroma regions, with some overlap, and vary significantly regarding distribution in different tumor types. These are useful target glycans to determine their distribution empirically with each new tissue analyzed, as they are generally highly abundant. This is illustrated in the overlay panel image in Figure 4 for the sialylated $m/z = 2122$ and 1976 glycans. The doubly sialylated N-glycans also have these same distributions, but are generally detected at much lower levels (images not shown). The presence of the core fucose appears to be one of the major factors for differential localization relative to the biantennary structures lacking the fucose. The presence of the core fucose of the indicated structures can be confirmed by collision-induced dissociation directly from tissues but requires significant amounts of tissue to perform the experiment [Powers et al., 2015].

An example of this phenomena is shown in colorectal tumor tissue where the presence of significant epithelial crypt structures is indicative of normal colon tissue regions. As shown in Figure 6A, there was both a structural and localization uniqueness to the most abundant N-glycans detected in the crypt epithelial cells. The common structural theme was the presence of N-glycans with putatively bisecting N-acetylglucosamine residues, including both fucosylated and non-fucosylated examples. Also shown is an example of a hybrid N-glycan structure at $m/z 1794$. These structures have been reported to be decreased in colon tumor tissues [Holst et al. 2016], consistent with their non-tumor localization shown in Figure 6A. This is consistent with the activity of GnT-III, the glycosyltransferase responsible for catalyzing the addition of the bisecting GlcNAc, being primarily associated with expression reflecting an anti-tumorigenesis activity [Taniguchi & Kizuka, 2015].

4.4. N-glycan branching and sialylation

Branching of complex biantennary glycans to tri- and tetra-antennary structures is catalyzed by GnT-IV and GnT-V and are frequently associated with more tumorigenic processes. Figure 6B shows the localization of the basic tri-antennary structures with or without a fucose, as well as a common tetra-antennary glycan with a single fucose of $m/z = 2539$.

Additionally, the tri-antennary structures with a single sialic acid are also shown. The distribution of the two non-fucosylated tri-antennary structures at $m/z = 2028$ and 2341 are similar to the abundant non-fucosylated biantennary glycan distributions shown for m/z 1663 and 1976 in Figures 4 and 5. The fucosylated tri-antennary glycan at $m/z = 2174$ and tetra-antennary glycan at $m/z = 2541$ have a largely adenocarcinoma tumor localization in the adenocarcinoma region of the colon tissue, analogous to the high mannose glycan distributions. Like that of high mannose glycans, a tumor-centric distribution of these two glycans is similar in many other tissue types, and are usually abundant in signal intensity, such that they are useful targets to examine in any new tumor tissues being analyzed.

A complication in the detection of sialylated glycans by MALDI is their well-known lability during the ionization process. Holst et al have demonstrated that stabilization of sialylated N-glycans *in situ* by ethylation reactions increases their detection and overall signal intensities. The Solarix series MALDI-FTICR instruments use a different source configuration allowing softer ionization and a cooling gas which facilitates detection of sialylated glycans, as can be seen in the example images used in this chapter. If a more standard MALDI instrument is used for N-glycan imaging, without any type of sialic acid stabilization, more non-sialylated complex glycans will be detected (e.g., $m/z = 1809$ and 1663) than is reflective of the actual amount of sialylated glycan present. At least for the bi-antennary structures with single or di-sialylated glycans, this may not be that critical regarding tissue localizations, as these sialylated structures generally co-localize with the non-sialylated precursors. This is not ideal and would have to be empirically determined for each tissue analyzed. Additionally, even with the capabilities of the MALDI-FTICR source configuration, the presence of up two sialic acids is generally the current limit to what N-glycans can be detected. For example, it cannot be concluded whether 3–4 sialic acid structures of the $m/z = 2174$ or 2539 glycans are present in the model colon tissue. An emerging solution is that of the *in situ* ethylation modification of sialic acid residues prior to PNGaseF release [Holst et al., 2016]. The ethylation modification has the added benefit of reacting differently with the two possible isomers of sialic acid, which are added as terminal residues in either α -2,3 or α -2,6 configurations. Ethylation of α -2,6 linked sialic acids results in a mass shift of 28 m.u., while an α -2,3 linkage will result in a lactonization reaction and decrease of 18 m.u. relative to the non-modified parent glycan. These shifts are readily detectable by any MALDI instrument. This is an emerging technique that is still being refined methodologically, and represents a significant improvement in the ability to study tissue sialylation for not only N-glycan structures, but other O-linked and glycosphingolipid species.

4.5. Fucosylation and the glycan isomer problem

In the mucinous tumor region, specific classes of complex glycans were detected, representing branched and fucosylated structures (Figure 6C). There is a clear structural theme of tetra-antennary glycans incorporating different numbers of galactose residues, as well as fucose. The presence of core fucose in the multi-fucosylated structures is assumed, but has not been confirmed. The five structures shown in Figure 6C were selected to illustrate this biosynthetic scheme, but there were many other glycans detected specifically in this region representing tri- and tetra-antennary structures with multiple fucose residues

not shown, overall highlighting a role for branching and multi-fucosylation as indicators of mucinous tumor.

While the high resolution capability of the MALDI-FTICR instrument can be used to determine the structural composition of N-glycans, there are multiple anomeric details that are lacking in regards to the specificity of the linkages between each sugar. For example, fucose residues can be attached via α -1,2 (FUT1,2), α -1,3 (FUT3,4,6,7,9,10,11) or α -1,4 (FUT3,4) linkages for outer arm modifications, or α -1,6 for core fucosylation linkages (FUT8). There are no differences in masses for these fucose linkages, and beyond the core fucose modification for simple bi-antennary complex glycans that can be confirmed by CID, the presence of 2 or more fucose residues precludes identification of the specific linkages or which arm of the glycan the attachment occurs. For sialylation, the ethylation modifications does directly address isomer identification, and for bi-antennary structures, the α -2,3 and α -2,6 linkages can be determined. For tri- or tetra-antennary glycans with 1–2 terminal sialic acid residues, it is not possible to determine which arm of the structure carries the modifications. Even with ethylation, only the numbers of total α -2,3 and α -2,6 sialic acid linkages can be inferred, and not their specific structural arm positions. Biologically, the locations of the terminal sialic acid residues are important as they serve as attachment sites for proteins, and their negative charges also affect folding and activity of their carrier protein. An emerging analytical approach that could assist with the challenges of determining fucosylation and sialylation isomers is ion mobility mass spectrometry, an analytical technique that measures the mobility of gas-phase ions through an electric field in the presence of a buffer gas. The mobility of ions is based on their charge, shape and size, and the method is increasingly being applied to the separation of glycan isomers [Gray et al., 2016]. In relation to glycosylated analytes, DESI and MALDI ionization coupled to ion mobility separations were recently reported for tissue imaging of multi-sialylated gangliosides and other glycosphingolipids [Škrášková et al., 2016]. This should be an equally robust approach for N-linked glycan imaging, as well as new MSI comparisons of glycosphingolipid distributions [Angel, Spraggins, Baldwin, & Caprioli, 2012; Angel, Jones et al., 2014] with N-glycans.

5. Emerging Applications

5.1. Combined Glycan and Peptide MS Imaging

The N-glycan classes described herein were all released by PNGaseF from a protein carrier. Using the glycan distribution maps obtained by MALDI imaging to identify the glycoprotein carriers of glycans of interest is one potential experimental extension of the approach. The most direct approach would be to target regions of interest for digestion with trypsin, followed by extraction and enrichment of glycopeptides from these regions. While great progress has been made in direct glycopeptide analysis to determine both peptide and glycan sequences using high resolution tandem MS [Banazadeh, Veillon, Wooding, Zabet, & Mechref, 2016; Thaysen-Andersen & Packer, 2014], there is still a requirement for large amounts of sample above what can be obtained by isolation of small, focal regions of tissue. A current alternative approach is to use MS imaging of peptides in situ, combined with extracted peptide sequencing identification, to map protein expression to tissue localization.

This distribution map of the peptides can be combined with the N-glycan map to correlate areas of overlap. Additionally, having the protein identification allows bioinformatic analysis to determine which glycoproteins are present in a given area. A recent study from Heijs et al. 2016 describes a sequential version of this approach, one in which a FFPE tissue is first treated with PNGaseF to release N-glycans, which are then imaged, followed by trypsin digestion of the same tissue and peptide imaging. An adjacent tissue slice is treated similarly, but glycans and peptides are extracted for tandem MS analysis and peptide sequencing. In one data example, a colorectal cancer FFPE tissue treated with sequential PNGaseF and trypsin digestions compared to trypsin alone increased the number of unique proteins identified from 236 (corresponding to 948 unique peptides) to 509 proteins (corresponding to 1334 unique peptides)[Heijs et al., 2016]. The sequential digestion improved protein identification, most likely due to removal of N-glycans that would otherwise block trypsin access. Cleavage of N-glycans by PNGaseF results in a deamidation of the asparagine residue to aspartic acid, a modification easily determined in LC-MS/MS peptide analysis, but that can also potentially arise as an artifact without careful experimental preparation [Hao, Ren, Alpert, & Sze, 2011]. There were 20 de-amidated peptides identified in the colorectal cancer tissue using the sequential PNGaseF/trypsin approach, compared to none detected with trypsin alone [Heijs et al., 2016].

To further illustrate this, an adjacent tissue slice of the example colorectal cancer specimen shown in Figures 2–6 was treated with trypsin and peptide profiling done by MALDI imaging. As shown in Figure 7, peptide masses indicative of the same tissue regions highlighted in the preceding glycan examples were selected. The high spatial correlation between adenocarcinoma and mucinous glycan and peptide localizations are evident, as well as the two stroma and crypt areas. The glycan examples shown are the same as those in Figure 2, and peptide identification from this tissue by tandem MS is ongoing. Although still correlative, and with protein ID as described, protein localizations can be validated with immunohistochemistry confirmation on tissues. As these are generally going to be abundant proteins, it is likely that appropriate antibody reagents are available. This abundance also lends itself to clinical biomarker assay development. From the glycomic side of characterizing the glycoprotein carriers, knowing the structural class of the types of glycans of interest can allow use of affinity enrichment of glycopeptides or glycoproteins using lectins or anti-carbohydrate epitope antibodies. It is possible that using targeted enrichment of tissue regions based on the presence of glycans of interest, combined with affinity enrichment based on glycan structure, could lead to improved amounts of glycopeptides amenable to current tandem MS analysis workflows. Overall, the approach described by Heijs et al. represents an effective MALDI-IMS proteomic-glycomic characterization strategy, and provides a template for continuing to refine these types of novel analyses.

5.2. Custom Multi-tumor TMA and other enzymes

In order to facilitate method development for N-glycan MSI applications, we have generated two custom TMAs comprised of multiple tissue core pairs of non-tumor and tumor regions representing eighteen different tumor types (pancreatic, colon, prostate, breast, lung, melanoma, sarcoma, head/neck, kidney, liver, glioma, ovarian, thyroid, uterine, cervical, testicular, gastric, bladder). An example of MSI N-glycan data from one TMA (pancreatic,

colon, prostate, breast, kidney, liver, thyroid, bladder) is shown for a high mannose Man₆ glycan ($m/z = 1419$) in Figure 8. In Figure 8A, the intensities are shown for the entire TMA, with data normalized across all of the tissue cores. The tumor types present in each row are listed in the figure legend. This allows a comparison of relative expression of the abundance of each individual glycan across the different tumor types. In Figure 8B, cores from each individual tumor type were analyzed as distinct image files, allowing normalization to be done for each tumor independently. For this high mannose glycan, there is consistently higher tumor levels for colon, prostate, lung, breast and bladder cancers, while the other tumor types are variable (Figure 8B). Data from each of the TMA core pairs is being compared systematically to what N-glycans are detected in the source FFPE blocks. The goal is to generate a reference database of expected MSI N-glycans for each tumor tissue type. This will also provide a framework including ethylated sialic acid structures as well as isomer information as it is obtained. Other images from different platforms like DESI/MALDI ion mobility could also be included.

Other glycosidase enzymes specific to galactose (galactosidase), fucose (fucosidase) and sialic acid (sialidase or neuraminidase) residues could be applied to tissue in different combinations with PNGaseF to aid in structural confirmations. Many of these glycosidases are linkage specific, thus in particular for fucose modifications, their use could provide added structural/isomeric information. Use of other enzymes with modified PNGaseF substrate specificity, for example an enzyme that only cleaves N-glycans with core fucose residues, could also find utility in the MSI N-glycan workflows.

5.3. Linkage to genomic studies

Because different glycosyltransferases and glycosidases determine the sequential biosynthesis of N-glycans, the gene expression levels of these enzyme's function could potentially regulate the composition of the N-glycome in each region. An extensive study of glyco-gene transcripts in different mouse organs, compared with the most abundant N-glycans detected in each tissue, illustrated that the levels of many transcripts (but not all) can be correlated with the levels of specific glycan structures [Nairn et al., 2008]. The glycan structural series presented in Figures 3–6 strongly suggests specific regulatory control of sequential glycosyltransferase networks linked with individual histopathology regions, consistent with transcriptional level control. Several studies have been published that have evaluated the transcriptome profiles of glycan biosynthesis genes to predict what glycan structures would be present [Kawano, Hashimoto, Miyama, Goto, & Kanehisa 2005; Suga, Yamanishi, Hashimoto, Goto, & Kanehisa, 2007], including glycosylation reaction network analysis strategies [Liu & Neelamegham, 2014]. Combining these approaches with the specific glycan structures identified in the N-glycan tissue imaging profiles has not been reported. The abundance of genomic data available via the Cancer Genome Atlas (TCGA) network for many types of cancer represents a wealth of N-glycan gene expression profiling data. Linking the N-glycan imaging maps to tissues with corresponding transcriptomic data represents a direct opportunity to more fully link glycome and genomic datasets to better understand the regulation and function of N-glycans in different cancer types.

5.4. Potential clinical diagnostic applications of N-glycan MSI data

There are several clinical diagnostic directions that N-glycan imaging of FFPE samples has made possible, as the panels of N-glycans obtained can be used to develop classification algorithms. One of the more direct possibilities is to use a new MALDI-MSI platform, the rapifleX MALDI TissueTyper™ time-of-flight instrument (from Bruker Daltonics) [Ogrinc Poto nik,, Porta, Becker, Heeren, & Ellis, 2015]. This system was designed to significantly decrease the time required to acquire and process tissue imaging data, essentially by 5-fold or more. Additionally, the laser spot size and raster distances can be done routinely at 10–20 micron resolutions, while maintaining the rapid analysis times. Pilot MALDI-MSI of N-glycans from FFPE tissues has been successful on this new instrument, and will be reported separately. These improved acquisition times bring the analysis of surgery-derived biopsy tissues and other standard pathology samples by MALDI IMS into a feasible time frame for clinical laboratory assays.

Knowing the glycan structures and their tissue locations, also facilitates development of specific lectin-based assays for direct lectin histochemistry tissue assays. Although less available, use of anti-carbohydrate epitope antibodies could also be used, as fluorescent or light microscopy visualization of cancer biomarkers in tissue is standard in the clinical pathology laboratory. The potential down side to their use is that the lectins or antibodies recognize glycan structural motifs and classes which could also be present on glycosphingolipids or O-glycosylated proteins. Specificity for detection of N-glycan glycoproteins could be lost or confounding.

6. Summary

The capability of profiling and identifying multiple N-glycan species in their native locations within the tumor microenvironment of FFPE tissue samples provides a long missing spatial component in our understanding of the role of N-glycans in tumor development and function. In the field of imaging mass spectrometry, targeting N-glycans is a recent development, and as highlighted herein, there is a wealth of new information that can be obtained for any cancer type. We expect that the lessons learned from these efforts will facilitate development of MSI strategies for O-linked glycans, which lacks an enzyme mediator to release them, as well as heparin and chondroitin sulfate glycosaminoglycans [Shao, Shi, Phillips, & Zaia, 2013]. The use of FFPE tissues as the primary source and the defined number of detectable N-glycan species greatly facilitates the potential use of the method, or information derived from it, in subsequent clinical assay measurements related to development of new tumor diagnostics. The limitations of the current MSI approaches for N-glycans are largely addressable, and new innovations in methodology and instrumentation, as well as a focus on imaging of other glycan classes, will continue to aid in the evolution of this approach.

Literature Citations

Adamczyk B, Tharmalingam T, Rudd PM. Glycans as cancer biomarkers. *Biochimie et Biophysica Acta*. 2012; 1820(9):1347–1353.

- Angel PM, Spraggins JM, Baldwin HS, Caprioli R. Enhanced sensitivity for high spatial resolution lipid analysis by negative ion mode matrix assisted laser desorption ionization imaging mass spectrometry. *Analytical Chemistry*. 2012; 84(3):1557–1564. [PubMed: 22243218]
- Angel PM, Caprioli RM. Matrix-assisted laser desorption ionization imaging mass spectrometry: in situ molecular mapping. *Biochemistry*. 2013; 52(22):3818–3828. [PubMed: 23259809]
- Balluff B, Rauser S, Meding S, Elsner M, Schöne C, Feuchtinger A, et al. MALDI imaging identifies prognostic seven-protein signature of novel tissue markers in intestinal-type gastric cancer. *American Journal of Pathology*. 2011; 179(6):2720–2729. [PubMed: 22015459]
- Banazadeh A, Veillon L, Wooding KM, Zabet M, Mechref Y. Recent advances in mass spectrometric analysis of glycoproteins. *Electrophoresis*. 2016; doi: 10.1002/elps.201600357
- Briggs MT, Kuliwaba JS, Muratovic D, Everest-Dass AV, Packer NH, Findlay DM, Hoffmann P. MALDI mass spectrometry imaging of N-glycans on tibial cartilage and subchondral bone proteins in knee osteoarthritis. *Proteomics*. 2016; 16(11–12):1736–1741. [PubMed: 26992165]
- Christiansen MN, Chik J, Lee L, Anugraham M, Abrahams JL, Packer NH. Cell surface protein glycosylation in cancer. *Proteomics*. 2014; 14(4–5):525–546. [PubMed: 24339177]
- Dam TK, Roy R, Das SK, Oscarson S, Brewer CF. Binding of multivalent carbohydrates to concanavalin A and Dioclea grandiflora lectin: thermodynamic analysis of the “multivalency effect.”. *Journal of Biological Chemistry*. 2000; 275:14223–14230. [PubMed: 10799500]
- Dapson RW. Macromolecular changes caused by formalin fixation and antigen retrieval. *Biotechnic & Histochemistry*. 2007; 82(3):133–140. [PubMed: 17987439]
- Drake RR, Schwegler EE, Malik G, Diaz J, Block T, Mehta A, et al. Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers. *Molecular and Cellular Proteomics*. 2006; 5(10):1957–1967. [PubMed: 16760258]
- Drake RR, Jones EE, Powers TW, Nyalwidhe JO. Altered glycosylation in prostate cancer. *Advances in Cancer Research*. 2015; 126:345–382. [PubMed: 25727153]
- Everest-Dass AV, Briggs MT, Kaur G, Oehler MK, Hoffmann P, Packer NH. N-Glycan MALDI Imaging Mass Spectrometry on Formalin-Fixed Paraffin-Embedded Tissue Enables the Delineation of Ovarian Cancer Tissues. *Molecular and Cellular Proteomics*. 2016; 15(9):3003–3016.
- Fenn LS, McLean JA. Structural resolution of carbohydrate positional and structural isomers based on gas-phase ion mobility-mass spectrometry. *Physical Chemistry Chemical Physics*. 2011; 13(6):2196–2205. [PubMed: 21113554]
- Gomori G. A new histochemical test for glycogen and mucin. *American Journal of Clinical Pathology*. 1946; 10:177–179. [PubMed: 20273799]
- Gray CJ, Thomas B, Upton R, Migas LG, Eyers CE, Barran PE, et al. Applications of ion mobility mass spectrometry for high throughput, high resolution glycan analysis. *Biochimie Biophysica Acta*. 2016; 1860(8):1688–1709.
- Gustafsson OJ, Briggs MT, Condina MR, Winderbaum LJ, Pelzing M, McColl SR, et al. MALDI imaging mass spectrometry of N-linked glycans on formalin-fixed paraffin-embedded murine kidney. *Analytical Bioanalytical Chemistry*. 2015; 407:2127–2139. [PubMed: 25434632]
- Hao P, Ren Y, Alpert AJ, Sze SK. Detection, evaluation and minimization of nonenzymatic deamidation in proteomic sample preparation. *Molecular and Cellular Proteomics*. 2011; 10(10):O111.009381.
- Hardesty WM, Kelley MC, Mi D, Low RL, Caprioli RM. Protein signatures for survival and recurrence in metastatic melanoma. *Journal of Proteomics*. 2011; 74(7):1002–1014. [PubMed: 21549228]
- Harvey DJ. Analysis of carbohydrates and glycoconjugates by matrix-assisted laser desorption/ionization mass spectrometry: an update for 2009–2010. *Mass Spectrometry Reviews*. 2015; 34(3):268–422. [PubMed: 24863367]
- Heijs B, Holst S, Briaire-de Bruijn IH, van Pelt GW, de Ru AH, van Veelen, et al. Multimodal Mass Spectrometry Imaging of N-Glycans and Proteins from the Same Tissue Section. *Analytical Chemistry*. 2016; 88:7745–7753. [PubMed: 27373711]
- Heimburg-Molinario J, Lum M, Vijay G, Jain M, Almogren A, Rittenhouse-Olson K. Cancer vaccines and carbohydrate epitopes. *Vaccine*. 2011; 29(48):8802–8826. [PubMed: 21964054]

- Hirabayashi J. Lectin-based structural glycomics: glycoproteomics and glycan profiling. *Glycoconjugate Journal*. 2004; 21:35–40. [PubMed: 15467396]
- Holst S, Heijs B, de Haan N, van Zeijl RJ, Briaire-de Bruijn IH, van Pelt GW, et al. Linkage-Specific in Situ Sialic Acid Derivatization for N-Glycan Mass Spectrometry Imaging of Formalin-Fixed Paraffin-Embedded Tissues. *Analytical Chemistry*. 2016; 88(11):5904–5913. [PubMed: 27145236]
- Jain P, Mondal SK, Sinha SK, Mukhopadhyay M, Chakraborty I. Diagnostic and prognostic significance of different mucin expression, preoperative CEA, and CA-125 in colorectal carcinoma: A clinicopathological study. *Journal of Natural Science, Biology, and Medicine*. 2014; 5:404–408.
- Jones EE, Dworski S, Canals D, Casas J, Fabrias G, Schoenling D, et al. On-tissue localization of ceramides and other sphingolipids by MALDI mass spectrometry imaging. *Analytical Chemistry*. 2014; 86(16):8303–8311. [PubMed: 25072097]
- Kawano S, Hashimoto K, Miyama T, Goto S, Kanehisa M. Prediction of glycan structures from gene expression data based on glycosyltransferase reactions. *Bioinformatics*. 2005; 21:3976–3982. [PubMed: 16159923]
- Kindblom LG, Angervall L. Histochemical characterization of mucosubstances in bone and soft tissue tumors. *Cancer*. 1975; 36:985–994. [PubMed: 241481]
- Kudelka MR, Ju T, Heimbürg-Molinari J, Cummings RD. Simple sugars to complex disease-mucin-type o-glycans in cancer. *Advances in Cancer Research*. 2015; 126:53–135. [PubMed: 25727146]
- Lis H, Sharon N. Lectins: carbohydrate-specific proteins that mediate cellular recognition. *Chemistry Reviews*. 1998; 98:637–674.
- Liu G, Neelamegham S. A computational framework for the automated construction of glycosylation reaction networks. *PLoS One*. 2014; 9(6):e100939. [PubMed: 24978019]
- Loke I, Kolarich D, Packer NH, Thaysen-Andersen M. Emerging roles of protein mannosylation in inflammation and infection. *Molecular Aspects of Medicine*. 2016; 51:31–55. [PubMed: 27086127]
- Mechref Y, Novotny MV, Krishnan C. Structural characterization of oligosaccharides using MALDI-TOF/TOF tandem mass spectrometry. *Analytical Chemistry*. 2003; 75(18):4895–4903. [PubMed: 14674469]
- McLean JA, Fenn LS, Enders JR. Structurally selective imaging mass spectrometry by imaging ion mobility-mass spectrometry. *Methods in Molecular Biology*. 2010; 656:363–383. [PubMed: 20680602]
- McManus JF, Cason JE. Carbohydrate histochemistry studied by acetylation techniques: I. Periodic acid methods. *The Journal of Experimental Medicine*. 1950; 91(6):651–654. [PubMed: 15422090]
- Miwa HE, Song Y, Alvarez R, Cummings RD, Stanley P. The bisecting GlcNAc in cell growth control and tumor progression. *Glycoconjugate Journal*. 2012; 29(8–9):609–618. [PubMed: 22476631]
- Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: diversity, synthesis and function. *Nature Reviews Molecular Cell Biology*. 2012; 13(7):448–462. [PubMed: 22722607]
- Mowry RW. Alcian blue technics for the histochemical study of acidic carbohydrates. *Journal of Histochemistry & Cytochemistry*. 1956; 4:403–407.
- Myers, RB., Fredenburgh, JL., Grizzle, WE. Carbohydrates. In: Bancroft, J., Gamble, DM., editors. *Theory and Practice of Histological Techniques*. Elsevier; Philadelphia, PA: 2008.
- Nairn AV, York WS, Harris K, Hall EM, Pierce JM, Moremen KW. Regulation of glycan structures in animal tissues: transcript profiling of glycan-related genes. *Journal of Biological Chemistry*. 2008; 283(25):17298–17313. [PubMed: 18411279]
- Nonaka M, Fukuda MN, Gao C, Li Z, Zhang H, Greene MI, et al. Determination of carbohydrate structure recognized by prostate-specific F77 monoclonal antibody through expression analysis of glycosyltransferase genes. *Journal of Biological Chemistry*. 2014; 289(23):16478–16486. [PubMed: 24753248]
- Nyalwidhe JO, Betesh LR, Powers TW, Jones EE, White KY, Burch TC, et al. Increased bisecting N-acetylglucosamine and decreased branched chain glycans of N-linked glycoproteins in expressed prostatic secretions associated with prostate cancer progression. *Proteomics Clinical Applications*. 2013; 7:677–689. [PubMed: 23775902]

- O'Connor PB, Costello CE. A high pressure matrix-assisted laser desorption/ionization fourier transform mass spectrometry ion source for thermal stabilization of labile biomolecules. *Rapid Communications in Mass Spectrometry*. 2001; 15:1862–1868.
- O'Connor PB, Mirgorodskaya E, Costello CE. High pressure matrix-assisted laser desorption/ionization Fourier transform mass spectrometry for minimization of ganglioside fragmentation. *Journal of the American Society of Mass Spectrometry*. 2002; 13:402–407.
- Ogrinc P, Potočnik N, Porta T, Becker M, Heeren RM, Ellis SR. Use of advantageous, volatile matrices enabled by next-generation high-speed matrix-assisted laser desorption/ionization time-of-flight imaging employing a scanning laser beam. *Rapid Communications in Mass Spectrometry*. 2015; 29(23):2195–2203. [PubMed: 26522310]
- Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nature Reviews Cancer*. 2015; 15(9):540–555. [PubMed: 26289314]
- Powers TW, Jones EE, Betesh LR, Romano PR, Gao P, Copeland JA, et al. Matrix assisted laser desorption ionization imaging mass spectrometry workflow for spatial profiling analysis of N-linked glycan expression in tissues. *Analytical Chemistry*. 2013; 85:9799–9806. [PubMed: 24050758]
- Powers TW, Neely BA, Shao Y, Tang H, Troyer DA, Mehta AS, et al. MALDI imaging mass spectrometry profiling of N-glycans in formalin-fixed paraffin embedded clinical tissue blocks and tissue microarrays. *PLoS One*. 2014; 9(9):e106255. [PubMed: 25184632]
- Powers TW, Holst S, Wührer M, Mehta AS, Drake RR. Two-Dimensional N-Glycan Distribution Mapping of Hepatocellular Carcinoma Tissues by MALDI-Imaging Mass Spectrometry. *Biomolecules*. 2015; 5(4):2554–2272. [PubMed: 26501333]
- Ravn V, Dabelsteen E. Tissue distribution of histo-blood group antigens. *Acta pathologica, microbiologica, et immunologica Scandinavica*. 2000; 108(1):1–28.
- Reid P, Owen D, Kruk P, Maitland ME. Histochemical classification based upon reaction types and its application to carbohydrate histochemistry. *The Histochemical Journal*. 1990; 22:299–308. [PubMed: 2133465]
- Reiding KR, Blank D, Kuijper DM, Deelder AM, Wührer M. High-throughput profiling of protein N-glycosylation by MALDI-TOF-MS employing linkage-specific sialic acid esterification. *Analytical Chemistry*. 2014; 86:5784–5793. [PubMed: 24831253]
- Richter S, Makovitzky J. Topo-optical visualization reactions of carbohydrate-containing amyloid deposits in the respiratory tract. *Acta histochemica*. 2006; 108:181–191. [PubMed: 16542712]
- Rini, J., Esko, J., Varki, A. Glycosyltransferases and Glycan-processing Enzymes. In: Varki, A. Cummings, RD. Esko, JD. Freeze, HH. Stanley, P. Bertozzi, CR. Hart, GW., Etzler, ME., editors. *Essentials of Glycobiology*. 2. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. Chapter 5
- Ruhaak LR, Miyamoto S, Lebrilla CB. Developments in the identification of glycan biomarkers for the detection of cancer. *Molecular and Cellular Proteomics*. 2013; 12(4):846–855. [PubMed: 23365456]
- Scalia CR, Boi G, Bolognesi MM, Riva L, Manzoni M, DeSmedt L, et al. Antigen Masking During Fixation and Embedding, Dissected. *Journal of Histochemistry & Cytochemistry*. 2016 pii: 0022155416673995. [Epub ahead of print].
- Schey KL, Anderson DM, Rose KL. Spatially-directed protein identification from tissue sections by top-down LC-MS/MS with electron transfer dissociation. *Analytical Chemistry*. 2013; 85:6767–6774. [PubMed: 23718750]
- Schuerenberg, M., Deininger, SO. Chapter 7: Matrix application with ImagePrep. In: Setou, M., editor. *Imaging Mass Spectrometry: Protocols for Mass Microscopy*. Springer; 2010. p. 87-91.
- Schultz MJ, Swindall AF, Bellis SL. Regulation of the metastatic cell phenotype by sialylated glycans. *Cancer Metastasis Reviews*. 2012; 31(3–4):501–518. [PubMed: 22699311]
- Scott JE, Quintarelli G, Dellovo MC. The chemical and histochemical properties of Alcian blue. *Histochemie*. 1964; 4:73–85. [PubMed: 4157595]
- Shao C, Shi X, Phillips JJ, Zaia J. Mass spectral profiling of glycosaminoglycans from histological tissue surfaces. *Analytical Chemistry*. 2013; 85(22):10984–10991. [PubMed: 24099043]

- Škrášková K, Claude E, Jones EA, Towers M, Ellis SR, Heeren RM. Enhanced capabilities for imaging gangliosides in murine brain with matrix-assisted laser desorption/ionization and desorption electrospray ionization mass spectrometry coupled to ion mobility separation. *Methods*. 2016; 104:69–78. [PubMed: 26922843]
- Spicer SS. A correlative study of the histochemical properties of rodent acid mucopolysaccharides. *Journal of Histochemistry & Cytochemistry*. 1960; 8:18–35. [PubMed: 13833306]
- Stanley, P., Schachter, H., Taniguchi, N. N-Glycans. In: Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. *Essentials of Glycobiology*. 2. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. Chapter 8
- Suga A, Yamanishi Y, Hashimoto K, Goto S, Kanehisa M. An improved scoring scheme for predicting glycan structures from gene expression data. *Genome Informatics*. 2007; 18:237–246. [PubMed: 18546491]
- Taniguchi N, Kizuka Y. Glycans and cancer: role of N-glycans in cancer biomarker, progression and metastasis, and therapeutics. *Advances in Cancer Research*. 2015; 126:11–51. [PubMed: 25727145]
- Toghi Eshghi S, Yang S, Wang X, Shah P, Li X, Zhang H. Imaging of N-linked glycans from formalin-fixed paraffin-embedded tissue sections using MALDI mass spectrometry. *ACS Chemical Biology*. 2014; 9(9):2149–2156. [PubMed: 25029481]
- Thaysen-Andersen M, Packer NH. Advances in LC-MS/MS-based glycoproteomics: getting closer to system-wide site-specific mapping of the N- and O-glycoproteome. *Biochimie Biophysica Acta*. 2014; 1844(9):1437–1452.
- Uemura T, Shiozaki K, Yamaguchi K, Miyazaki S, Satomi S, Kato K, et al. Contribution of sialidase NEU1 to suppression of metastasis of human colon cancer cells through desialylation of integrin beta4. *Oncogene*. 2009; 28(9):1218–1229. [PubMed: 19151752]
- Varki A. Biological roles of glycans. *Glycobiology*. 2016; *In press*. doi: 10.1093/glycob/cww086
- Wang D. N-glycan cryptic antigens as active immunological targets in prostate cancer patients. *Journal of Proteomics and Bioinformatics*. 2012; 5:90–95.
- Wang D, Dafik L, Nolley R, Huang W, Wolfinger RD, Wang LX, et al. Anti-oligomannose antibodies as potential serum biomarkers of aggressive prostate cancer. *Drug Development Research*. 2013; 74:65–80. [PubMed: 25152555]
- Yang J, Caprioli RM. Matrix sublimation/recrystallization for imaging proteins by mass spectrometry at high spatial resolution. *Analytical Chemistry*. 2011; 83(14):5728–5734. [PubMed: 21639088]
- Zoldoš V, Novokmet M, Be eheli I, Lauc G. Genomics and epigenomics of the human glycome. *Glycoconjugate Journal*. 2013; 30(1):41–50. [PubMed: 22648057]

Figure 1.

A. Five glycans and their symbols, used throughout the chapter, and four N-glycans representative of different structure classes; B. Site of action of peptide N-glycosidase F (PNGaseF); C. Hematoxylin and Eosin stain (H&E) of a colon cancer FFPE tissue slice. Indicated are areas of adenocarcinoma (red font), normal crypt epithelial cells (blue font), mucinous tumor and stroma (yellow font); D. Alcian blue stain of the colon tumor.

Figure 2.

Examples of regiospecificity of different N-glycans detected by MALDI-FTICR imaging mass spectrometry. The colon tumor FFPE tissue was antigen retrieved and incubated with PNGaseF, and N-glycans detected by MALDI-IMS as previously described [Powers et al., 2014]. A. H&E stain; B. a stroma glycan (green), Hex5HexNAc4Fuc1NeuAc (+2Na), $m/z = 2122.810$; C. a high mannose tumor glycan (red), Man6, $m/z = 1419.515$; D. a stroma glycan (yellow), Hex5HexNAc4NeuAc, $m/z = 1976.745$ (+2Na); E. a crypt glycan (aqua blue) Hex3HexNAc5Fuc1, $m/z = 1688.647$; F. a mucinous tumor glycan (pink), Hex6HexNAc6Fuc1, $m/z = 2377.798$.

Figure 3.
High mannose and paucimannose N-glycans. The indicated glycans and their masses were detected primarily in the adenocarcinoma regions of the colon tumor.

Figure 4.

Core fucosylated, biantennary N-glycans. The indicated glycans and their masses were detected primarily in the stroma regions, and adenocarcinoma regions of the colon tumor. The bottom left panel shows an overlay of two stroma glycans at 2122 (red) and 1976 (green). All masses include one sodium, except where indicated.

Figure 5.

Non-fucosylated, biantennary N-glycans. The indicated glycans and their masses were detected primarily in the stroma regions, and adenocarcinoma regions of the colon tumor. All masses include one sodium, except where indicated.

Figure 6.

Other glycan examples. A. Hybrid and bisecting N-acetylglucosamine glycans present primarily in the epithelial crypt cell region. B. Tri- and tetraantennary N-glycan examples in the stroma and adenocarcinoma regions. C. Tetraantennary N-glycans that are present primarily in the mucinous tumor region. All structures shown are representative of glycan composition, but not specific linkages or individual branch modifications.

Figure 7.

Illustration of co-localization of N-glycans and peptides detected by MALDI-FTICR imaging mass spectrometry. The same glycans from Figure 2 are included in the indicated panels, and a corresponding peptide image. For peptides, tissue was sprayed with trypsin and digested, followed by MALDI-IMS similar to published protocols [Heijs et al., 2016]. A. stroma (green) glycan $m/z = 2122.810$; peptide $m/z = 1055.491$; B. stroma (yellow), glycan $m/z = 1976.745 (+2Na)$; peptide $m/z = 886.439$; C. Epithelial crypt (aqua blue) glycan, $m/z = 1688.647$; peptide $m/z = 911.444$; D. adenocarcinoma (red), glycan $m/z = 1419.515$; peptide $m/z = 890.449$; E. mucinous tumor (pink), glycan $m/z = 2377.798$; peptide $m/z =$

931.431; F. glycan overlay of the shown mucinous tumor glycan (pink), adenocarcinoma glycan (red) and stroma glycan (green); peptide overlay of each peptide mass and color shown in A.–E.

Figure 8.
Example N-glycan MALDI-MSI data for a tissue microarray representing eight different tumor types. A. MALDI-IMS intensity of the Man6 glycan across each tissue core. Non-

tumor (N) regions and tumor (T) regions are shown for each tumor type: Rows 1 and 2 – uterus (6 cores); Rows 2 and 3 – thyroid (12 cores); Row 3 – prostate (6 cores); Rows 3 and 4 – pancreas (10 cores); Rows 4 and 5 – lung (12 cores); Rows 5 and 6 – liver (8 cores); Row 6 – kidney (6 cores); Rows 6 and 7 – colon (14 cores); Rows 7 and 8 – breast (6 cores); Row 8 – bladder (8 cores). B. Each tissue type in the microarray analyzed as an individual imaging file. Shown is data for the Man6 N-glycan.