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Imaging Cellular Glycan Epitopes and Glycoproteins Using Glycosyltransferases

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Abstract

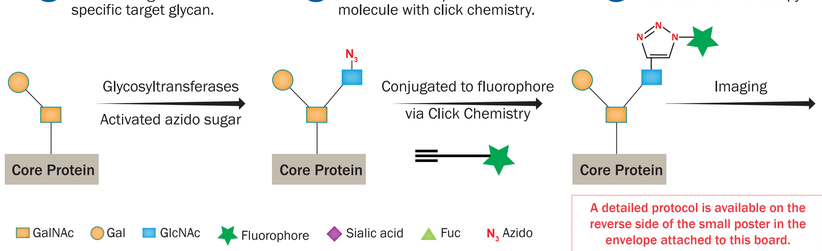
Glycans are basic molecules in all living organisms. However, they are not well characterized due to the unavailability of specific and high-sensitivity detection reagents. We describe here a method for enzyme-based glycan imaging using glycosyltransferases. Azido-sugars are incorporated into target glycans followed by click chemistry-based conjugation with fluorescent reporters. Since glycosyltransferases have well defined substrate specificities, this method offers precise detection of target glycans. Examples include imaging of O-GlcNAc, O-GalNAc (Tn antigen), T antigen, sialyllactosamine (sLN), hyaluronan (HA), and heparan sulfate epitopes in different cell lines. This highly specific technique will facilitate the detection and characterization of biologically important glycans.

Introduction

Glycosylation is one of the most common types of post-translational modifications. Glycans are assembled onto core proteins by various glycosyltransferases in the endoplasmic reticulum and Golgi apparatus. They are typically presented on the cell surface or secreted for association with the extracellular matrix. Glycans play various biological roles in protein folding and quality control, as well as recognition events with glycan-binding lectins, growth factors, and cytokines. Glycans can be attached to protein polypeptides by covalent modification of serine or threonine residues (O-linked glycans and proteoglycans), asparagine residues (N-linked glycans), or can be secreted as free carbohydrates (hyaluronan).

1. Core-1 glycan (Galβ1-3GalNAc-R) is also known as T antigen and is the substrate of GCNT1. The precursor of T antigen, also known as Tn antigen (O-GalNAc) is the substrate of B3GNT6. Both T and Tn antigens are involved in cancer etiology.
2. O-GlcNAc is recognized by a mutant B4GalT1Y285L. It is a cytoplasmic and nuclear epitope that is involved in the regulation of metabolism and the development of diabetes and cancer.
3. Sialyl lactosamine (sLN) is recognized by FUT6 and FUT7 to generate Lewis X (sLex). sLex is essential for leukocyte trafficking and homing.
4. Heparan sulfate (HS) exists as HS proteoglycans and is abundant in extracellular matrix (ECM). It is synthesized by exostosins (EXTs) which are dual enzymes with both GlcA and GlcNAc transferase activities.
5. Hyaluronan (HA) is a non-sulfated free polysaccharide and is polymerized by HA synthase.

Procedure



A detailed protocol is available on the reverse side of the small poster in the envelope attached to this board. Please take one for reference and additional information.

Table 1. Enzymes used in this study

All enzymes are of human origin except where indicated otherwise.

Application	Use Enzyme & Small Molecule	Catalog #	Information
Detect **Ser/Thr** residues in O-glycosylation sites	GALNT2	7507-GT	Adds azide to protein Ser/Thr residues in O-glycosylation sites
	UDP-azido-GalNAc	ES103	
Add **O-GalNAc** to Ser/Thr in O-glycosylation sites	GALNT2	7507-GT	Adds GalNAc to Ser/Thr residues, can be used with B3GNT6 to form Tn antigen
	UDP-GalNAc		
Remove **O-GlcNAc** from Ser/Thr residues	O-GlcNAcase (OGA)	6779-GH	Removes O-GlcNAc
Detect **Tn antigen** Only	B3GNT6	6505-GT	Adds azide onto O-GalNAc (Tn antigen)
	UDP-azido-GlcNAc	ES104	
Detect **Terminal O-GlcNAc** and **Tn antigen**	B4GalT1Y285L	7040-GT	Adds azide to terminal GlcNAc, including O-GlcNAc
	UDP-azido-GalNAc	ES103	
Convert **Tn antigen** to **T antigen** (Core-1 O-glycan)	C1GalT1	8659-GT	Adds Galactose to O-GalNAc to form T antigen
	UDP-Gal		
Detect **T antigen (Core-1 O-glycan)**	GCNT1	7248-GT	Adds azide to Core-1 O-glycan (T antigen)
	UDP-azido-GlcNAc	ES104	
Detect **Siallylactosamine (sLN)**	FUT7	*6409-GT*	Adds azide to Sialyllactosamine (sLN)
	GDP-azido-Fuc	ES101	
Detect Both **sLN** and **LN**	FUT6	*Inquire*	Adds azide to Lactosamine and Sialyllactosamine
	GDP-azido-Fuc	ES101	
Detect **Heparan Sulfate (HS)**	EXT1/2	8567-GT	Adds azide to Heparan Sulfate
	UDP-azido-GlcNAc & UDP-GlcA	ES104	
Cleave **Heparan Sulfate** and leave Terminal GlcNAc	Heparanase (HPSE)	7570-*GH*	Cleaves HS and exposes a GlcNAc residue at the newly created non-reducing end
Cleave **Heparan Sulfate** and leave Terminal GlcA	Heparinase III (Hep III)	6145-GH	Cleaves HS and leaves an unsaturated GlcA residue at the newly created non-reducing end
Detect **Hyaluronan (HA)**	Hyaluronan Synthase (HAS)	*Inquire*	Adds azide to Hyaluronan
	UDP-azido-GlcNAc & UDP-GlcA	ES104	
Cleave **Hyaluronan** at β–1,4 bonds	Hyaluronan Lyase	5150-GH	Cleaves HA and leaves an unsaturated residue at the newly created non-reducing end
Detect complex **N-glycans**	ST6GAL1	7620-GT	Adds azide to beta1-4 linked Gal residues in complex N-glycans
	CMP-azido-Sialic Acid	ES102	
Remove terminal **Sialic Acid**	Neuraminidase	5080-NM	Exposes terminal Gal residues on N-glycans

Results

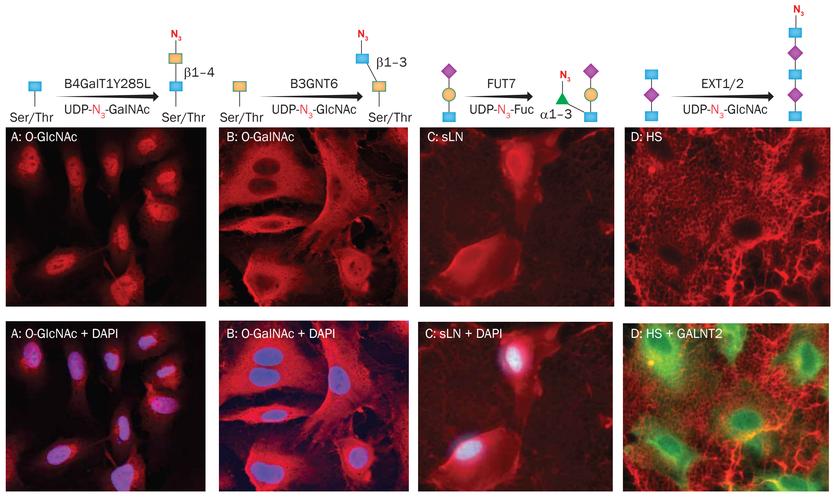


Figure 1. HUVEC cells stained by different glycosyltransferases. HUVEC cells were cultured in a 24-well plate to confluence and then stained with different enzymes. Glycans are in red (via Alexa Fluor® 555). **A**) O-GlcNAc; **B**) O-GalNAc (introduced by GALNT2); **C**) Sialyllactosamine (sLN); **D**) Heparan sulfate (HS). Nuclei are stained in blue (DAPI). In **D**, cells were revealed by GALNT2 staining.

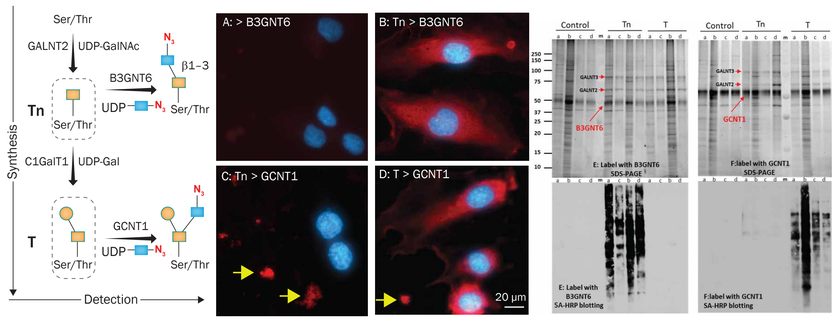


Figure 2. Specificity of T and Tn Antigen staining. Shown at the left is a schematic view of introducing Tn and T antigens onto HUVEC cells and their cellular extracts, and their staining using B3GNT6 and GCNT1. (**A**), Direct staining of HUVEC cells with B3GNT6. Introduced Tn antigen was imaged using B3GNT6 (**B**) or GCNT1 (**C**). (**D**), Introduced T antigen was imaged using GCNT1. Glycan staining is in red; DAPI staining of cell nuclei is in blue. Yellow arrows indicate the de novo synthesized and secreted T antigen by the cells. (**E**) and (**F**), SDS-PAGE (upper panels) and SA-HRP blots (lower panels) of the synthesized T and Tn antigens in cellular extracts, detected by B3GNT6 (**E**) or GCNT1 (**F**). Lane a, nuclear extract from HUVEC cells; b, nuclear extract from HEK cells; c, cytoplasmic extract from HUVEC cells; d, cytoplasmic extract from HEK 293 cells; m, molecular weight marker.

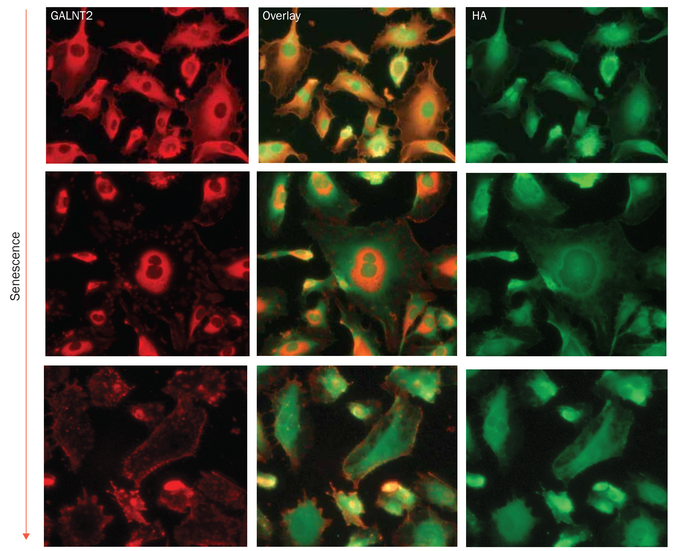


Figure 3. GALNT2 staining reveals protein synthesis activity in cells. HUVEC cells at different stages of senescence were imaged with GALNT2 (red, detected by Alexa Fluor® 555) and hyaluronan (HA, in green with Cy5). Cells gradually lost their staining by GALNT2 but not HA. Since GALNT2 only stains polypeptides that have unmodified Ser/Thr residues, the staining reflects the activity of protein synthesis in the cells. Senescent cells lost the majority of their protein synthesis activity. To visualize weak signals, the gamma rating was raised to 2.0 for all images.

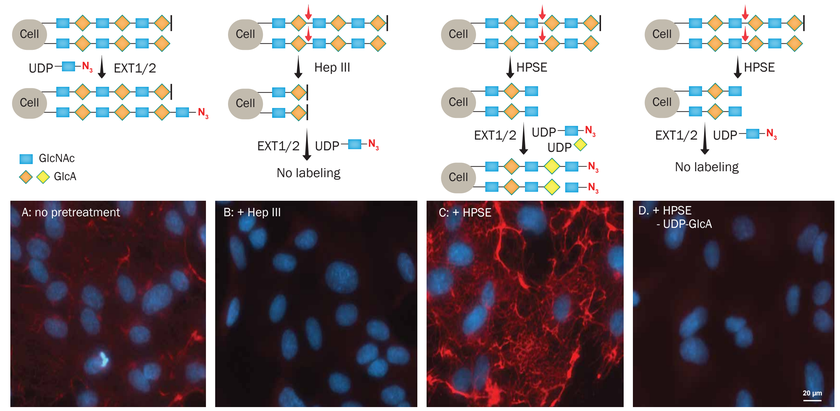


Figure 4. Specificity of Heparan Sulfate staining using EXT1/2. Confluent HUVEC cells were imaged without pretreatment (**A**), after pretreatment with Hep III (**B**), after pretreatment with HPSE (**C**), or after pretreatment with HPSE in the absence of UDP-GlcA (**D**). HS staining is shown in red (Alexa Fluor® 555), and DAPI staining of cell nuclei is shown in blue. This experiment demonstrates the strict specificity of EXT1/2 enzymes for heparan sulfate.

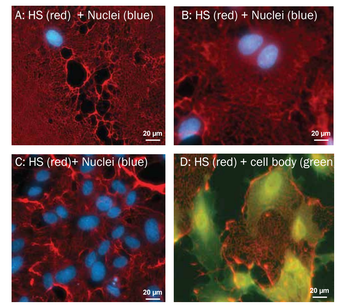


Figure 5. Morphology of ECM of confluent HUVEC cells revealed by HS staining. HS was stained in red; cell bodies in (**D**) were stained in green by GALNT2. Nuclei are in blue (DAPI). The ECM resembles a carpet (**A**), a mattress (**B**), or a tangled mesh (**C**).

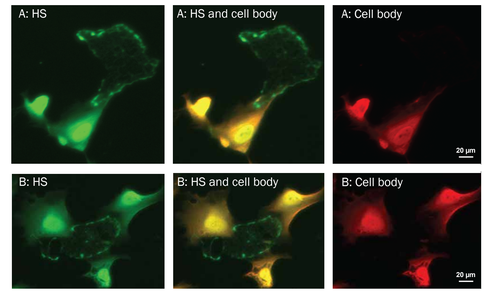


Figure 6. Intermediate structure of ECM revealed by HS staining. HS of sub-confluent HUVEC cells was stained in green, and cell bodies were stained in red by GALNT2. In each case, ECM being extruded by HUVEC cells resembles the shape of a casting net.

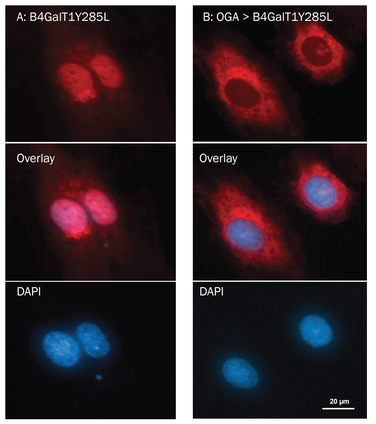


Figure 7. Specificity of O-GlcNAc staining. Sub-confluent HUVEC cells were imaged using B4GalT1Y285L either directly (**A**) or after treatment with O-GlcNAc specific glycosidase OGA (**B**). Images were normalized to the highest pixel value without changing gamma settings. OGA treatment abolished nuclear but not cytoplasmic staining.

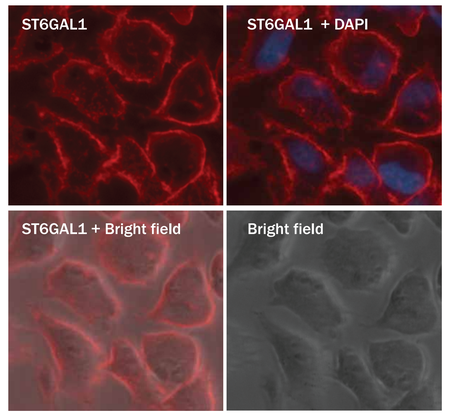


Figure 8. Specific staining of complex N-glycans on HeLa cells. Cells were first treated with *C. perfringens* Neuraminidase to remove the terminal sialic acid. Then azido-sialic acid was added back with ST6GAL1, followed by fixation and click labeling. The glycocalyx at the cell surface is strongly labeled.

Summary

- O-GlcNAc, O-GalNAc, Tn and T antigens, Lewis X precursors, Hyaluronan and Heparan sulfate are imaged using glycan-specific enzymes.
- Unprecedented ease and specificity of glycan imaging is achieved.
- Enzymatic synthesis of T antigen is demonstrated in cells and cellular extracts.
- O-GalNAcylation by GALNT on cells reveals the dynamics of protein synthesis.
- HS staining reveals the morphology of ECM as well as its mechanism of formation.

Imaging and Synthesis of Cellular Glycan Epitopes Using Glycosyltransferases

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Remove terminal **Sialic Acid**	Neuraminidase	5080-NM	Exposes terminal Gal residues on N-glycans

Materials and Methods

GDP-Azido-Fucose, UDP-Azido-GalNAc, UDP-Azido-GlcNAc, Biotinylated Alkyne, and DAPI were from Tocris Bioscience. Recombinant Human B4GalT1 (Y285L) Protein, Recombinant Human GALNT2 Protein, Recombinant Human Fucosyltransferase 6/FUT6 Protein, Recombinant Human Fucosyltransferase 7/FUT7 Protein, Recombinant Human GCNT1 Protein, Recombinant Human B3GNT6 Protein, Recombinant Human Exostosin 1/2 Heterodimer, Recombinant Human C1GalT1/C1GalT1C1 Protein, Recombinant Human Active Heparanase/HPSE Protein, Recombinant *B. thetaiotaomicron* O-GlcNAcase/OGA Protein, and Recombinant *P. heparinus* Heparinase III Protein were from R&D Systems. Streptavidin-Alexa Fluor® 555 and Streptavidin-Alexa Fluor® 488 were from Thermo Fisher Scientific. UDP-Gal, UDP-GlcA, and all other small chemicals were from Sigma-Aldrich.

Cell Growth and Fixation

C3H/10T1/2 cells (ATCC Catalog #CCL-226) were grown in MEM NEAA 1X Earle's Salts (Irvine Scientific) supplemented with 10% fetal bovine serum (Corning), 2 mM L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Sigma). Clonetics™ HUVEC cells (Lonza Catalog #C2517A) were grown in Clonetics™ EGM™-2 BulletKit™ (Lonza). Cells were plated in a 24-well cell culture plate and grown to the desired confluence. Cell cultures were rinsed with sterile PBS and fixed in 4% paraformaldehyde for 30 minutes at room temperature followed by washing 5 times with sterile phosphate buffered saline (PBS).

Pretreatment of Cells for Imaging

The following treatments were performed in a single well of a 24-well plate. For O-GlcNAc removal, cells were treated with 2 µg of OGA in 200 µL of buffer containing 50 mM MES and 100 mM NaCl at pH 5.5 for 1 hour at 37 °C. For HS removal, cells were treated with 2 µg of Heparinase III in 200 µL of buffer containing 50 mM Tris and 2 mM $CaCl_2$ at pH 7.5 for 1 hour at 37 °C. For HS modification by HPSE, cells were treated with 2 µg of HPSE in 200 µL of buffer containing 0.1 M NaOAc at pH 5.0 for 1 hour at 37 °C. For introduction of O-GalNAc to cells, 50 nmol of UDP-GalNAc and 2 µg of GALNT2 in 200 µL of buffer containing 25 mM Tris, 150 mM NaCl, 10 mM $MnCl_2$ at pH 7.5 were added to each well, and the cells were incubated at 37 °C for one hour.

Incorporation of Clickable Carbohydrates Using Glycosyltransferases

To introduce clickable carbohydrates to cells, 200 µL of buffer containing 25 mM MES, 0.5% (w/v) Triton® X-100, 2.5 mM $MgCl_2$, 10 mM $MnCl_2$, 1.25 mM $CaCl_2$ and 0.75 mg/mL BSA at pH 7.0 was added to each well of a 24-well plate. Following this step, different enzymes and azido sugars were used to label distinct carbohydrate epitopes. For imaging lactosamine, 20 nmol of GDP-Azido-Fucose and 2 µg of FUT6 or FUT7 were added. For O-GlcNAc imaging, 20 nmol of UDP-Azido-GalNAc and 2 µg of B4GalT1(Y285L) were added. For O-GalNAc imaging, 20 nmol of UDP-Azido-GlcNAc and 2 µg of B3GNT6 were added. For core-1 O-glycan imaging, 20 nmol of UDP-Azido-GlcNAc and 2 µg of GCNT1 were added. For HS imaging, 20 nmol of UDP-Azido-GlcNAc, 50 nmol of UDP-GlcA, and 4 µg of Exostosin 1/2 were added. The plate was then incubated at 37 °C for 1 to 2 hours, or at room temperature for overnight.

Conjugation of Clickable Carbohydrates to Biotin and Fluorescent Dye

For each click chemistry reaction, 20 nmol of Cu^{2+}, 10 nmol of Biotinylated Alkyne, and 200 nmol of ascorbic acid were combined and then diluted with 200 µL of 25 mM Tris, 150 mM NaCl at pH 7.5. The mixture was then applied to the cells in a 24-well plate for 30 minutes at room temperature. The reaction solution was then removed and Streptavidin-Alexa Fluor® 555 or Streptavidin-Alexa Fluor® 488 at 10 µg/mL in PBS was applied for 15 minutes. DAPI at 10 µM was added if needed. Cells were then washed thoroughly with PBS and stored in PBS.

Imaging of the Fluorescent Labeled Cells

Pictures were captured on an AXIO Observer microscope (ZEISS) with a ZEISS Axiocam 506 mono camera and Zen 2 Pro software simultaneously through the channels of Alexa Fluor® 555 (or Alexa Fluor® 488) and DAPI. For most images, exposure time was automatically set and contrast was adjusted using Best Fit. For comparison of the results of different pretreatment conditions, identical exposure and contrast adjustment parameters were applied to all images.

Cytoplasmic and Nuclear Extract Preparation and Pretreatment

HUVEC cells were cultured in Endothelial Cell Growth Media (R&D Systems Catalog # CCM027) in a 5% CO_2 incubator. HEK293T cells were cultured in IMDM media, supplemented with 5% FBS and penicillin/streptomycin. About 30 x 10^6 cells were scraped and collected by centrifugation at 2000 RPM for 5 minutes. Cell fractionation was performed using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Catalog #78835) according to the manufacturer's instructions. For T antigen synthesis, 10 µL of cell extracts was incubated with 1 µg each of GALNT2 and GALNT3, 1 µg of C1GalT1, 10 nmol of UDP-GalNAc, 10 nmol of UDP-Gal in buffer containing 25 mM Tris, 10 mM Mn^{2+}, 150 mM NaCl at pH 7.5 at 37 °C for 1 hour. For Tn antigen synthesis, UDP-Gal was omitted. For OGA treatment, 10 µL of cell extracts was treated with 10 µL 0.1 M MES at pH 5.0 and 1 µg of OGA and incubated at 37 °C for 30 minutes.

SDS-PAGE and Blotting

After the click chemistry reaction, proteins were separated on a 12% SDS-PAGE containing TCE which reacts with the indole ring of the tryptophan. Gels were visualized using UV excitation. Gels were blotted onto nitrocellulose paper under 30 volts for 30 minutes. The blots were then blocked with 10% fat-free milk for 10 minutes followed by probing with Streptavidin-HRP at 10 µg/10 mL for 20 minutes. The blots were washed thoroughly with 25 mM Tris, pH 7.6, 137 mM NaCl, and 0.01% Tween for 30 minutes with three buffer changes. The membrane was visualized with enhanced chemiluminescence (ECL) peroxidase substrate and exposed to X ray film.

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