

Novel Tools for Spatial Profiling of Protein Expression and Glycosylation in Brain Pathology

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Introduction

Multiplexed protein analysis in native cellular contexts holds great promise for uncovering the composition, interactions, and functions of distinct cell types in complex biological systems. However, current multiplexed protein imaging technologies often face limitations in detection sensitivity or involve technically demanding protocols. To address these issues, we have developed an ultra-sensitive, multiplexed *in situ* protein profiling approach that combines reiterative staining with off-the-shelf antibodies and the newly developed Cleavable Fluorescent Probe (CFP™) cleavable TSA fluorophores.

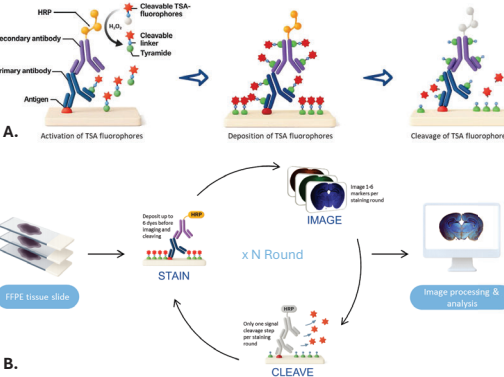


Figure 1. Ultrasensitive and multiplexed protein imaging with cleavable TSA fluorophores. **(A)** Illustration of the CFP signal amplification and cleavage system. Samples are incubated with a primary antibody, followed by an HRP-conjugated secondary antibody. The HRP enzyme catalyzes the conversion of cleavable TSA fluorophores into highly reactive radicals, which covalently bind to tyrosine residues on or near the enzyme site. Following imaging, the fluorophores can be released using mild cleavage reagents. **(B)** Workflow of reiterative protein staining. In each round, multiple targets can be detected. Through reiterative analysis cycles, a large number of distinct proteins can be quantitatively profiled in single cells *in situ*.

In addition to protein analysis, glycans can also be profiled using this approach. Glycosylation play critical roles in cell function in both healthy and diseased states from controlling proliferation to modulating the immune response. Lectins are glycan-binding proteins that recognize specific glycan structures. Their glycan-specific nature makes them important tools to profile, characterize, and capture complex glycans in biological systems.

We demonstrated the compatibility of CFP cleavable TSA fluorophores with lectins from Vector Laboratories. In addition, we investigated the spatial glycosylation state of the protein using Glysite Explorer *in situ* PLA Glycan Detection Kit developed by Vector Laboratories. These assay facilitate the exploration of glycan complexity, enabling the detection of major glycan motifs.

Methods

Target proteins are recognized by primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase (HRP), which catalyzes the covalent deposition of CFP fluorophores on or close to the target proteins. After imaging, the fluorophores are chemically cleaved, enabling

subsequent rounds of staining, antibody stripping, imaging, and signal cleavage. This approach allows for the interrogation of many targets in individual cells while maintaining spatial resolution and sample integrity.

The similar concept can also be applied to HRP-labelled lectins targeting specific glycans. This approach allows for the simultaneous interrogation of many glycans and proteins in individual cells while maintaining spatial resolution.

Results

Multiplexed Protein Detection

Applying this method, ten (10) different proteins in a mouse formalin-fixed paraffin-embedded (FFPE) brain tissue were stained on Biocare ONCORE Pro X system. This method can effectively detect high-plex proteins in real world biological samples.

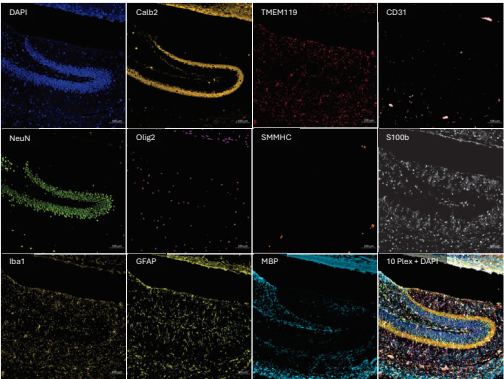


Figure 2. Same slide 10-plex immunofluorescence staining performed on FFPE mouse brain section using the Biocare ONCORE Pro X stainer. Scale bar, 100 µm.

Marker	Vendor	Cat No.	Dilution	Detection
Calretinin	ABclonal	A21965	1:20000	CFP 555
TMEM119	ABclonal	A27148	1:6000	CFP 645
NeuN	ABclonal	A19068	1:2000	CFP 490
Olig2	ABclonal	A26746	1:200	CFP 750
SMMHC (MYH11)	ABclonal	A4046	1:4000	CFP 555
S100B	ABclonal	A19108	1:10000	CFP 645
MBP	ABclonal	A25814	1:20000	CFP 750
CD31	ABclonal	A19014	1:2000	CFP 555
GFAP	ABclonal	A19058	1:1000	CFP 555
Iba1	ABclonal	A20844	1:2000	CFP 645

Table 1. Primary antibodies used for 10-plex immunofluorescence. In each round, up to four markers were stained and imaged.

Multiplexed Protein & Glycan Co-Detection

Three (3) fluorophores (CFP 490, 555, and 645) were used to detect seven (7) proteins (NeuN, HLA-DRA, MAP2, GFAP, EAAT1, Iba1, MBP) and four (4) lectins (WFL, PHA-L, LCA, SNA) on the same human hippocampal tissue section.

After imaging, fluorophores were cleaved and antibodies/lectins stripped to allow repeated staining cycles. This process was applied to 5 normal and 5 Alzheimer's disease (AD) FFPE hippocampal tissues to compare glycan expression and cell marker co-localization.

Lectin	Name	Binding Specificity	Binding Motif
LCA	Lens Culinaris Agglutinin	Core Fucose	
SNA, EBL	Sambucus Nigra Lectin	Sialic Acid	
PHA-L	Phaseolus Vulgaris Leucoagglutinin	Complex N-Glycans	
WFA, WFL	Wisteria Floribunda Lectin	GalNAc	

Legend: Galactose (Gal), N-Acetylgalactosamine (GalNAc), Fucose (Fuc), N-Acetylglucosamine (GlcNAc), Mannose (Man), Sialic acid (Sia)

Table 2. Lectin properties with major binding motifs. Figure created by BioRender.com.

Expression of NeuN, GFAP, Iba1, and MBP reveals the structural integrity and distribution of neurons, astrocytes, microglia, and oligodendrocytes in the dentate gyrus (DG), with changes indicating neuroinflammation, gliosis, or neurodegeneration. Fluorescent patterns from WFL, PHA-L, LCA, and SNA lectin staining highlight differential glycan abundance across cellular compartments.

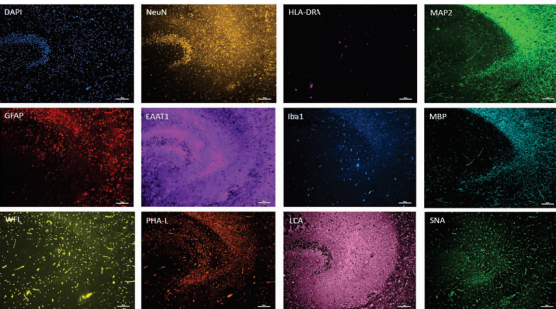


Figure 3. Same slide 11-plex protein and glycan co-detection on FFPE human AD hippocampus tissues. Illustration of distinct fluorescence patterns highlight the spatial organization of proteins and glycans in the dentate gyrus (DG). Scale bars, 200 µm.

Marker	Vendor	Cat No.	Dilution	Detection
NeuN	ABclonal	A0951	1:50	CFP 555
HLADRA	ABclonal	A10863	1:200	CFP 645
MAP2	ABclonal	A22206	1:200	CFP 555
GFAP	ABclonal	A19058	1:400	CFP 645
EAAT1	ABclonal	A9712	1:200	CFP 555
Iba1	ABclonal	A19776	1:200	CFP 645
MBP	ABclonal	A24860	1:200	CFP 555
WFL	Vector Labs	B-1355-2	1:200	CFP 645
PHA-L	Vector Labs	B-1115-2	1:200	CFP 645
LCA	Vector Labs	B-1045-5	1:200	CFP 555
SNA	Vector Labs	B-1305-2	1:200	CFP 490

Table 3. Primary antibodies from ABclonal and biotinylated lectins from Vector Laboratories were used for the 11-plex protein and glycan co-detection assay.

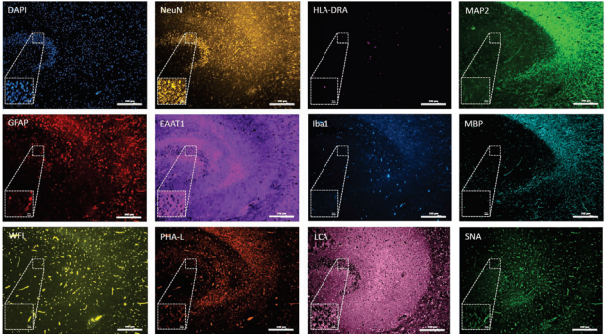


Figure 4. Same slide 11-plex protein and glycan co-detection on FFPE human AD hippocampus tissues. Whole frame with a zoomed-in view of the DG, a key region for assessing cellular protein and glycan changes and highly susceptible to AD-related neurodegeneration. Scale bars, 200 µm.

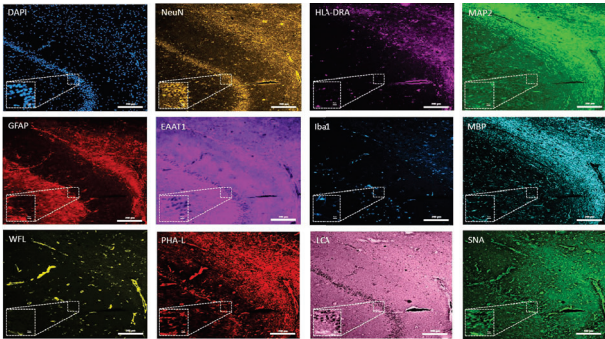


Figure 5. Same slide 11-plex protein and glycan co-detection on FFPE normal human hippocampus tissues. Whole frame with a zoomed-in view of the DG. Scale bars, 200 µm.

According to the initial data analysis, LCA and WFL lectins show a higher percentage of cell numbers in AD tissue, suggesting that their binding increases in the AD hippocampus due to elevated expression of core fucosylated *N*-glycans, and *N*-Acetylglactosamine (GalNAc), respectively. These glycosylation changes in vulnerable cells within the AD-affected dentate gyrus (DG) could influence pathology by altering cell signaling, neuroinflammation, and protein aggregation. The co-staining method precisely identifies glycan changes across specific cell populations, comparing pathological to normal conditions, thus clarifying cell-type-specific glycosylation alterations. Such detailed cellular insights are crucial for developing glycosylation-based biomarkers and therapeutic targets.

Glycosylation of Neural Cell Marker

Glial Fibrillary Acidic Protein (GFAP) is a known biomarker for neurodegenerative diseases, and investigating the protein glycosylation is critical to enhance our understanding of the disease biology.

A novel proximity-based detection technology - Glysite Explorer *in situ* PLA (isPLA) Glycan Detection Kit from Vector Laboratories was utilized to identify protein glycosylation through the use of DNA-labeled probes and a DNA amplification technology. Anti-GFAP antibody was paired with Glysite Explorer Lectins on FFPE human normal brain tissues to identify GFAP protein glycosylation.

Glysite Explorer isPLA Kit provides deeper biological insights, enriching data from traditional IHC/IF as well as Spatomics's spatial multiplexing CFP platform. Altogether, these technologies emphasize the transformative potential of glycosylation-focused research in advancing precision medicine.

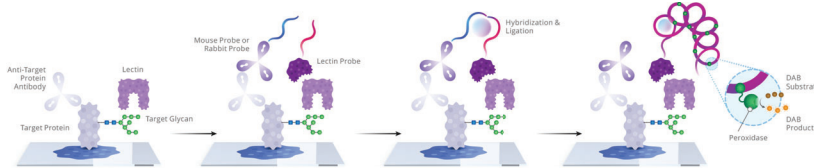


Figure 6. Schematic of Glysite Explorer *in situ* PLA Glycan Detection Kit developed by Vector Laboratories. Glysite Explorer Lectin binds to the target glycan, and primary antibody binds to the target protein. Secondary oligo-labeled probes bind to the primary antibody and Glysite Explorer Lectin. When secondary probes are in proximity (within ~40 nm), ligation occurs. Amplification and detection produces a colored precipitate at the site of proximity.

Reagent	Specificity	Vendor	Cat No.	Dilution
Anti-GFAP Primary Antibody	GFAP protein	Leica	NCL-L-GFAP-GA5	1:200
Glysite Explorer GNL Lectin	Mannose	Vector Labs	GEK-1249	1:50
Glysite Explorer SNA Lectin	Sialic Acid	Vector Labs	GEK-1309	1:50
Glysite Explorer Jacalin Lectin	O-glycans	Vector Labs	GEK-1159	1:50
Glysite Explorer ECL Lectin	LacNAc	Vector Labs	GEK-1149	1:50
Glysite Explorer AAL Lectin	Fucose	Vector Labs	GEK-1399	1:50

Table 4. Anti-GFAP primary antibody from Leica and Glysite Explorer Lectins from Vector Labs were used for the *in situ* proximity protein glycosylation assay on FFPE human normal brain tissue.

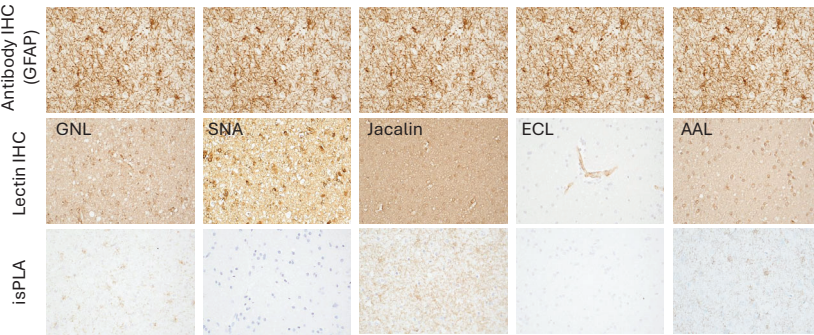


Figure 7. *In situ* proximity protein glycosylation assay on FFPE human normal brain tissues. Whole frame with 20x objective. Top panel shows IHC staining by Anti-GFAP primary antibody. Middle panel (from left to right) shows IHC staining by B-GNL Lectin, SNA Lectin, Jacalin Lectin, ECL Lectin and AAL Lectin respectively. Bottom panel (from left to right) shows abundant isPLA staining of GFAP Ab + Glysite Explorer GNL Lectin, minimal isPLA staining of GFAP Ab + Glysite Explorer SNA Lectin, abundant isPLA staining of GFAP Ab + Glysite Explorer Jacalin Lectin, minimal isPLA staining of GFAP Ab + Glysite Explorer ECL Lectin and subtle isPLA staining of GFAP Ab + Glysite Explorer AAL Lectin respectively. Positive signals: brown staining. Hematoxylin: blue staining.

Summary

- We have developed a highly sensitive, multiplexed single-cell *in situ* analysis method, enabling simultaneous detection of proteins and glycans in the same biological samples, including FFPE tissues. This novel glycoproteomics approach, leveraging CFP cleavable TSA fluorophores, allows visualization of more than 10 protein and glycan markers at the subcellular level and can be automated using Biocare ONCORE Pro X and Leica BOND Rx platforms.
- Further experiments are planned to quantitatively analyze the glycan expression pattern between normal and AD samples.
- Initially demonstrated in Alzheimer's disease research, this versatile method is also applicable to cancer biology, immunology, and infectious disease research.
- We utilized *in situ* Proximity Ligation Assay to explore the glycosylation changes of a specific neural cell marker in human normal brain tissues. Further experiments are planned to study protein glycosylation in AD samples. Collectively, this data will add to the growing understanding of the impact of protein glycosylation on brain pathology.

For more information about the CFP technology, please refer to [Spatomics.com](#). To learn more about Glysite Explorer *in situ* PLA Glycan Detection Kit, please visit [vectorslabs.com](#).