Multiplexed spatial profiling of protein and glycan expression using CFP Fluor cleavable TSA fluorophores

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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.

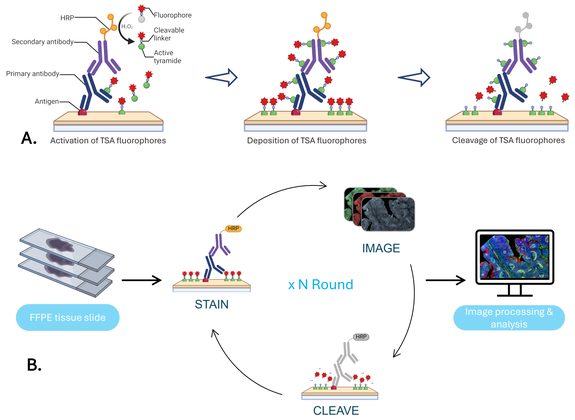


Figure 1. Ultrasensitive and multiplexed protein imaging with cleavable TSA fluorophores. (**A**) Illustration of the CFP signal amplification and cleavage system. Samples are incubated with a primary antibody, followed by an HRP-conjugated secondary antibody. The HRP enzyme catalyzes the conversion 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*. Figure created by BioRender.com.

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 a subgroup of glycan-binding proteins that recognize glycan motifs and mediate their functionality. 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 developed by Vector Laboratories. This assay facilitates the exploration of glycan complexity, enabling the detection of major glycan motifs, including sialylation, fucosylation, galactosylation, and complex *N*-glycosylation for characterizing glycan abundance in target specimens.

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. 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, we stained twelve (12) different proteins in a human formalin-fixed paraffin-embedded (FFPE) tonsil tissue on Biocare ONCORE Pro X system. This method can effectively detect high-plex proteins in real world biological samples.

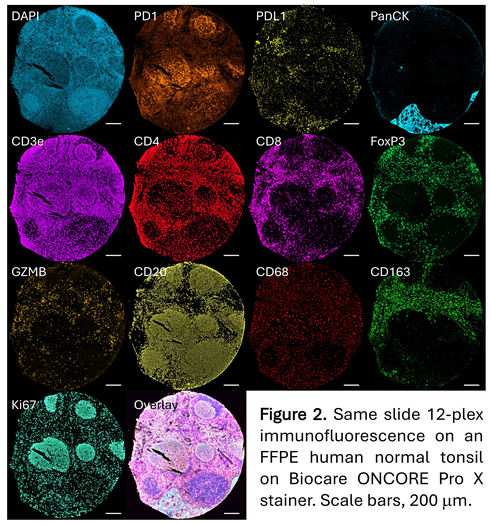


Figure 2. Same slide 12-plex immunofluorescence on an FFPE human normal tonsil on Biocare ONCORE Pro X stainer. Scale bars, 200 μm.

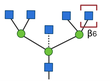
Marker	Vendor	Cat No.	Dilution	Round	Detection
PD1	SinoBio	10377-MM23	1:50	1	CFP 647
PDL1	SinoBio	10084-T24	1:1000	1	CFP 488
CD20	Abcam	64088	1:100	1	CFP 555
FOXP3	Abcam	20034	1:500	1	CFP 750
CD4	ABclonal	19018	1:200	2	CFP 647
GZMB	SinoBio	10345-R002	1:1000	2	CFP 555
CD8	CST	85336	1:400	2	CFP 488
PanCK	Abcam	ab7753	1:983	2	CFP 750
CD68	Abcam	AB213363	1:500	3	CFP 647
CD3e	Abcam	AB5690	1:100	3	CFP 750
Ki67	Abcam	16667	1:250	3	CFP 555
CD163	Abcam	AB182422	1: 500	3	CFP 488

Table 1. Primary antibodies used for 12-plex immunofluorescence. In each round, four (4) markers were stained and imaged.

Multiplexed Protein & Glycan Co-Detection

Three (3) fluorophores (CFP 488, 555, and 647) 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	

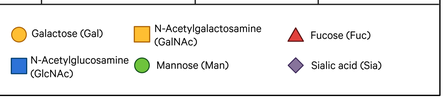


Table 2. Lectin properties with major binding motifs. Figure created by BioRender.com.

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.

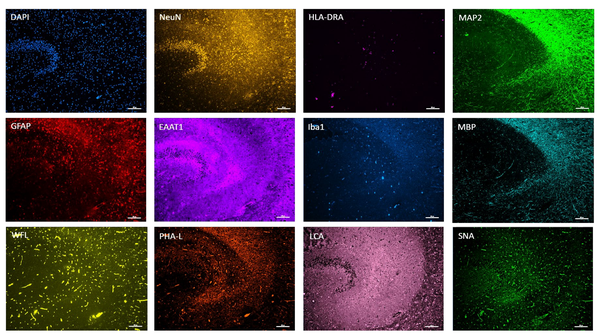


Figure 3. Same slide 11-plex protein and glycan co-detection on FFPE human AD hippocampus tissues. Illustration of distinct fluorescence patterns highlight the spatial organization of proteins and glycans in the dentate gyrus (DG). Scale bars, 200 μm.

Marker	Vendor	Cat No.	Dilution	Detection
NeuN	ABclonal	A0951	1:50	CFP 555
HLADRA	ABclonal	A10863	1:200	CFP 647
MAP2	ABclonal	A22206	1:200	CFP 555
GFAP	ABclonal	A19058	1:400	CFP 647
EAAT1	ABclonal	A9712	1:200	CFP 555
Iba1	ABclonal	A19776	1:200	CFP 647
MBP	ABclonal	A24860	1:200	CFP 555
WFL	Vector Labs	B-1355-2	1:200	CFP 647
PHA-L	Vector Labs	B-1115-2	1:200	CFP 647
LCA	Vector Labs	B-1045-5	1:200	CFP 555
SNA	Vector Labs	B-1305-2	1:200	CFP 488

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

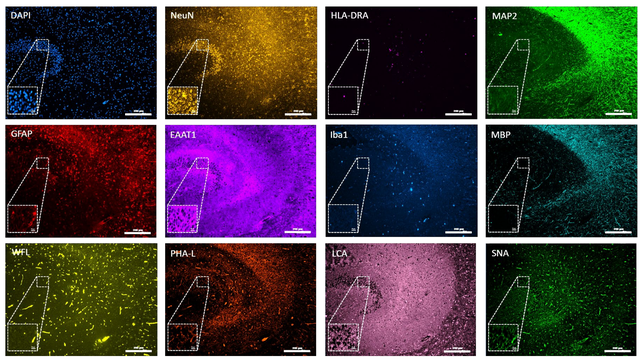


Figure 4. Same slide 11-plex protein and glycan co-detection on FFPE human AD hippocampus tissues. Whole frame with a zoomed-in view of the DG, a key region for assessing cellular protein and glycan changes and highly susceptible to AD-related neurodegeneration. Scale bars, 200 μm.

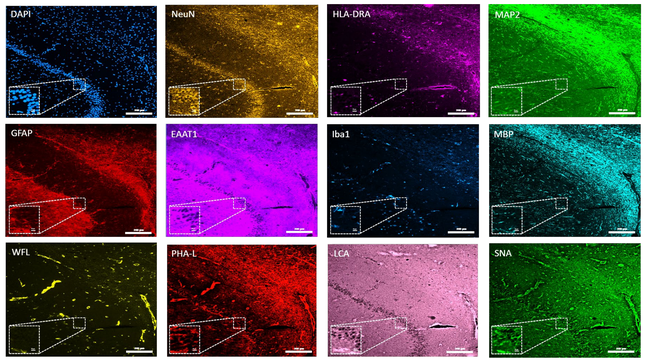


Figure 5. Same slide 11-plex protein and glycan co-detection on FFPE normal human hippocampus tissues. Whole frame with a zoomed-in view of the DG. Scale bars, 200 μm.

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*-Acetylgalactosamine (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.

Summary

1. 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.

2. Further experiments are planned to quantitatively analyze the glycan expression pattern between normal and AD samples.

3. Initially demonstrated in Alzheimer's disease research, this versatile method is also applicable to cancer biology, immunology, and infectious disease research.

Note: For more information about the CFP technology, please refer to www.spatomics.com