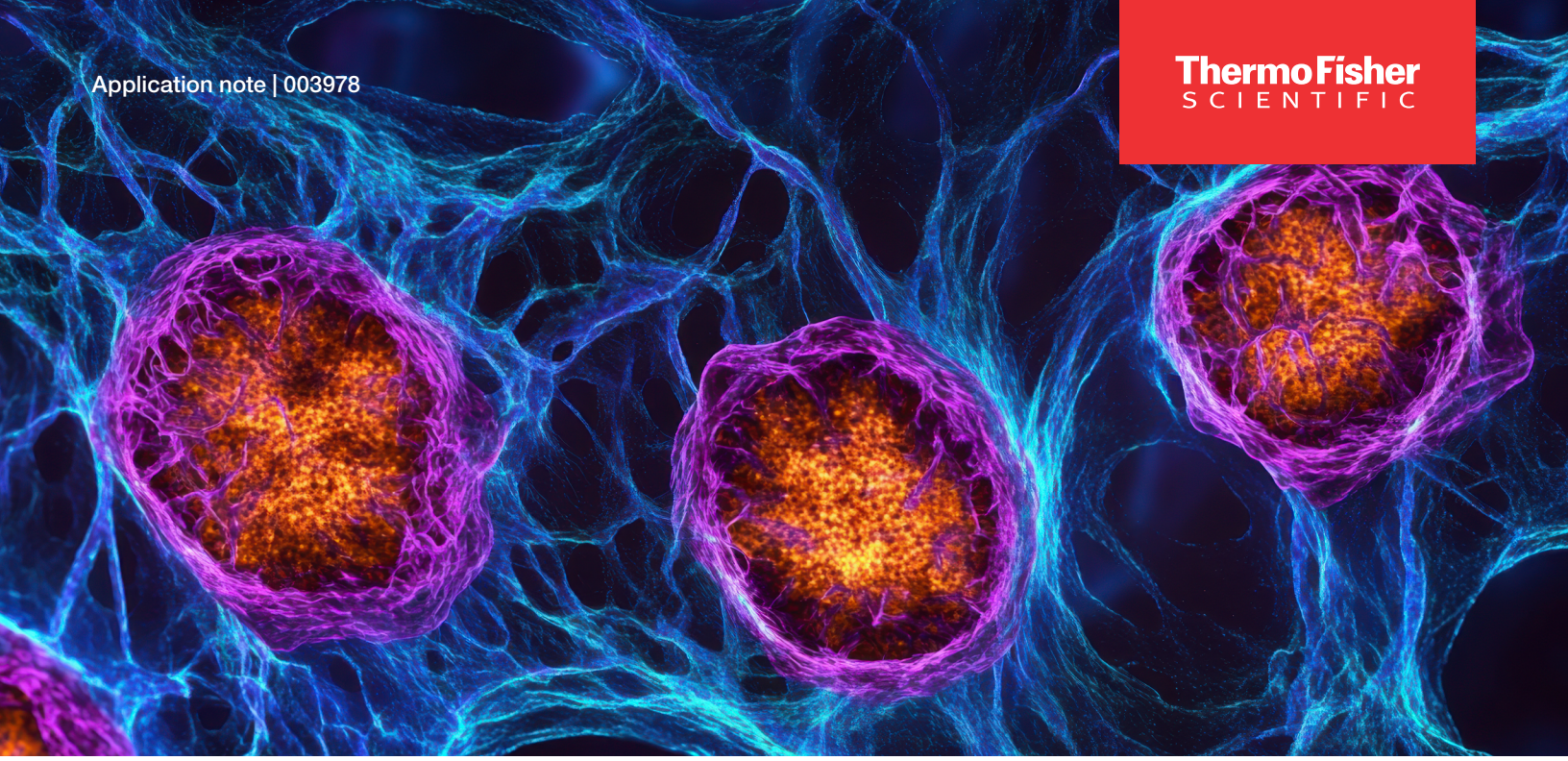

Application note | 003978

High-sensitivity subcellular spatial proteomics of ciliary organelles using the Microscoop and Orbitrap Astral mass spectrometer

Authors

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Goal

Demonstrate the application of the Syncell® Microscoop® platform in combination with the Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer for high-resolution spatial proteomic analysis of ciliary samples. By leveraging targeted photolabeling to enrich ciliary proteins, followed by sensitive mass spectrometric detection, this approach helps enable the identification of cilia-specific proteins and provides deeper insights into their molecular composition and functional organization.

Introduction

Understanding the spatial distribution of proteins within cells is critical for unraveling the complexities of cellular function and signaling, particularly in highly specialized organelles such as cilia. Cilia are dynamic, microtubule-based structures that play essential roles in motility, sensory transduction, and signaling pathways.[1] Given their small size and structural complexity, precise mapping of the ciliary proteome poses a significant technical challenge.[2]

To overcome the limitations of traditional spatial proteomics reliant on predefined markers,[3] we employed the Microscoop system for hypothesis-free, submicron-resolution proteomic analysis. Using photoactivatable biotin-based probes (Synlight kit), the platform enables selective labeling of proteins within defined regions of interest (ROIs), guided by minimal prior marker input and real-time imaging. Automated, laser-guided photolabeling helps ensure high-throughput precision, followed by protein

enrichment via streptavidin-biotin pulldown (Synpull kit). This workflow is compatible with both fresh and FFPE tissues, enabling unbiased discovery of spatially localized proteins.

Finally, we integrated this highly spatially resolved labeling method with an Orbitrap Astral mass spectrometer, equipped with Thermo Scientific™ FAIMS Pro Duo interface and a front-end Thermo Scientific™ Vanquish™ Neo UHPLC system. The FAIMS Pro Duo interface enhances ion selectivity through differential compensation voltages (CV), reducing chemical noise and enabling sensitive detection of low-abundance ions. The Orbitrap Astral mass spectrometer offers high acquisition speed, ultra-high sensitivity, and deep proteome coverage, making it well-suited for spatial proteomics from limited sample inputs. This combined Microscoop and Orbitrap Astral mass spectrometer workflow enabled robust detection of sub-organelle protein signatures, including low-copy-number species, providing unprecedented insights into the molecular composition and organization of cilia (Figure 1).

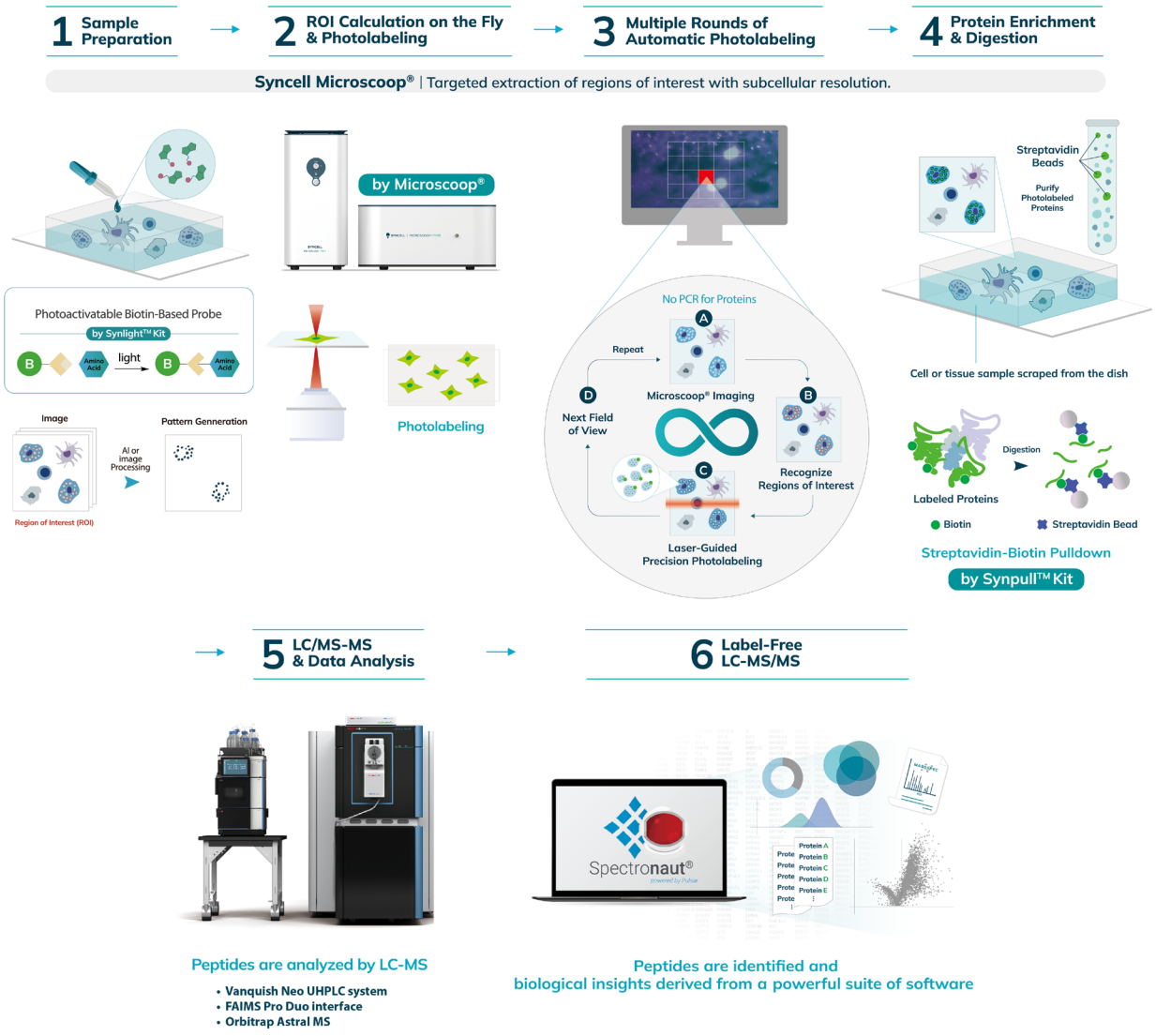

Figure 1: Workflow of targeted subcellular protein analysis using Microscoop integrated with Orbitrap Astral mass spectrometer. The process includes: (1) sample preparation using photoactivatable biotin-based probes, (2) AI-guided detection of regions of interest (ROIs) and photolabeling, (3) multiple rounds of automated laser-guided photolabeling by Microscoop, (4) protein enrichment and digestion using streptavidin-biotin pulldown, (5) LC-MS/MS analysis with Orbitrap Astral MS for high-resolution mass spectrometry, and (6) label-free identification and data analysis using powerful software to derive biological insights.

Experimental approach

Understanding the spatial distribution of proteins within cells is critical for unraveling the complexities of cellular function and signaling, particularly in highly specialized organelles such as cilia.

Consumables

- Synlight-Rich Kit (P/N SYN-RI0206, Syncell)
- Synpull Kit (P/N SYN-PU0106, Syncell)
- Chambered glass (P/N C1-1.5H-N, CellVis)
- RPE-1 cells
- ARL13B antibody (P/N sc-515784, Santa Cruz)
- Water with 0.1% formic acid (FA) (v/v), Thermo Scientific™ Optima™ LC-MS grade, Thermo Scientific™ (P/N LS118-500)
- 80% Acetonitrile (ACN), 20% water with 0.1% formic acid, Optima LC-MS, Fisher Chemical™ (P/N LS122500)
- Formic acid, 99.0+%, Optima LC-MS grade, Fisher Chemical™ (P/N A117-50)

Samples

- Photolabeled (PL) RPE-1 cells (ciliary pattern scanning)
- Unlabeled (UL) RPE-1 cells (immersed with the reagents of Synlight-Rich Kit)

LC columns

- IonOpticks Aurora® Ultimate™ 25×75 TS C18 UHPLC column (P/N AUR3-25075C18-TS)

Instrumentation

- Vanquish Neo UHPLC system (P/N VN-S10-A-01)
- (P/N 6036.1180)
- Orbitrap Astral mass spectrometer (P/N BRE725600)
- Thermo Scientific™ EASY-Spray™ NG ion source (P/N ES082)
- FAIMS Pro Duo interface (P/N FMS03-10001)
- Microscoop® Mint (Syncell)

Software

- Spectronaut® software (Biognosys AG)

Sample preparation

Retinal pigment epithelium (RPE1) cells were grown on chambered glass slides to ~90% confluency, then serum-starved for 48 hours to induce primary cilia formation before methanol fixation. ARL13B immunofluorescence was used to visualize cilia, after which cells were incubated with the species-agnostic Synlight-Rich reagents (compatible with FFPE, methanol-fixed, PFA-fixed, and fresh-frozen samples). Using Microscoop, we performed microscopy-guided two-photon illumination (≈350 nm xy resolution) that triggered a photochemical reaction of Synlight-Rich to biotinylate individual ciliary structures within each field of view. This automated procedure was repeated across ~1 × 10^6 cells (≈14 structures/second), yielding ~100 µg of total lysate in a 20-hour session. A sample with identical ingredients (cells and reagents) but without the two-photon illumination (unlabeled, or UL, vs. the illuminated one, or photolabeled, PL) was used for normalization by obtaining the ratio of PL/UL counts for each protein to account for the background effect of endogenously biotinylated proteins and nonspecific binding during the Synpull workflow. Both UL and PL samples were scraped and subjected to the Synpull Kit for lysing, pulldown, and washing to isolate subcellular, region-specific biotinylated proteins. Approximately 20 ng of each eluate (UL and PL) was then analyzed via LC-MS using the Orbitrap Astral mass spectrometer to enable deep subcellular proteome profiling and quantification.

LC-MS analysis

All LC-MS runs for ciliary peptides were separated and analyzed using a Vanquish Neo UHPLC system coupled to an Orbitrap Astral mass spectrometer that was equipped with a FAIMS Pro Duo interface. Peptide separation was achieved on the Vanquish Neo UHPLC system using a direct injection configuration with an Aurora TS analytical column (25 cm, 75 µm, 1.7 µm particle size). Chromatographic gradients were formed using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 80% acetonitrile as mobile phase B. Detailed liquid chromatography parameters and gradient settings are provided in Table 1 below. Mass spectrometer source parameters and scan parameters can be found below in Table 2.

Table 1. Vanquish Neo UHPLC system gradients and LC parameters for 25 minute run time

25 minute run time			
	Time (min.)	**% Mobile Phase B**	**Flow (µL/min.)**
Gradient	0	8	0.4
	0.1	8	0.4
	1.9	8	0.2
	2	28.0	0.2
	12	50.0	0.2
	19.5	99.0	0.2
	22	99.0	0.3
	25	99.0	0.3
LC parameters	LC configuration	Direct	
	Fast loading/equilibration mode	Pressure control	
	Loading/equilibration/wash pressure	Max pressure	
	Equilibration factor	3	
	Sampler temperature	7°C	
	Mobile phase A / weak wash	0.1% Formic acid in water	
	Mobile phase B / strong wash	0.1% Formic acid in 80% acetonitrile	
	Zebra wash	Not enabled	
	Zebra wash cycles	0	
	Analytical column temperature	50°C	
Column specifications	Analytical column	Aurora Ultimate 25×75 TS C18 UHPLC column (P/N AUR3-25075C18-TS)	

Table 2. 25 minute Orbitrap Astral mass spectrometer parameters
(A) Global source and mass spectrometer parameters with FAIMS on; (B) MS1 full scan experiment parameters; (C) MS2 DIA scan experiment parameters

Global parameters (source & MS)	
Positive Ion Voltage	1800 Volts
Ion Transfer Tube Temperature	290°C
Expected Peak Width	10 seconds
Default Charge State	2
Lock Mass Correction	EASY-IC™
Total Carrier Gas Flow	3.5
FAIMS Mode	Standard Resolution

MS1 full scan experiment parameters	
Orbitrap Resolution	240K
Scan Range (m/z)	400–800
Normalized AGC Target (%) / Absolute AGC Value	500% / 5.00e6
Maximum Injection Time (ms)	100
Microscans	1

MS2 DIA scan experiment parameters	
Precursor mass range (m/z)	400–800
Isolation window (m/z)	5
Window placement optimization	Off
AGC target	Custom
Normalized AGC target (%) / absolute AGC value	500% / 5.00e4
FAIMS CV	-45/-50/-65
Maximum injection time (ms)	10 Omit.
DIA scan range (m/z)	150–2000
HCD collision energy (%)	25
RF lens (%)	40
Time	0.6 seconds

Data processing and analysis

The raw DIA files from both the labeled and unlabeled samples were analyzed together using Spectronaut software in directDNA™ mode. Protein identification was conducted using the Pulsar search engine, and the reference database employed was UniProtKB/Swiss-Prot, specifically the human proteome database (Homo sapiens: 20,423 entries) with default search settings. All results were processed and filtered with a 1% precursor and 1% protein group false discovery rate (FDR). Exported output files were imported to R Studio (2023.09.0 Build 463) with R (v4.3.1) for downstream data analysis and visualization.

Results

Robust and deep protein quantification from subcellular ciliary samples

Using the Microscoop-based photolabeling approach combined with the Orbitrap Astral mass spectrometer, we successfully quantified over 5,300 proteins and 41,000 peptides from 20 ng of sample in triplicates. PL and UL replicates showed high consistency with each replicate detecting over 5,000 protein groups, demonstrating the method's robustness and sensitivity. PL samples enrich for ciliated proteins; however, the proteome depth of PL and UL is often expected to be similar due to the detection of endogenously biotinylated proteins, non-specific labeled proteins, and non-specific binders in the UL controls. Importantly, 84% of proteins (4,452/5,306) were confidently identified with at least two unique peptides, highlighting the depth and reliability of this spatial proteomics workflow for subcellular ciliary samples (Figure 2).

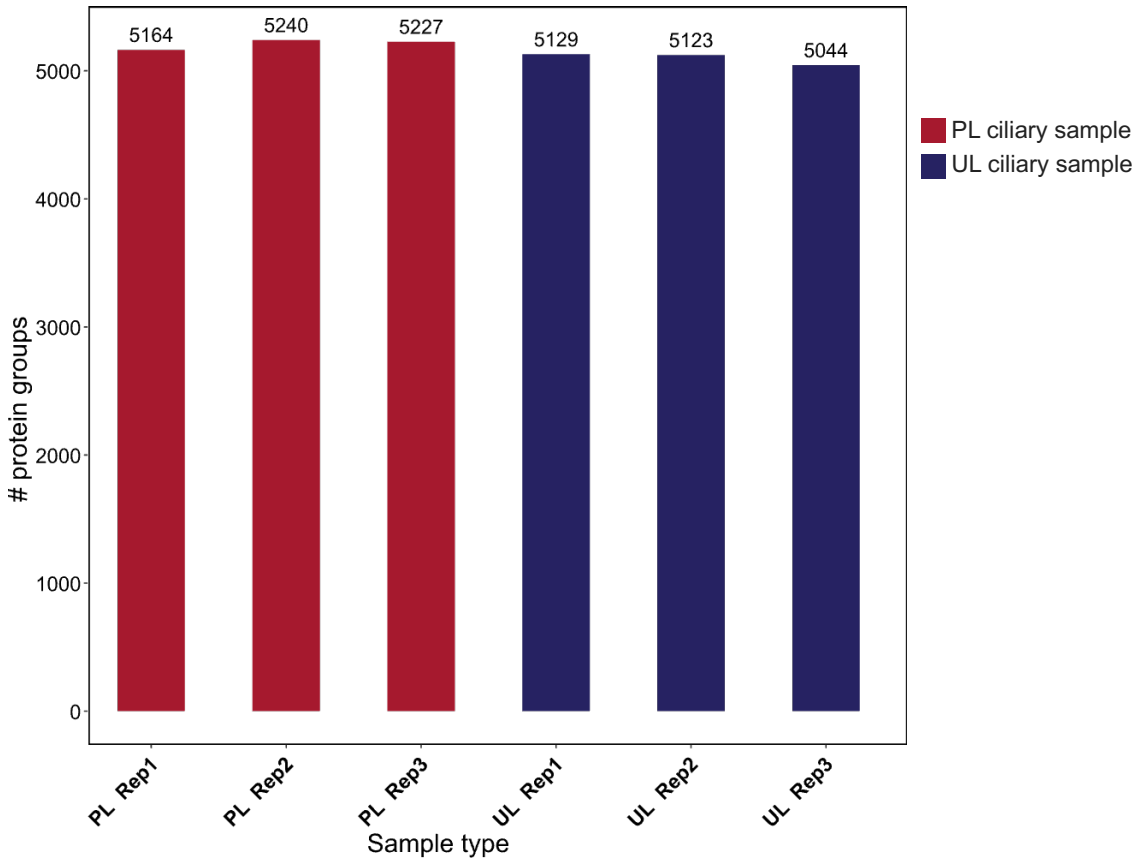

Figure 2: Deep proteome profiling from low-input ciliary samples. Three technical replicates of PL (red) and UL (blue) ciliary samples show consistent identification of >5,000 protein groups, demonstrating high sensitivity and reproducibility.

High reproducibility across photolabeled and unlabeled samples

Both PL and UL ciliary samples demonstrated excellent quantitative reproducibility with a median CV of 13% across replicates. About 69% (3,639 protein groups) in PL and about 67% (3,521 protein groups) in UL exhibited CVs below 20%, and nearly 40% (2,000 protein groups) in each group showed CVs below 10%. These results highlight the robustness and consistency of the Microscoop and Orbitrap Astral mass spectrometer workflow for precise protein quantification, even from low-input samples (Figure 3).

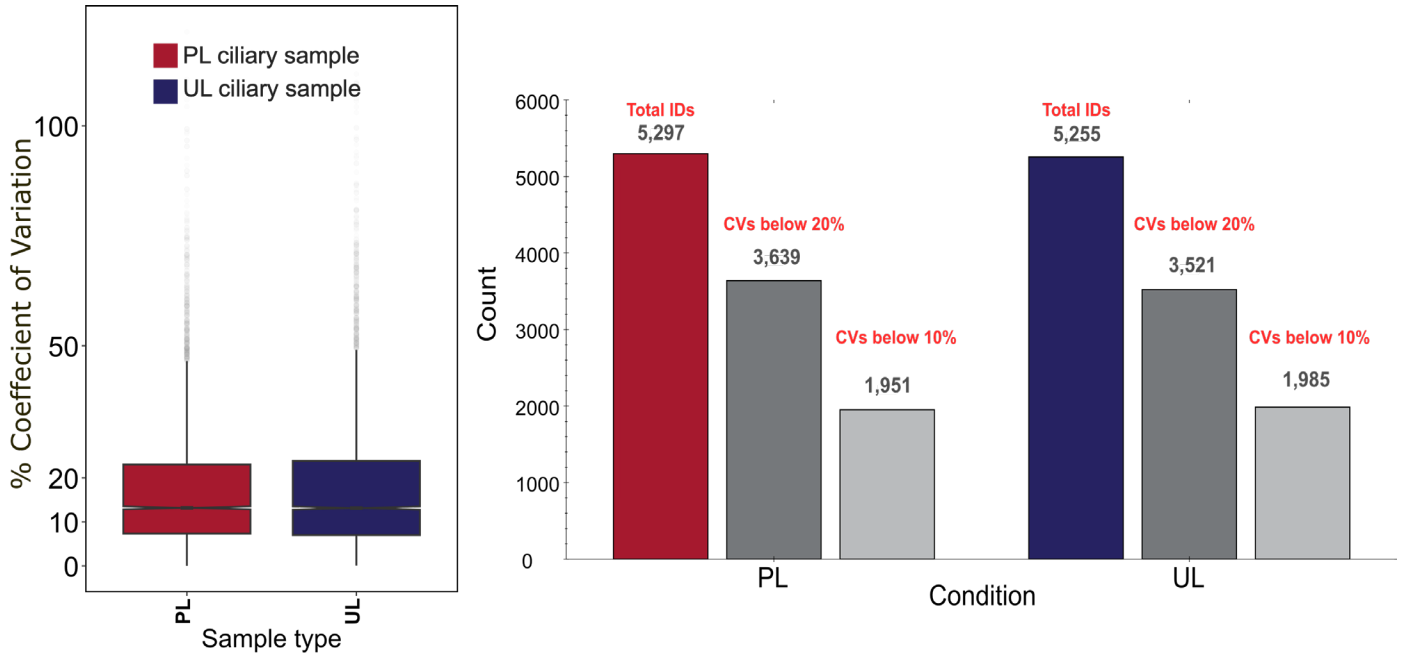

Figure 3: High reproducibility across photo-labeled and unlabeled samples. PL and UL ciliary samples show strong quantitative reproducibility with median CVs of 13% and thousands of proteins exhibiting CVs below 20%, demonstrating the quantitative consistency of the Microscoop and Orbitrap Astral mass spectrometer workflow.

Broad dynamic range of ciliary proteome

Dynamic range assessment of triplicate injections of PL samples revealed protein intensities spanning over 5.5 orders of magnitude. The most abundant protein detected was immunoglobulin heavy variable 6-1 (log_{10} median intensity: 5.3), while one of the least abundant was the methyl monooxygenase 1 (log_{10} median: 0.2) (Figure 4). This broad dynamic range underscores the method's sensitivity and capacity to detect proteins across a wide abundance spectrum, enabling comprehensive and spatially resolved profiling of ciliary organelles.

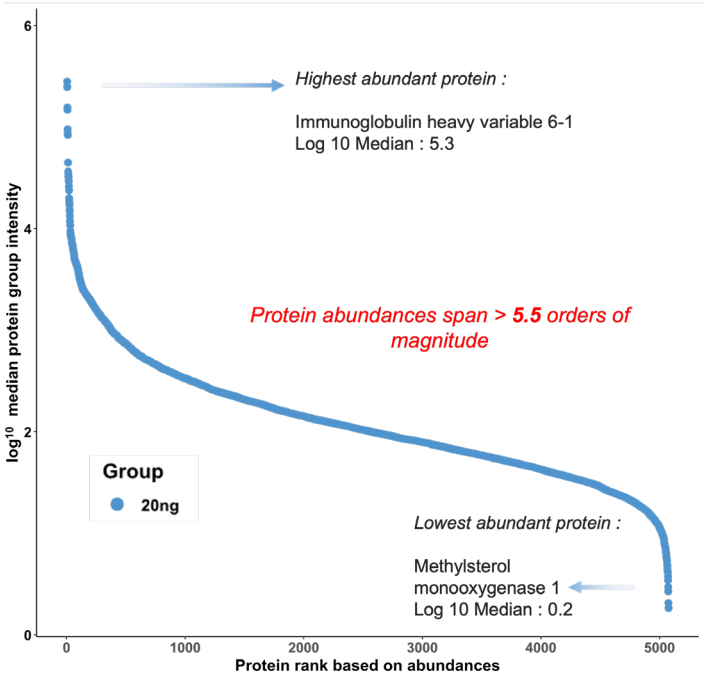

Figure 4: Extensive dynamic range for ciliary organelle. Protein intensities from triplicate injections of 20 ng of PL samples spanned over 5.5 orders of magnitude highlighting the method's depth and sensitivity.

Distinct spatial proteomic signatures in photolabeled cilia

Spatial proteomic profiling of PL and UL ciliary samples using the Microscoop platform coupled with the Orbitrap Astral mass spectrometer revealed distinct protein distribution patterns. Principal component analysis (PCA) showed clear separation between PL and UL groups, with PL samples tightly clustering and explaining over 64% of the variance along PC1, indicating reproducible and condition-specific proteomic profiles (Figure 5, left panel).

Further differential analysis using a volcano plot identified 1,781 proteins significantly enriched and 211 proteins depleted in the PL group (log2FC >0.58, $P < 0.05$), highlighting robust spatial enrichment of protein subsets within PL ciliary regions (Figure 5, right panel). These results demonstrate the power of this workflow to resolve organelle proteomic signatures with high precision.

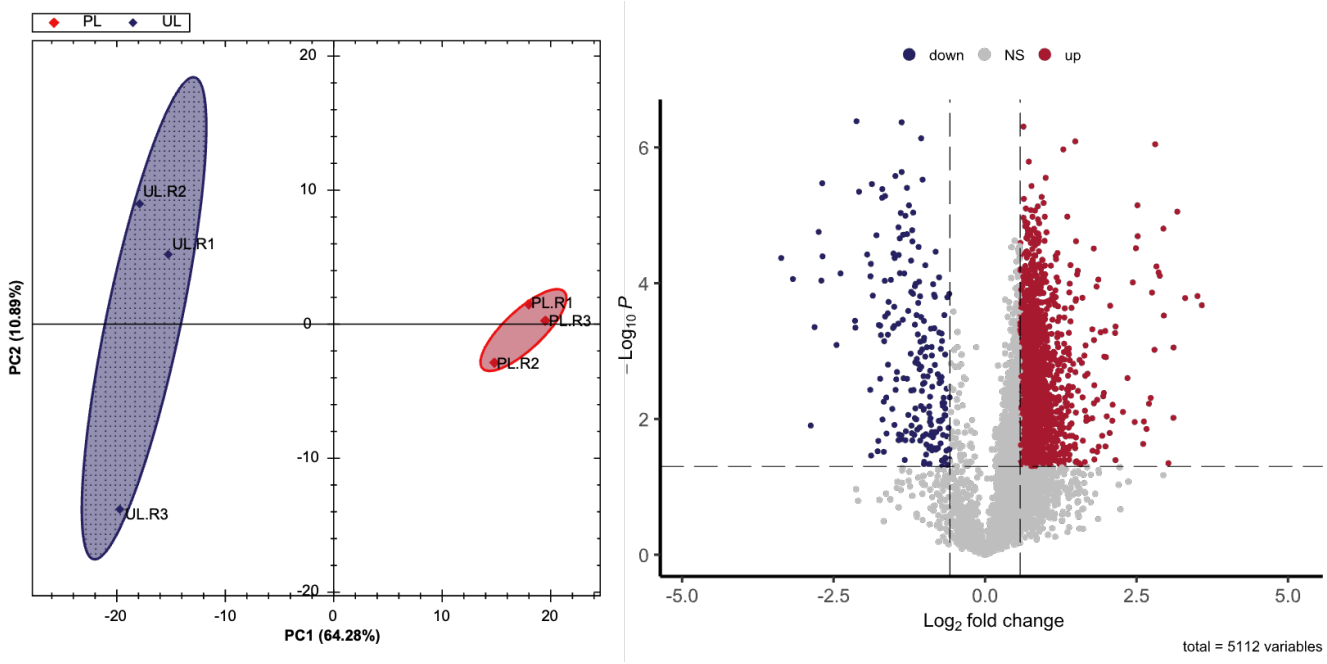

Figure 5. Spatial proteomic profiling reveals distinct ciliary signatures. PCA (left) of PL and UL ciliary samples shows clear separation and tight PL clustering with PC1 explaining over 64% of variance. Volcano plot analysis (right) identified 1,781 enriched and 211 depleted proteins in PL samples ($P < 0.05$), demonstrating precise spatial proteomic resolution using the Microscoop-Orbitrap Astral mass spectrometer workflow.

Photolabeling reveals enrichment of ciliary and centrosomal proteins

Ontology analysis of the PL-enriched proteins revealed a strong enrichment in proteins associated with ciliary and centrosomal compartments (Figure 6). Notably, intraciliary transport particles, ciliary base, centriole, ciliary tip, and ciliary transition zone showed high fold enrichment and statistical significance, indicating successful and selective photolabeling of proteins localized to the cilium. Additionally, substantial enrichment was observed in the centrosome, microtubule organizing center, and ciliary basal body, further supporting effective subcellular targeting. These data validate the spatial precision of the photolabeling approach and demonstrate its power in profiling localized proteomes with high specificity.

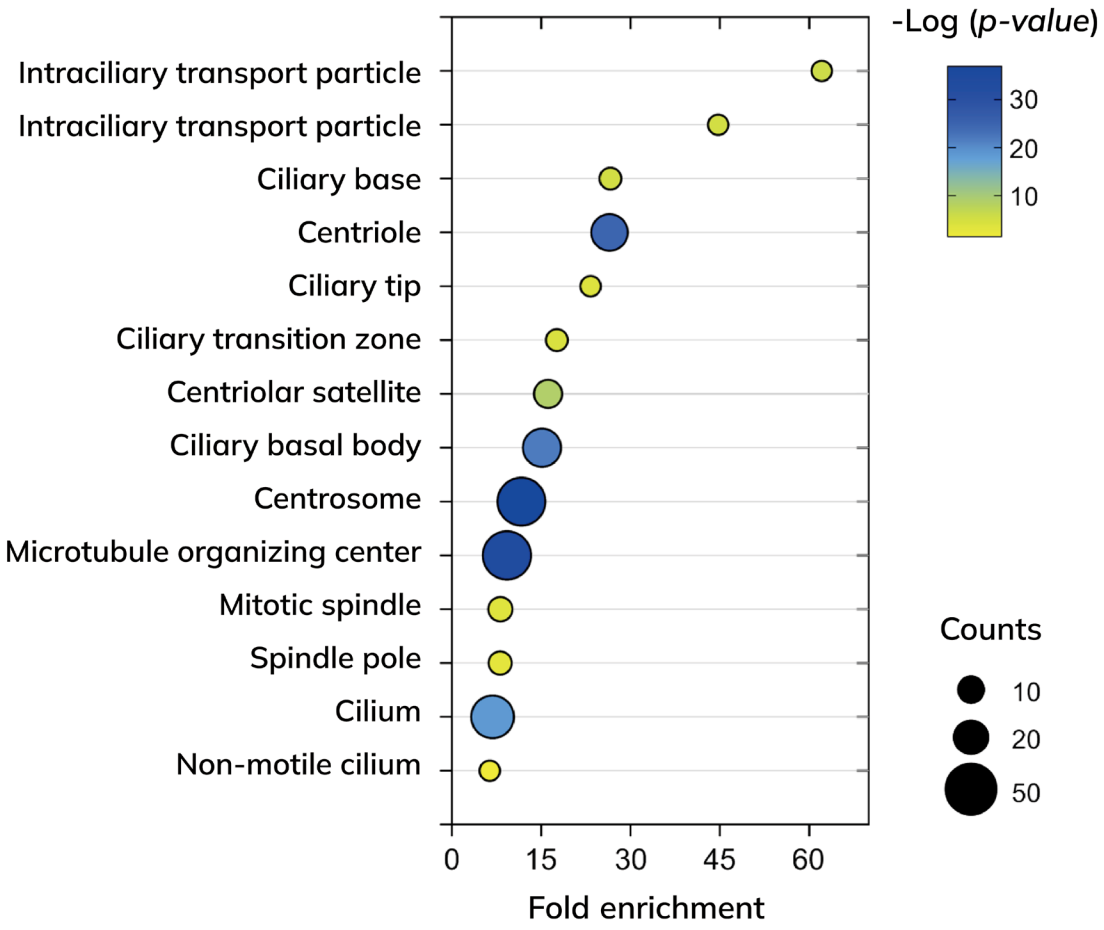

Figure 6. Subcellular component ontology analysis of enriched photolabeled proteins. The bubble plot displays enriched cellular compartments with fold enrichment on the x-axis. Bubble size indicates the number of proteins associated with each term and color reflects statistical significance ($-\log_{10}$ p-value) with blue indicating higher confidence. Key enriched compartments include ciliary and centrosomal structures.

Summary

Robust protein identification

- Identified over >5,300 proteins at 20 ng of ciliary organelle samples.

High reproducibility across all photolabeled and unlabeled samples

- Median CV of 13%; over 60% of proteins had CV <20%, showing strong consistency.

Wide dynamic range

- 20 ng samples covered >5.5 orders of magnitude in abundance in ciliary organelle.

Distinct spatial proteomic signatures

- PCA showed clear separation between PL and UL groups (PC1 >64%).
- 1,781 proteins enriched in PL cilia, revealing organelle resolution.

Targeted photolabeling uncovers ciliary and centrosomal protein enrichment

- Photolabeling combined with high-sensitivity proteomic analysis revealed a distinct enrichment of proteins associated with ciliary and centrosomal structures, highlighting the method's specificity for spatially resolved proteome profiling of the cilia organelle.

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