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Spatial Mapping of Proteins in Nuclear and Nucleolar Compartments

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Authors

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Introduction

Spatial proteomics is an emerging field that integrates the study of protein localization, distribution, and interactions within the complex architecture of cells and tissues[1]. Unlike traditional proteomics, which often focuses on the global quantification of proteins, spatial proteomics aims to map the precise locations of proteins within specific cellular compartments and substructures. This spatial resolution provides critical insights into the functional organization of the proteome and its dynamic changes in response to various biological processes [2, 3]. Within this field, nuclear and nucleolar proteomics focus on the proteins within the nucleus and nucleolus, the central hubs of cellular control and regulation. These subcellular compartments are not only integral to the preservation and expression of genetic information but also play critical roles in the maintenance of cellular homeostasis and response to environmental stimuli.

Investigating the protein composition of the nucleus and nucleolus reveals key insights into the intricate regulatory systems that control cellular function. By delving into the proteomic profiles of these critical cellular compartments, we can uncover novel biomarkers, therapeutic targets, and fundamental principles of cellular organization and function. The nucleus, the cell's "control center," contains DNA and is the site of replication, transcription, and regulatory processes that dictate cellular function and identity. Within the nucleus, the nucleolus stands out as a prominent sub-nuclear structure, primarily responsible for ribosome biogenesis, a process crucial for protein synthesis and cell growth. While the nucleolus is increasingly recognized as a multifunctional hub involved in stress responses, cell cycle regulation, and various aspects of cellular metabolism, its isolation from the nucleus remains a significant challenge.

Submicron-scale photolabeling enables spatially-resolved proteomic analysis of nuclear and nucleolar structures

Fueled by recent advancements in high-resolution mass spectrometry and bioinformatics, proteomics has now reached unprecedented resolution and sensitivity, enabling comprehensive mapping of the nuclear proteomes, revealing the dynamic interplay of proteins. Techniques like subcellular fractionation, affinity purification, and proximity labeling have been refined to isolate and characterize proteins from these compartments[4-6]. While these methods effectively validate known high-abundance proteins, they face challenges in achieving the high sensitivity and discover low-abundance proteins.

Addressing these challenges, the Microscoop™ platform is specifically designed to study subcellular structures, including the nucleus and nucleolus, with high precision through photolabeling at the submicron-scale. It incorporates a motorized epifluorescence microscope, a high-resolution sCMOS camera, and a two-photon light source (Fig. 1), all enhanced by a photochemical probe for precise targeting and isolation of nuclear components for proteomic analysis. This technology follows a workflow involving several steps executed millions of times: first, the microscopy identifies nuclear structures within the sample. Next, images are captured and processed to eliminate background noise. Subsequently, the system employs pattern recognition techniques to identify specific nuclear structures, followed by targeted illumination within these structures for photochemical labeling.

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Finally, the system transitions to the next Field of View (FOV) (Fig. 2), repeating the iterative process crucial for spatially isolating proteins within the nucleus and nucleolus.

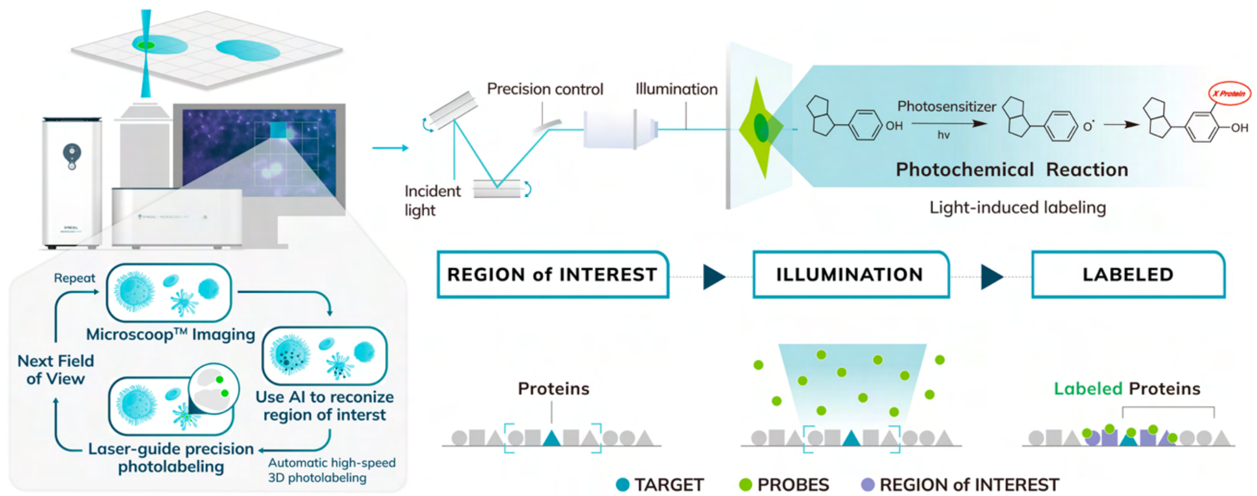


Fig. 1 | The Microscoop™ platform for submicron-scale photolabeling. The system integrates a motorized epifluorescence microscope, a high-resolution sCMOS camera, and a two-photon light source, enabling precise control of all components. Sub-millisecond temporal precision is achieved through automated imaging and intelligent labeling, facilitating high-speed, high-resolution spatial photolabeling of nuclear and nucleolar structures.

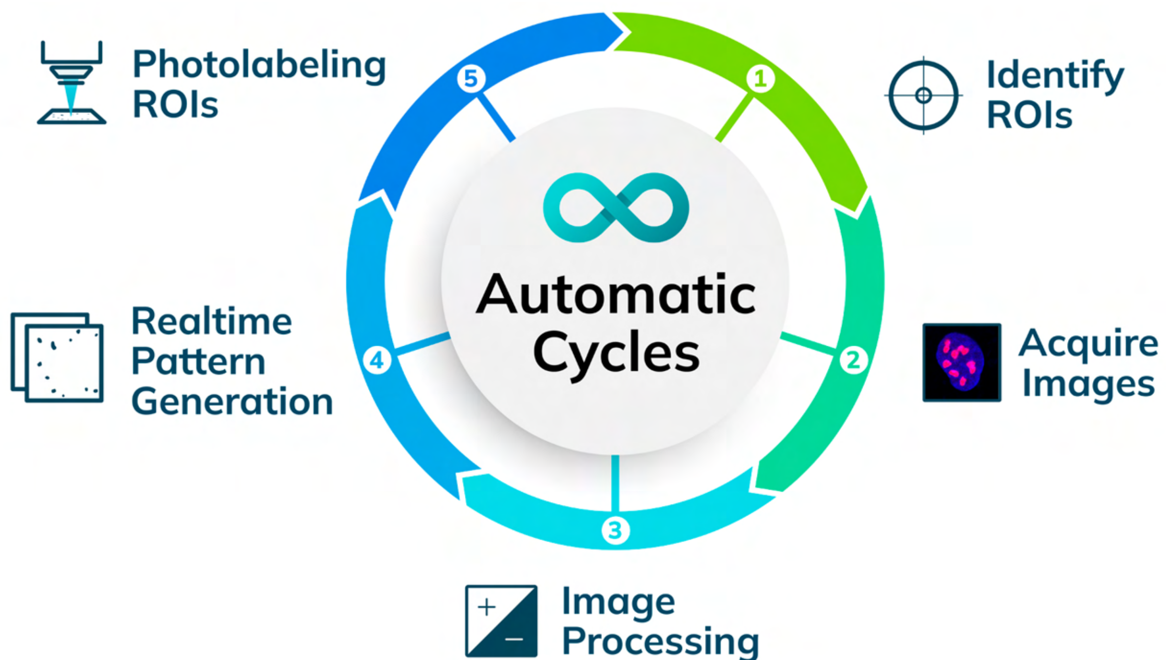


Fig. 2 | Workflow for ultrahigh-content targeted photo-biotinylation includes: (1) identifying and acquiring images of regions of interest by light microscope; (2) generating real-time patterns of ROIs; (3) illuminating the selected region within ROIs for protein photo-biotinylation; (4) moving the stage to the next FOV; and repeating steps 1-4 for each FOV until all FOVs have been processed.

Microscoop™ platform demonstrates broad applicability for subcellular biotinylation

To achieve efficient subcellular protein isolation, the labeling resolution needs to be at the submicron scale. The Microscoop™ platform utilizes high-precision photolabeling to achieve this, as demonstrated on U-2OS cells. By illuminating single lines defined by the movement of the femtosecond (fs) laser spot, we achieved a remarkable labeling resolution of 240 nm using a 40x objective (Fig.3). The versatility of the Microscoop™ platform was further demonstrated by photolabeling proteins at five distinct subcellular locations: nuclei, nucleoli, nuclear pore complexes, stress granules, and Golgi apparatuses. Image segmentation based on the characteristics of each structure allowed for targeted illumination and biotinylation of the desired region. The high spatial specificity of this technique was confirmed by the close match between the *in situ* biotinylated regions and the corresponding subcellular structures in both lateral (xy) and axial (z) directions (Fig. 4).

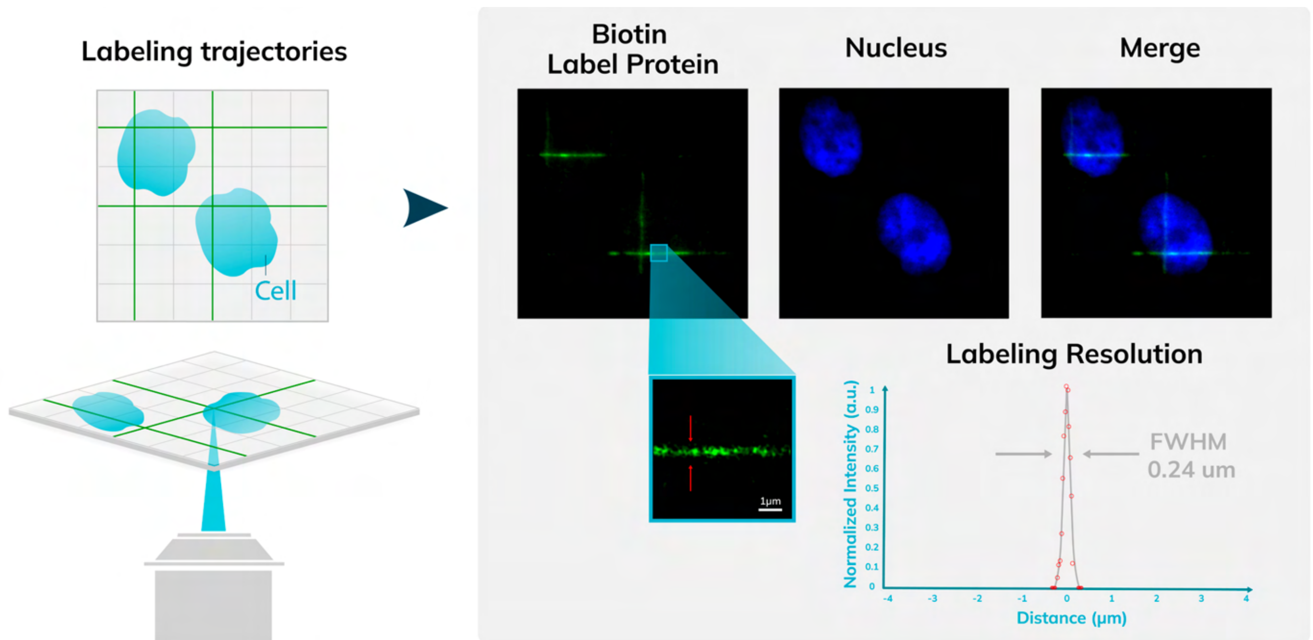
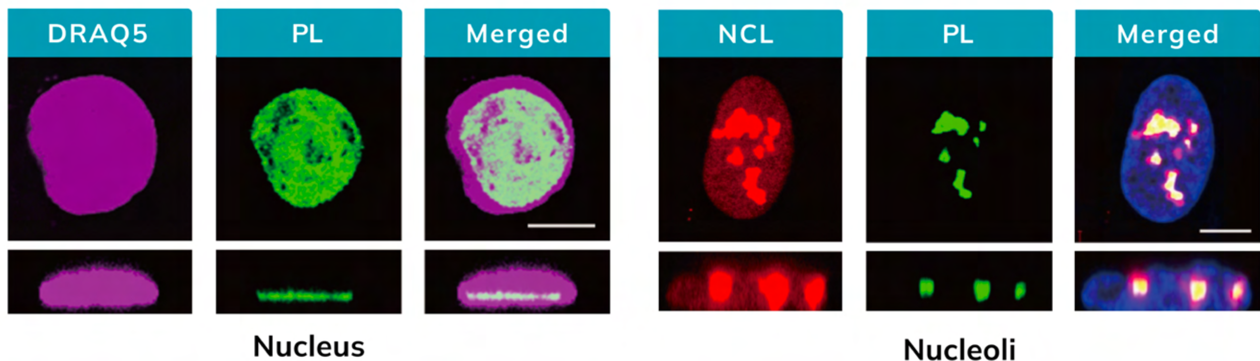


Fig. 3 | Resolution of photo-biotinylation. A line "cross" pattern is photolabeled on fixed U-2OS cells, and the biotinylated molecules are shown in green. DAPI: Blue, scale bar: 10 µm. 40x/0.95 NA objective.



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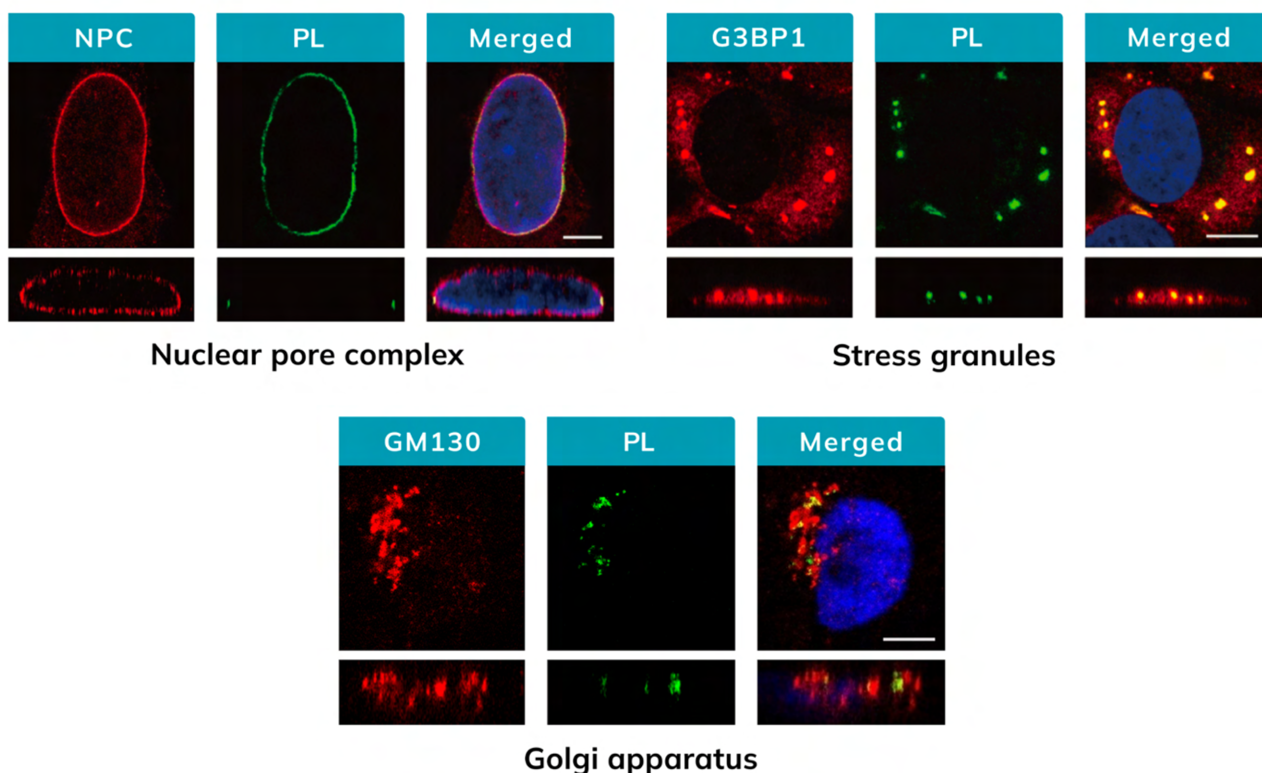

Fig. 4 | Photo-biotinylation in xy (top view) and z (side view) directions of various subcellular structures, visualized by confocal images. The ROIs are stained with Alexa Fluor 568 secondary antibody (red), the photolabeled signals are shown with Dy488-NeutrAvidin (green), while the nuclear stains DRAQ5 and DAPI are indicated in magenta and blue, respectively.

Microscoop™ Enables High-Sensitivity and High-Specificity Subcellular Spatial Proteomics

Protein spatial purification, facilitated by the Microscoop™ platform, unlocks a powerful approach for subcellular spatial proteomics with exceptional sensitivity and specificity. To demonstrate this, we targeted and photolabeled nuclear proteins in fixed U-2OS cells using DRAQ5, a far-red fluorescent DNA stain. After 16 hours of targeted photolabeling, the cells were harvested and lysed, followed by biotinylated protein enrichment through streptavidin bead pulldown and tryptic digestion for LC-MS/MS analysis (Fig. 5A). Dot blot analysis confirmed the successful photo-biotinylation of the sample, as biotinylated proteins were enriched in both the protein lysate and streptavidin bead pulldown fractions only where photolabeling illumination was applied (Fig. 5B). Additionally, the absence of the internal control α-tubulin in the pulldown fraction further validated the efficiency of nuclear protein isolation.

Photolabeled nuclei were harvested for LC-MS/MS analysis (Fig. 5C), identifying 4,820 proteins with high confidence. The distribution of protein abundances was analyzed based on the ratio of copies in photolabeled (PL) samples compared to control (CTL) samples, denoted as PL/CTL ratio (Fig. 5D). The specificity of the spatially labeled nuclear proteome was evaluated by determining the percentage of true positive proteins known as nuclear proteins among the obtained proteomes. Notably, out of the 1,316 proteins that showed differential enrichment, 1,207 were annotated as nuclear proteins, reflecting a 92% true positive rate compared to the existing nuclear protein database [7,8]. Notably, the distribution of protein abundance revealed that over 10% of the identified nuclear proteins were low-

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abundance, with less than 10,000 copies per cell (Fig 5E). This highlights the remarkable ability of Microscoop™ to detect low-abundance proteins, crucial for discovering novel biomarkers.

Furthermore, CORUM complex analysis using differentially enriched proteins as seed proteins revealed the presence of over-represented protein complexes within the targeted nuclear region, such as the spliceosome, histone complexes, and RNA polymerase complexes (Fig. 5F). This demonstrates that our systems not only identifies site-specific proteins but also reveals spatially related protein complexes, providing valuable insights into cellular organization and function.

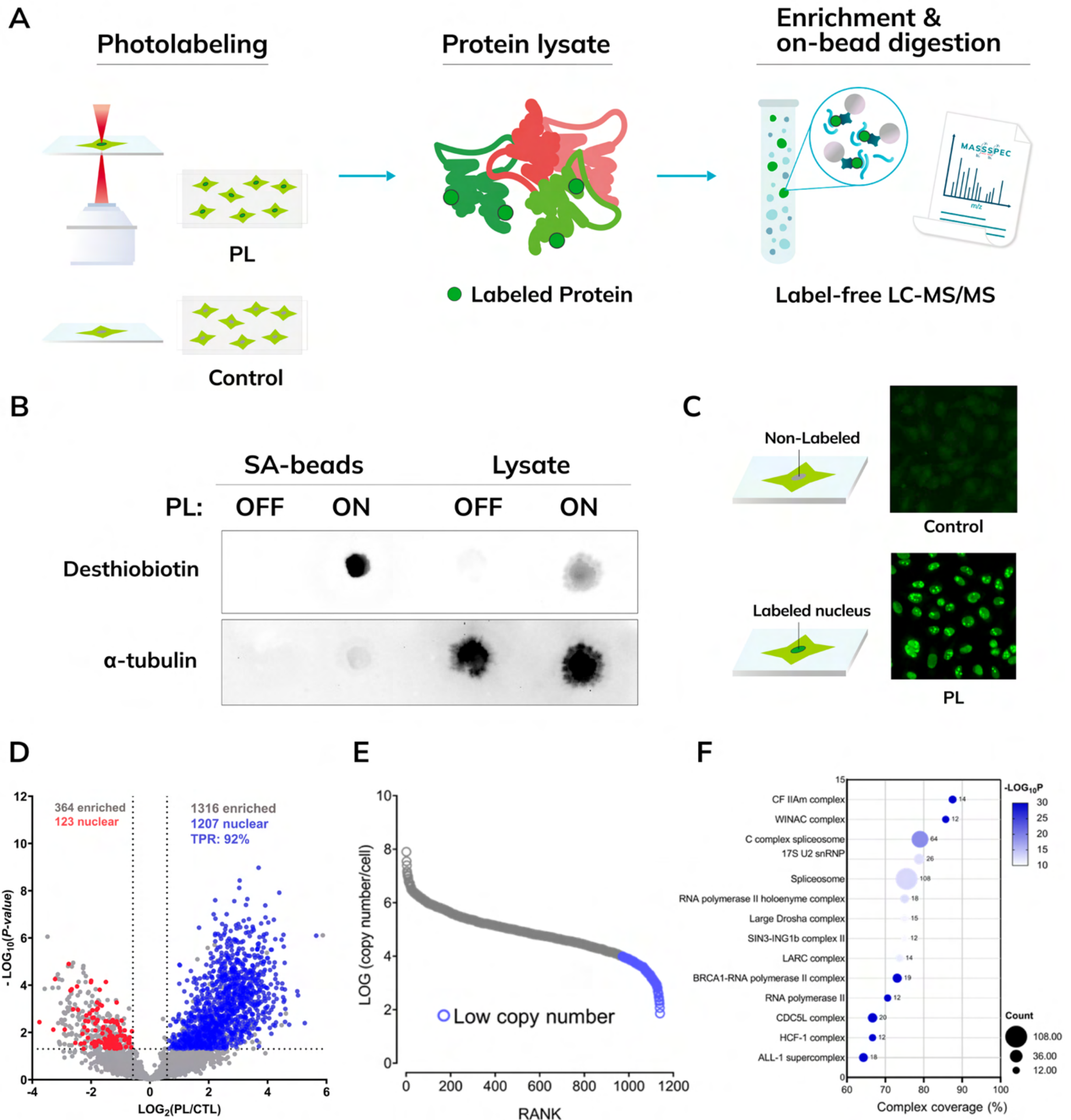

Fig. 5 | Subcellular proteomics by spatial purification. (A) Steps of proteomic profiling after targeted photo-biotinylation. Both photolabeled (PL: green) cells and control cells (without illumination: (gray) are lysed, enriched using streptavidin

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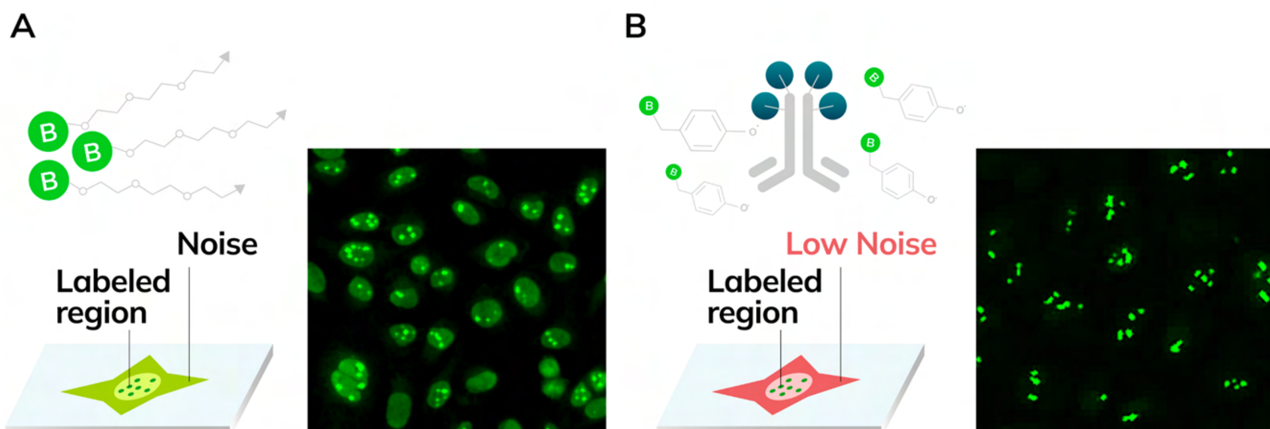
beads, and then digested with trypsin prior to LC-MS/MS measurement. (B) Effective protein photo-biotinylation as demonstrated by the dot-blot assay, showing desthiobiotin signals in photolabeled (ON) cells but not in control cells (OFF). (C) Photolabled nuclei were harvest for LC-MS/MS analysis. (D) High specificity of nuclear proteins obtained through spatial purification with targeted photo-biotinylation. A distribution of overall protein abundances is binned by the ratio of copies in a photolabeled (PL) sample to those in a control (CTL) sample annotated as PL/CTL ratio. High true positive rate of nuclear proteins in the PL enriched group (blue) compared to the CTL sample. (E) Distribution of protein copy numbers, with the those having a low copy number ones shown in blue (< 10,000 copy number per cell). (F) CORUM analysis of protein complexes, revealing major nuclear associated complexes.

High-precision nucleolar proteomics with antibody-conjugated photosensitizers

To demonstrate the versatility of the Microscoop™ platform for even smaller subcellular targets, we focused on nucleoli, organelles within the nucleus ranging from 0.5 to 3.5 μm. When using free-diffusing photosensitizers for labeling such tiny structures, the diffusion of the photosensitizer slightly expanded the biotinylated area, compromising the specificity of the proteomic analysis (Fig. 6A). To overcome this limitation, we employed antibody-conjugated photosensitizers. These photosensitizers were specifically targeted to the nucleoli using an anti-nucleolin (NCL) primary antibody and DBP in the media (Fig. 4B). This approach allowed for high-precision biotinylation confined to the nucleoli, as demonstrated by the low background labeling even within the densely packed nuclear environment (Fig. 4C). This highlights the broad applicability of antibody-conjugated photosensitizers for subcellular affinity labeling.

Mass spectrometry analysis of the photolabeled nucleoli revealed 3,162 proteins with high confidence. The distribution of protein abundances based on the ratio of photolabeled nucleoli (PL) to control samples (CTL) was plotted (Fig. 7A). The specificity of the spatially labeled nucleolar proteome was further confirmed by ranking the proteins based on the PL/CTL ratio. Notably, within the top 200 ranked proteins, 180 were annotated as nucleolar proteins, resulting in a 90% true positive rate compared to the known nucleolar proteome [7-11](Fig.7B). This demonstrates the effectiveness of antibody-conjugated photosensitizers in achieving high-precision subcellular proteomics for smaller targets like nucleoli. Cellular component analysis using differentially enriched proteins as seed proteins revealed the presence of over-represented proteins within the nucleolar compartment, such as nucleolus, ribonucleoprotein complex, and chromosome (Fig. 7C). This high-precision approach enables the selective isolation and analysis of nucleolar proteins, previously challenging due to their close proximity to other nuclear structures, offering valuable insights into their unique functions and contributions to cellular processes.



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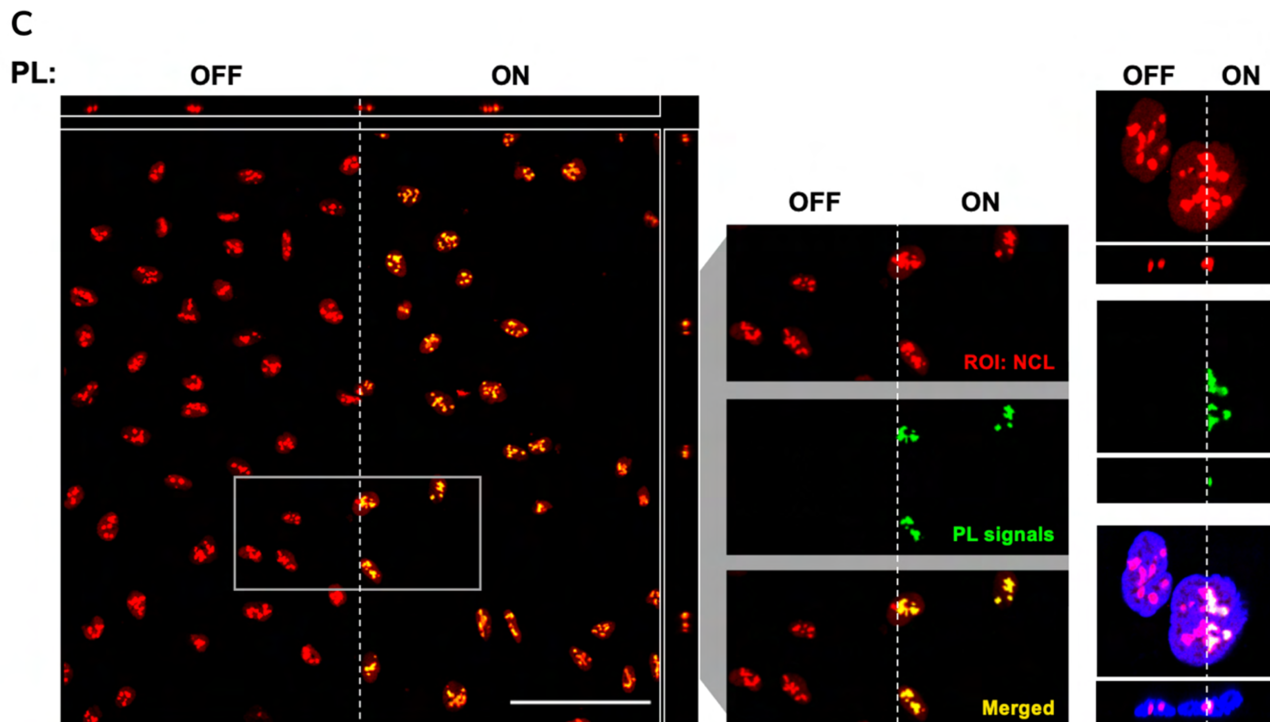

Fig. 6 | (A)(B) Method comparison using a free photoactive probe (A) and an antibody-conjugated photoactive probe (B). Non-specific biotinylation is reduced when using the antibody-conjugated photoactive probe (B) for photolabeling small compartment (nucleoli: 0.5-3.5 μm). (C) Precision photo-biotinylation using the antibody-conjugated probe. Confocal images show photolabeled (ON) and non-labeled (OFF) regions of interest (ROIs) within nucleoli (red), showing precise labeling in xy and z directions using the antibody-conjugated photoactive probe. Nucleus (DAPI: blue), nucleoli (NCL: red), and photo-biotinylation (Dy488-NeutrAvidin: green). Scale bar: 10 μm.

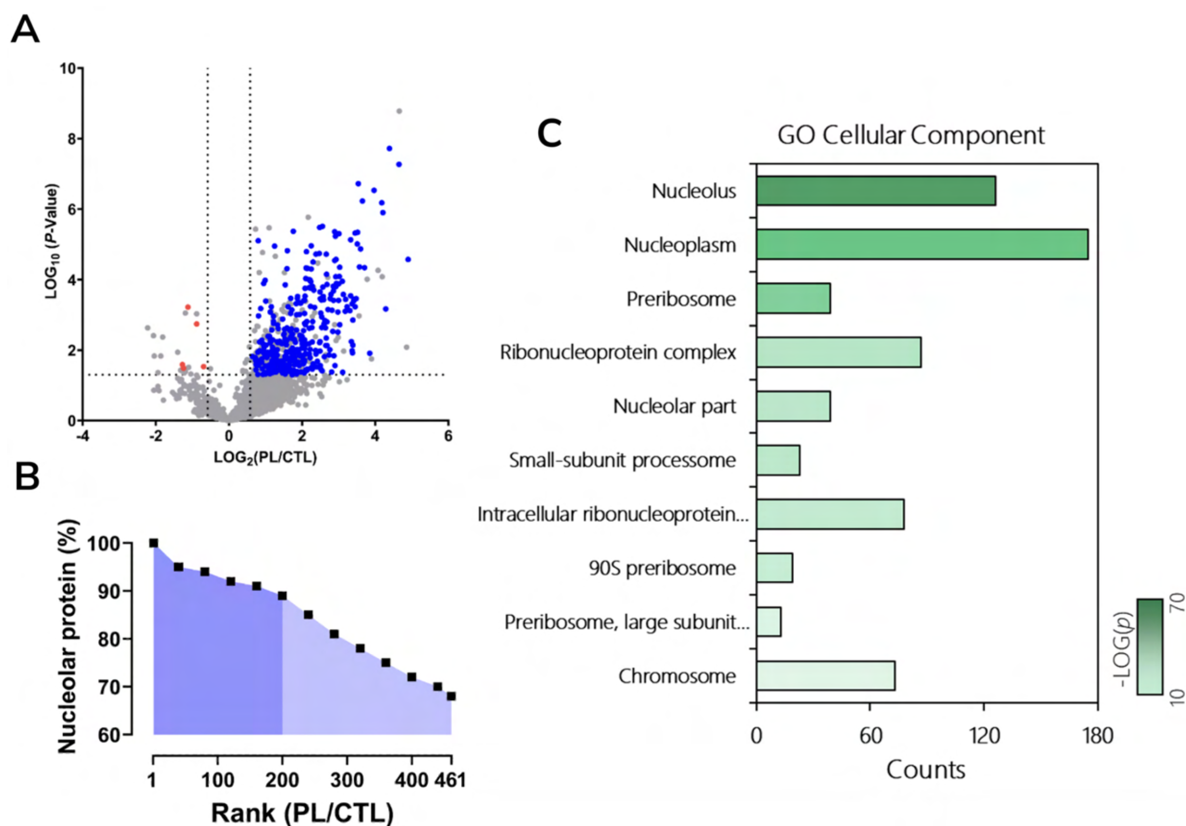

Fig. 6 | (A) Distribution of overall protein abundance according to the PL/CTL ratio for the nucleolar proteome study. (B) The true positive rate of nucleolar proteins against the rank of PL/CTL for enriched proteins. (C) Gene ontology analysis of nucleolar proteome.

Conclusion

The Microscoop™ platform represents a significant advancement in subcellular proteomics, particularly for studying the nucleus and nucleolus. This innovative technology offers unmatched precision and detail in spatial photolabeling, enabling researchers to uncover valuable information about cellular processes and disease mechanisms. Microscoop™ allows researchers to map intricate protein networks in these subcellular structures, providing insights into cellular mechanisms and disease development. It overcomes traditional limitations by detecting low-abundance proteins, crucial for understanding cellular processes and identifying potential biomarkers. By revealing the spatial organization of protein complexes, Microscoop™ enhances our understanding of cellular functions, achieving high-precision photolabeling even in tiny structures like nucleoli. Its high-throughput capability enables extensive and rapid analysis, deepening biological insights from spatial proteomic studies.

The application of Microscoop™ has led to discover in nuclear and nucleolar proteomics, identifying novel proteins and expanding our understanding of these critical subcellular structures. By mapping protein interactions, Microscoop™ has elucidated how proteins regulate gene expression, a fundamental cellular process. Studying changes in nuclear and nucleolar proteomes associated with diseases like cancer and neurodegenerative disorders provides valuable insights into disease mechanisms and informs new therapeutic strategies, potentially leading to innovative treatments. The future of spatial studies with Microscoop™ is promising, especially with advancements in disease models. It has great potential to enhance our understanding of nuclear dynamics in health and disease, leading to significant discoveries in biomedical science.

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