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WHITE PAPER

Unveiling Primary Ciliary Proteins with Microscoop™

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Authors

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Introduction

Primary cilia are microtubule-based organelles extending from the surface of most mammalian cells, playing pivotal roles in signal transduction and cellular functions[1]. Malfunctions of primary cilia are implicated in a number of genetic disorders termed ciliopathies, including polycystic kidney disease[2], Bardet-Biedl syndrome[3], and Joubert syndrome[4]. These conditions adversely affect organs such as the kidneys, retina, and brain, influencing key biological processes like cell cycle regulation and tissue development. The study of primary cilia, despite their significance, is filled with challenges. The organelles' diminutive size and structural delicacy significantly complicate their analysis. Additionally, the dynamic nature of their assembly further complicates their study, necessitating high-resolution techniques for detailed analysis.

Addressing these challenges, the Microscoop™ platform emerges as an advancement in biological research technology, particularly enhancing the capability for spatial photolabeling at the microscale. It enables the in-depth investigation of primary cilia, providing the necessary precision and detail to comprehensively explore their biological function. With the potential to transform our understanding of ciliary functions in both health and disease, its application in proteomic analysis demonstrates its utility in unraveling the complex protein constitutes of primary cilia, essential for elucidating cellular mechanisms and pathogenesis.

Capturing and analyzing primary cilia with Microscoop™

The Microscoop™ platform is designed for the isolation and identification of proteins within submicron cellular regions, specifically aiming to target and analyze subcellular organelles such as primary cilia. It combines a motorized epifluorescence microscope, a high-resolution sCMOS camera, and a two-photon light source. This system is further enhanced by a specially developed photochemical probe (Fig. 1), allowing for precise targeting and isolation of primary cilia for proteomic analysis. To facilitate visualization, primary cilia are pre-stained with the well-known marker polyglutamylation modification (GT335). Real-time image analysis is employed to segment the primary cilia and filter out non-specific signals. This ensures efficient segmentation of each Field of View (FOV) image for identifying primary cilia, thereby enabling effective path planning and labeling control. Through the use of two-photon illumination, the system triggers photochemical agents to photo-biotinylate proteins within the primary cilia. The Microscoop™ platform controls the sequential photo-biotinylation of individual primary cilia via mechatronic position control, processing millions of primary cilia to collect sufficient proteins for downstream proteomic analysis. The photo-biotinylated proteins are then subjected to streptavidin pull-down, followed by hypothesis-free mass spectrometry (LC-MS/MS) analysis, facilitating a comprehensive proteomics discovery. By integrating ultrahigh-content, high-speed microscopy with targeted photo-biotinylation, the Microscoop™ platform revolutionizes the study of primary cilia, enabling spatial isolation for proteomics discovery.

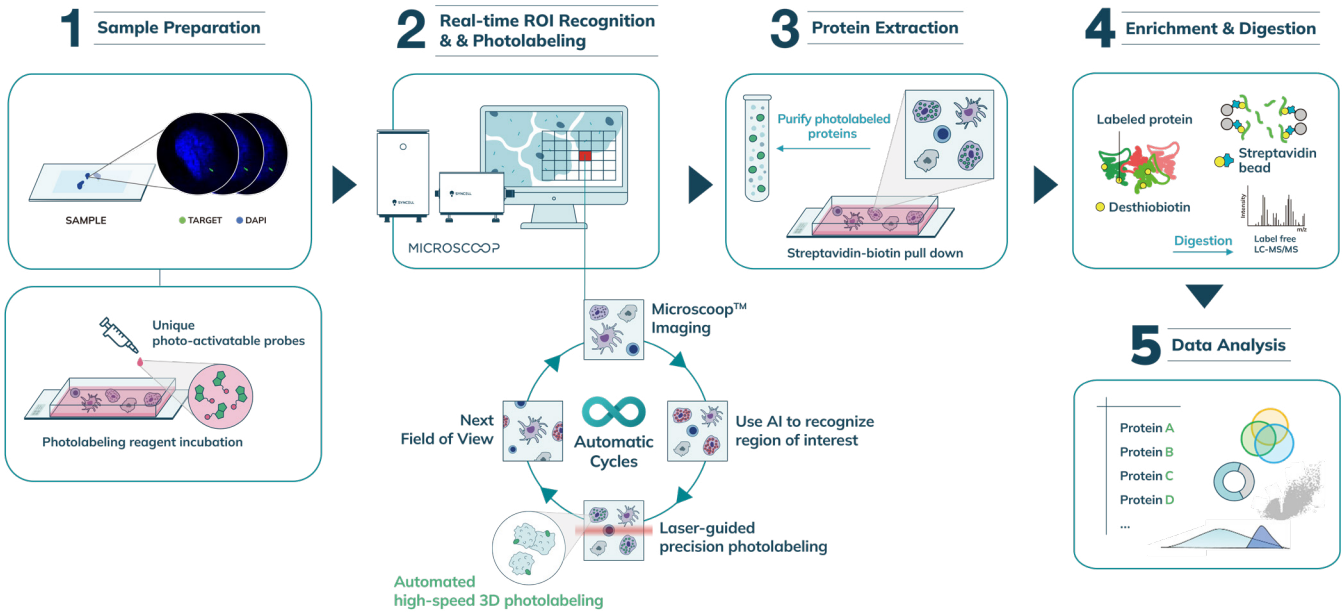

Fig. 1 | Schematic workflow for mapping the primary cilia proteome. A microscopy-guided protein discovery platform integrates image acquisition, photochemistry, microscopy, optics, and mechatronics enables ultrahigh-content *in situ* photolabeling followed by mass spectrometry analysis.

Microscoop™ has been engineered to enable photolabeling at the subcellular level, targeting ROIs such as primary cilia, which are presumed to have uniform protein constituents identifiable by distinct morphological features and contrast under microscopy. This method involves several steps executed millions of times: 1) employing microscopy to identify primary cilia; 2) capturing images; 3) processing images to eliminate background noise; 4) recognizing primary cilia patterns; 5) illuminating within primary cilia for photochemical labeling; 6) transitioning to the next FOV (Fig. 2). This repetitive process is crucial for spatially isolating proteins, thereby gathering enough proteins to address the challenge of protein amplification. Remarkably, existing technologies lack the capability for such extensive and rapid repetition across locations and timespans.

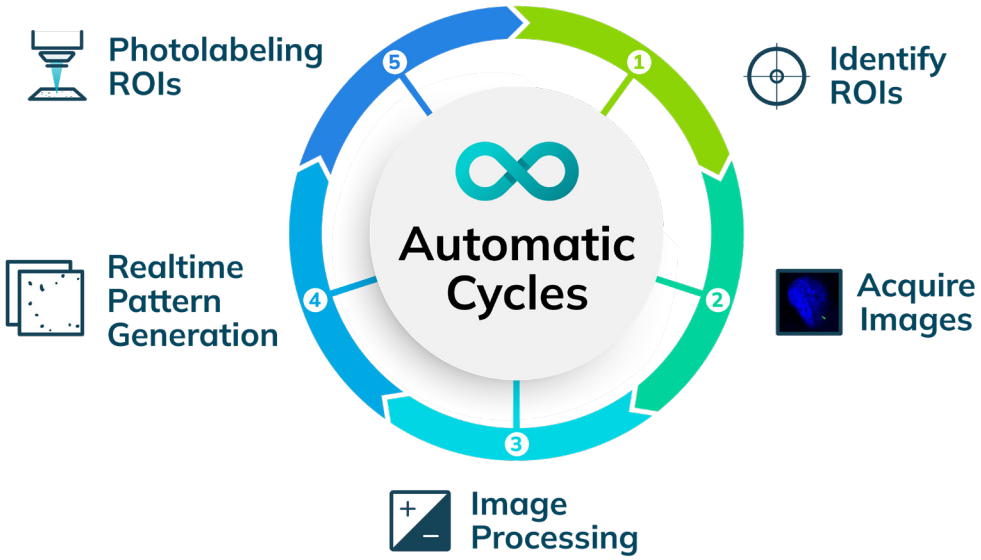

Fig. 2 | The process and design of the microscopy-guided protein isolation platform. The process includes: (1) identifying the primary cilia by light microscopy; (2) acquiring images of primary cilia; (3) processing images to identify primary cilia as ROIs; (4)

generating realtime patterns of ROIs; (5) illuminating the selected region within ROIs for protein photo-biotinylation; (6) moving the stage to the next FOV; and repeating steps 1-5 for each FOV until all FOVs of interest have been processed.

The images are processed in real-time to segment the primary cilia using image processing techniques, including thresholding, filtering, size and length exclusion, and morphological recognition. These steps are uniformly applied across all FOVs, with pre-processing or post-processing adjustments to ensure consistent image quality. The segmentation results, depicted in Figure 3, require 0.1 to 1 second for completion, varying with the image's complexity and quality. Following segmentation, the coordinates of the primary cilia's grid points are determined. A planned path for photochemical activation is then optimized and used to guide the galvanometers (galvos) across these points. The galvos and the Acousto-Optic Modulator (AOM) synchronize to within approximately 100 microseconds, enabling precise control over the locations and duration, thus ensuring a consistent photochemical reaction across all spots. For locations with multiple primary cilia, the scanning path sequentially targets each cilium, initiating at the periphery and spiraling clockwise towards the center before proceeding to the next cilium's starting point. This method reduces travel time and minimizes mechanical stress on the galvos. Achieving this level of intricacy within a feasible timeframe relies on speed optimization, seamless automation, and accurate mechatronic control throughout the process.

To validate the labeling accuracy and precision for primary cilia as small as 0.2 μm in width and 1 μm in length, primary cilia of RPE1 cells were pre-stained with GT335, followed by photo-biotinylation using Microscoop™. The platform accurately recognized primary cilia, enabling targeted spatial photolabeling with two-photon illumination. This method facilitated the isolation of primary cilia proteins with high specificity, as demonstrated by the congruence between the *in situ* biotinylated regions (green) and the primary cilia (red) fluorescence in both lateral (xy) and axial (z) directions (Fig. 3, right panel).

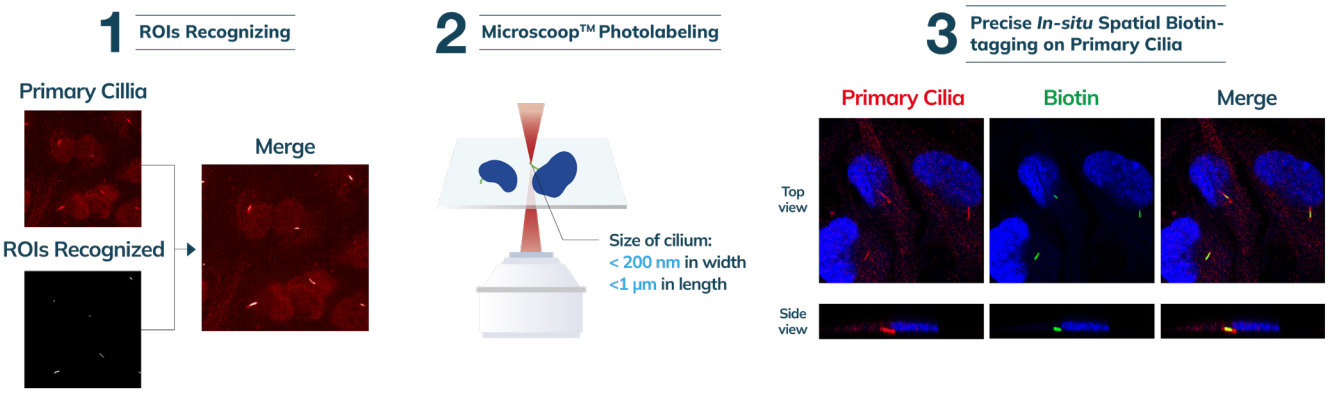

Fig. 3| Primary cilia are processed by filtering and segmentation by image processing (left), Confocal micrographs depicting precise and accurate photolabeled primary cilia at lateral (xy)- and axial (z) directions (right). Red: GT335, Green: NeutrAvidin-488, Blue: DAPI.

Unveiling ciliary proteins and functional insights

Photolabeling experiments were conducted on PFA fixed RPE-1 cells, specifically targeting regions marked with GT335 to elucidate the protein composition of primary cilia (Fig. 4A). Following hours of precise photolabeling, cells were harvested and lysed to extract proteins. The photolabeled proteins were subsequently enriched through streptavidin bead pulldown, digested with trypsin, and analyzed via LC-MS/MS. This comprehensive analysis resulted in the high-confidence identification of 4,233 proteins.

Notably, 524 ciliary proteins were distinctly marked in red on the plot, showing significant enrichment in the photolabeled group, indicative of their specific association with primary cilia (Fig. 4B). Among the identified proteins, key ciliary trafficking components such as intraflagellar transport proteins (IFTs), kinesins, dyneins, GTPases, and phosphatidylinositol phosphates (PIPs) were enriched, exhibiting high photolabeling to control (PL/CTL) ratios (Fig. 4C). Additionally, proteins involved in structural support and cellular organization, including microtubules, septins, and annexins, were also observed to be enriched within the photolabeled sample.

Gene ontology (GO) enrichment analysis further validated these findings, demonstrating a significant association of the enriched proteins with critical biological processes such as ciliary assembly, transportation, and signaling, underpinning the complex functionality of the ciliary proteome (Fig. 4D). Notably, proteins involved in intraciliary transport involved in cilium assembly, a key process in cilium assembly, were significantly overrepresented in the GO category specifically related to cilium assembly. These findings support the effectiveness of targeted photolabeling and proteomic analysis in revealing the network of proteins essential for ciliary function and structure.

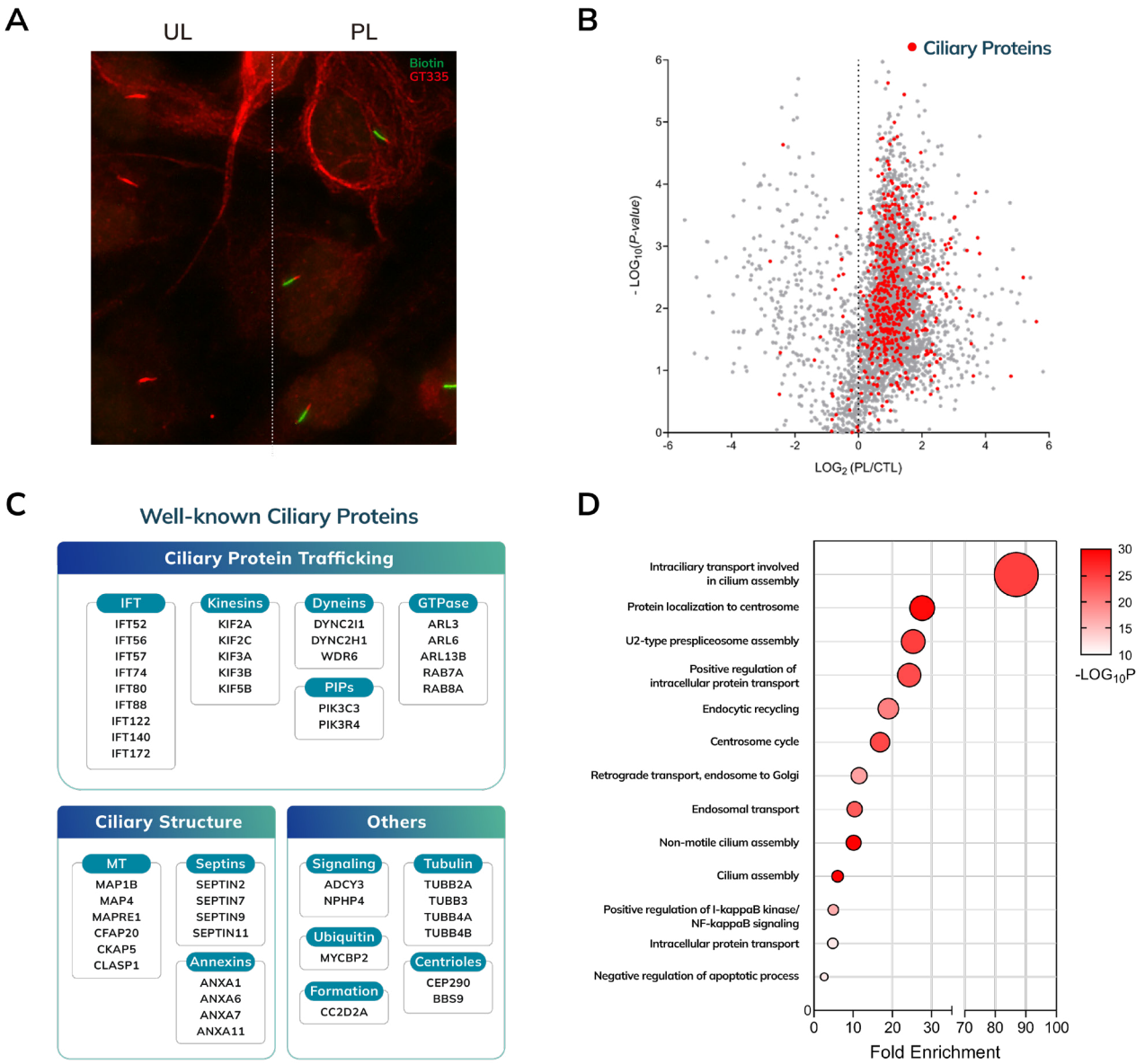

Fig. 4 | A, Confocal micrographs of unphotolabeled (UL) and photolabeled (PL) at user defined primary cilia. B, A distribution of overall protein abundances according to the PL/CTL ratio for the ciliary proteome. Ciliary proteins (red) are enriched in the PL

group compared to the CTL sample. C, Well-known cilia proteins identified by Microscoop™. D, The top 100 enriched proteins were subjected to Gene ontology to reveal cilia related biological process.

Conclusion

In this white paper, we present a comprehensive exploration of primary cilia, aiming to deepen our understanding of their composition and biological roles. Our research initiated with the precise targeting and labeling of primary cilia utilizing the Microscoop™ technology, renowned for its accuracy and specificity in photo-biotinylation within primary cilia. This enabled the provision of a comprehensive list of protein candidates associated with primary cilia. In conclusion, our findings demonstrate that the Microscoop™ technology effectively enables spatially specific photolabeling of primary cilia, facilitating the identification of both known and novel ciliary proteins and thus enhancing our understanding of this essential cellular component.

To propel cilia research forward, access to reliable and comprehensive proteomics databases is essential. The currently available open-source cilia databases are fragmented and exhibit inconsistencies, marked by variations in data quality and differences across platforms. This situation, combined with the complexity and small size of primary cilia, presents significant challenges to conducting effective research in this field. In an effort to address these issues, we have collected data from several cilia databases, including Gene Ontology[5], UniProt[6], and CiliaCarta[7], into the SYNCELL database. The ciliary proteome from SYNCELL offers a comprehensive and consistent dataset of ciliary proteins, designed to address the current gaps in research. We aim to empower researchers with the necessary tools for advancing our understanding of ciliary functions and mechanisms.

References

1. Satir, P. & Christensen, S.T. Overview of structure and function of mammalian cilia. *Annu Rev Physiol* **69**, 377-400 (2007).
2. Brown, J.M. & Witman, G.B. Cilia and Diseases. *Bioscience* **64**, 1126-1137 (2014).
3. Tobin, J.L. & Beales, P.L. Bardet-Biedl syndrome: beyond the cilium. *Pediatr Nephrol* **22**, 926-936 (2007).
4. Cantagrel, V. et al. Mutations in the cilia gene ARL13B lead to the classical form of Joubert syndrome. *Am J Hum Genet* **83**, 170-179 (2008).
5. Ashburner, M. et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* **25**, 25-29 (2000).
6. UniProt, C. UniProt: the Universal Protein Knowledgebase in 2023. *Nucleic Acids Res 51*, D523-D531 (2023).
7. van Dam, T.J.P. et al. CiliaCarta: An integrated and validated compendium of ciliary genes. *PLoS One* **14**, e0216705 (2019).

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