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## Abstract

Image-guided protein extraction at organelle-scale resolution holds significant promise for discovering novel protein constituents within disease- or functionrelated subcellular regions like primary cilia. Our firmware-integrated microscopy platform facilitates spatial protein purification through in situ subcellular photo-biotinylation at user-defined regions of interest (ROIs) one field of view (FOV) at a time, automatically processing thousands of FOVs. Illumination patterns of the ROI for each FOV are calculated in real-time using machine learning or traditional image processing. Light activation of amino acid crosslinkers is achieved by a two-photon laser in the platform, rendering precise protein biotinylation with 300-nanometer precision. A high-speed mechatronic control is implemented to coordinate imaging, pattern generation, targeted illumination, and FOV movement, allowing for the rapid biotinylation of millions of ROI spots within hours in cell or tissue samples. Once enough proteins are biotinylated, subsequent cell lysis, avidin pulldown and LC-MS/MS analysis unveil the subcellular proteome with exceptional sensitivity, specificity, and resolution. Using this technology, termed optoproteomics, we investigated the proteome of primary cilia in RPE-1 cells, identifying the proteome including 524 known ciliary proteins notably enriched. The top identified proteins encompassed key ciliary trafficking components and those involved in structural support and cellular organization. Gene ontology (GO) enrichment analysis highlighted the significant association of high-ranking proteins with critical ciliary processes such as assembly, transportation, and signaling, particularly including proteins involved in intraciliary transport. A group of novel protein constituents were identified, providing testable hypotheses for their roles in primary cilia. These findings underscore the efficacy of targeted photolabeling and proteomic analysis in unraveling the network of proteins essential for ciliary function and structure, showcasing optoproteomics' potential for comprehensive subcellular spatial proteome discovery and its broad utility in cell biology for discovering novel protein compositions or biomarkers.



Fig. 1 | Schematic workflow of SYNCELL Microscoop<sup>®</sup>. A total-sync ultra-content microscopic platform that integrates image acquisition, photochemistry, microscopy, optics, and FPGA-based mechatronics enable high-content in situ photolabeling followed by mass spectrometry analysis.



Fig. 2 | A, Workflow for ultrahigh-content targeted pohoto-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. B, 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.

# Subcellular spatial proteomics by microscopy-guided photo-biotinylation reveals novel protein constituents in primary cilia



# **Microscoop<sup>®</sup> : Photo-induced biotinylation within primary cilia**





ACTN4	ATP6V1A	CEP135	CROCC	DYNC1I2	GAK	IFIT3	KIFC1	NEK9
ACTR1A	ATP6V1D	CEP152	CSNK1A1	DYNC2H1	GANAB	IFT122	KIRREL1	NIN
ACTR2	ATXN10	CEP164	CSNK2B	DYNC2I1	GLE1	IFT140	LDHB	NME7
ACTR3	AURKA	CEP170	CTNNA1	EEF1A1	GLOD4	IFT172	LRBA	NOL6
ADD3	AXL	CEP192	CTNNB1	EFCAB7	GLRX3	IFT27	MACF1	NPHP
AKAP9	BBS1	CEP250	CTTN	EFTUD2	GMDS	IFT52	MAP1A	NUDC
AKT1	BBS2	CEP290	CUL3	EGFR	GNA11	IFT56	MAP1B	NUP21
ANKMY2	BBS4	CEP350	CYFIP2	EHD3	GOT2	IFT57	MAP1S	NUP21
ANXA11	BRI3BP	CEP43	CYLD	EIF2A	GRK2	IFT70A	MAP4	NUP3
ANXA6	C2CD3	CEP57	DCTN1	EIF2S1	GSK3B	IFT74	MAP4K3	NUP6
AP2A1	CALR	CEP63	DCTN2	EIF3A	GSN	IFT80	MAPKAP1	NUP9
AP3M2	CALU	CEP76	DCTN3	EIF4B	HAT1	IFT88	MAPKBP1	OCRL
APEX1	CAMK2D	CEP78	DCTN4	EIF4H	HAUS1	INPPL1	MAPRE1	ODF2
APP	CAMSAP1	CEP89	DDX1	EIF5A	HAUS3	INTS1	MDM1	OFD1
APPL1	CAPN2	CEP97	DDX21	EIPR1	HAUS4	INTS2	MED16	OGFF
ARF4	CAPZB	CFAP20	DDX56	ELMO2	HAUS5	IPO5	MICAL3	ORC1
ARHGAP1	CARS1	CHD4	DHX30	ELP4	HAUS6	IQCE	MLEC	P4HA
ARHGAP35	CASK	CHORDC1	DHX9	EML1	HDAC1	ITGA3	MROH2B	PACS
ARHGEF18	CAV1	СІТ	DLG5	EPB41L3	HHIP	KATNAL1	MSN	PAFAH1
ARL3	CC2D2A	CKAP2	DMD	EPS15	HK1	KATNAL2	MTCL1	PAPSS
ARMC9	CCDC61	CKAP5	DNAAF5	EPS8L2	HMGB2	KATNB1	MXRA8	PCM1
ASAP1	CCDC88A	CLASP1	DNAH11	ERC1	HNRNPLL	KDM3B	MYADM	PCNT
ASNS	CCP110	CLIC1	DNAJA1	EXOC3	HSP90AB1	KHSRP	MYH10	PDCD6
ATG16L1	CCT2	CLTB	DNAJB1	EXOC4	HSP90B1	KIF1B	MYO1E	PDE10
ATCR	CCT4	CLTC	DNM2	EXOC5	HSPA4	KIF20A	MY05A	PDGFR

ł	Gene name	Protein description	Gene name	Pre
-	PPIB	Peptidyl-prolyl cis-trans isomerase B	CD2AP	CD2
	ALDH1A3	Retinaldehyde dehydrogenase 3	NUP98	Nuc
	CAVIN1	Caveolae-associated protein 1	AP3B1	AP-
	SF3A3	Splicing factor 3A subunit 3	GOLGA4	Gold
	TRIM25	E3 ubiquitin/ISG15 ligase TRIM25	CNOT1	CCR
	AP2A2	AP-2 complex subunit alpha-2	COPB1	Coa
	SRP72	Signal recognition particle subunit SRP72	NPM1	Nuc
	CTNND1	Catenin delta-1	SERPINH1	Serp
	MARS1	MethioninetRNA ligase, cytoplasmic		Uve
	HNRNPDL	Heterogeneous nuclear ribonucleoprotein D-like	UACA	anky
		Patatin-like phospholipase domain-containing	TKT	Trar
	TNILAU	protein 6	AARS1	Alar
	CPNE3	Copine-3	FLOT2	Floti
	EPHA2	Ephrin type-A receptor 2	TJP1	Tigh
	SUPT16H	FACT complex subunit SPT16	NXF1	Nuc
	RPS7	Small ribosomal subunit protein eS7	ARPC1B	Acti

Fig. 4 | A, Confocal micrographs of unphotolabeled (UL) and photolabeled (PL) at user defined primary cilia. B, 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. Ciliary proteins (red) are enriched in the PL group compared to the CTL sample. C, The top 100 enriched proteins were subjected to Gene ontology to reveal cilia related biological process. D, Well-known ciliary proteins identified by Microscoop<sup>®</sup>. E, 427 ciliary proteins significantly enriched by Microscoop<sup>®</sup>. F, The 427 enriched ciliary proteins were subjected to Reactome to reveal cilia related pathways. G, The ranking of the top 50 protein abundances (PL/CTL), where ciliary proteins are indicated in red and nonciliary proteins are indicated in grey. H, The list of the top 30 non-ciliary proteins (putative ciliary proteins) enriched by Microscoop<sup>®</sup>. I, The top 30 putative ciliary proteins (H) were subjected to Gene ontology to reveal cilia related biological process. J, Top 100 ranked proteins were subjected to STRING to reveal protein-protein interaction networks, where the 30 putative ciliary proteins (H) are indicated in red.

## **References:**

Microscopy-guided subcellular proteomic discovery by high-speed ultra-content photo-biotinylation. Chen et al. bioRxiv 2023.12.27.573388.

Precise In-situ Spatial Biotine Microscoop<sup>®</sup> Photolabeling **U** tagging on Primary Cilia **Primary Cilia Biotin** Merge 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 Size of cilium: (z) directions (right). Red: GT335, Green: < 200 nm in width NeutrAvidin-488, Blue: DAPI. <1 µm in length

# Summary

• An innovative platform that combines microscopy, deep learning, two-photon illumination, and mechatronics for advanced image-guided photo-biotinylation hypothesis-free proteomics. • Fast and precise photo-biotinylation of spatially specific proteins from hundreds of thousands of cells enhances the sensitivity of mass spectrometry. • In mapping the ciliary proteome, 427 known ciliary proteins were enriched, and the validation of previously unreported proteins in primary cilia is underway.





