Identification of the novel immune synapse-localized proteome for immuno-oncology using Microscoop® -induced targeted photo-biotinylation

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Abstract

Although numerous immune synapse (IS) protein species have been identified, many IS-localized protein species remain unknown. Understanding the proteome of the IS between a target cell and a lymphocyte is crucial for advancing immuno-oncology. However, the low abundance of ISs and the absence of a definitive enrichment marker have hindered efficient proteomic profiling. In this study, we utilized Microscoop®, an innovative system that integrates microscopy, machine learning, and photochemical labeling to enable precise and spatially specific enrichment of IS proteins, thereby facilitating proteomic discovery for the IS. We employed Raji B cells as antigen-presenting cells (APCs) and induced IS formation with Jurkat T cells. The system first employed immunofluorescence imaging of CD3, a common IS marker in Jurkat T cells, and utilized a convolutional neural network-based deep learning algorithm to recognize IS formation from CMTPX-stained Raji B cells. Our automated system successfully achieved spatially targeted biotin-tagging of proteins at ISs through multiple rounds of imaging, deep learning-driven pattern generation, and photochemical labeling. Subsequent streptavidin pull-down and mass spectrometry analysis enabled the identification of IS-specific proteins. Remarkably, our spatial proteomic approach led to the isolation and identification of hundreds of different species at the IS interface, including proteins associated with key components of T-cell receptor (TCR) signaling pathways such as the TCR/CD3 complex, Src and Tec family tyrosine kinases, and pivotal NF-kB signaling proteins. Additionally, we identified a significant enrichment of proteins not previously associated with the IS. Our study not only illuminates previously unknown aspects of immune regulation at the IS interface but also has significant implications for cancer research, particularly in understanding and manipulating immune responses for therapeutic purposes.

Microscoop®: Ultrahigh-content microscopy-guided photo-biotinylation platform

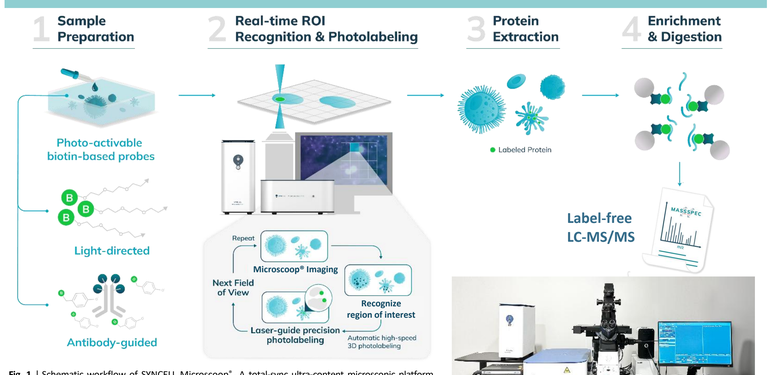


Fig. 1 | Schematic workflow of SYNCELL Microscoop®. A total-sync ultra-content microscopic platform that integrates image acquisition, photochemistry, microscopy, optics, and FPGA-based mechatronics enable high-content in situ photolabeling followed by mass spectrometry analysis.

Synchronized system for high-speed and accurate image masking

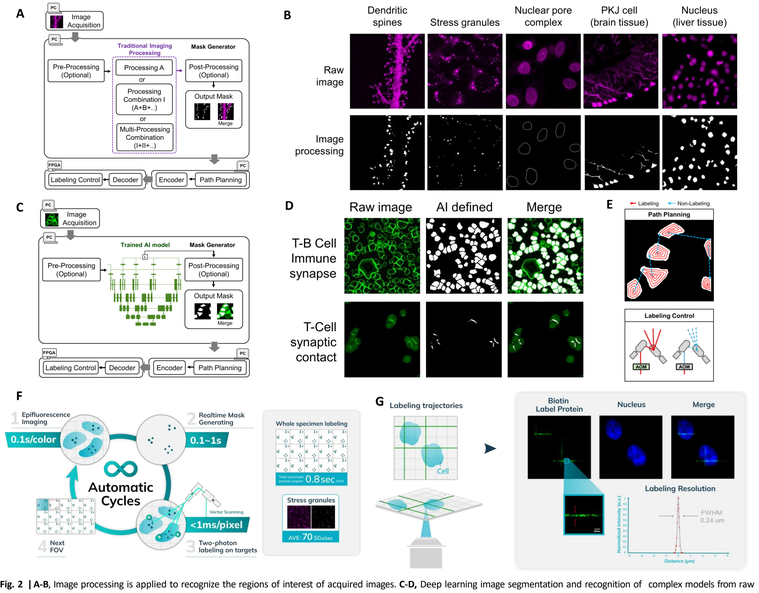
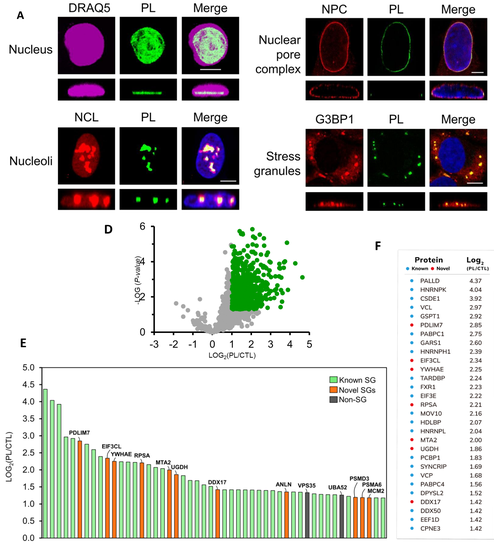


Fig. 2 | **A-B**, Image processing is applied to recognize the regions of interest of acquired images. **C-D**, Deep learning image segmentation and recognition of complex models from raw images. **E,** Algorithm generates the labeling path and the non-labeling path of an input mask, and labeling control of the galvanometer system and the AOM. **F,** Workflow for ultrahigh-content targeted photo-biotinylation includes: (1) identifying and acquiring images of regions of interest by light microscope; (2) generating realtime 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. **G,** 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.

Photo-induced spatial biotinlyation in subcellular compartments at a nanoscale resolution and discovery of novel proteins



Mapping the proteome of stress granules and discovery of novel biomarkers

Protein ● Known ● Novel	Log_2 (PL/CTL)
PALLD	4.37
HNRNPK	4.04
CSDE1	3.92
VCL	2.97
GSPT1	2.92
PDLIM7	2.85
PABPC1	2.75
GARS1	2.60
HNRNPH1	2.39
EIF3CL	2.34
YWHAE	2.25
TARDBP	2.24
FXR1	2.23
EIF3E	2.22
RPSA	2.21
MOV10	2.16
HDLBP	2.07
HNRNPL	2.04
MTA2	2.00
UGDH	1.86
PCBP1	1.83
SYNCRIP	1.69
VCP	1.68
PABPC4	1.56
DPYSL2	1.52
DDX17	1.42
DDX50	1.42
EEF1D	1.42
CPNE3	1.42
EIF3F	1.41
YWHAB	1.41
YWHAH	1.40
CLIC4	1.40
HNRNPM	1.39
HNRNPR	1.36
ANLN	1.35
EIF3D	1.35
EIF4G1	1.35
VPS35	1.33
CENPF	1.30
EIF4G2	1.28
DDX1	1.27
TRIM25	1.27
UBAS2	1.26
PCBP2	1.22
PSMD3	1.19
PSMA6	1.19
MCM2	1.18
SFN	1.18
MATR3	1.18
EIF4A3	1.16
FKBP4	1.16
CAP1	1.14
ROCK2	1.12
SND1	1.12
CAP2B	1.11
HSP90B1	1.08
PSMB3	1.08

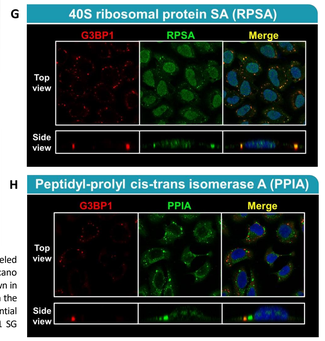


Fig. 3 | A, Top- (xy) and side- (z) views of photolabeled subcellular compartments. **B,** Confocal micrographs of photolabeled (PL) and non-photolabeled (UL) at user-defined stress granules (SG). **C,** Confocal micrographs depicting precise and accurate photolabeled SG at top- and side-view. **D,** Volcano plot of relative protein levels in photolabeled samples to control samples (PL/CTL ratio) in log_2 scale. Over-represented (enriched) proteins are shown in green. **E,** 74% of true positive SGs are found in the top 50 proteins ranked by PL/CTL ratio. Novel SG proteins (orange) and known SG (green) from the top 50 ranked proteins. **F,** List of novel SG proteins (red) discovered by Microscoop®. **G-H,** Confocal micrographs depicting SG formation of potential stress granule proteins in U-2OS cells with or without arsenite stress. Twelve potential SG proteins (green) are highly co-localized with G3BP1 SG markers. lens: 63x oil. PL: photolabed (biotinylated) proteins stained with NeutrAvidin-488, Red: G3BP1, Blue: DAPI. Scale bar: 10 μm.

Spatial proteomics of primary cilia: known and putative novel primary cilia proteins and their functional insights

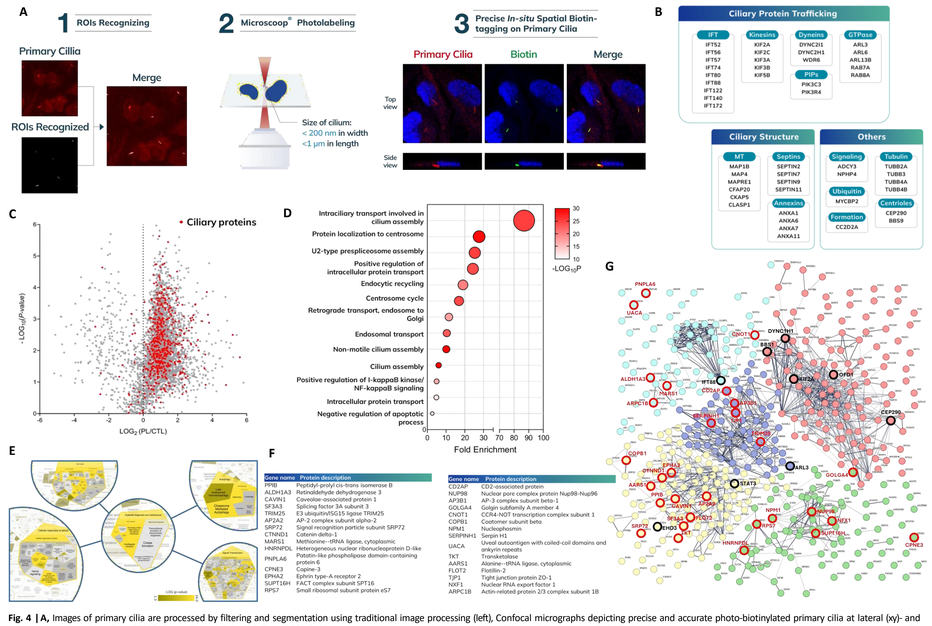
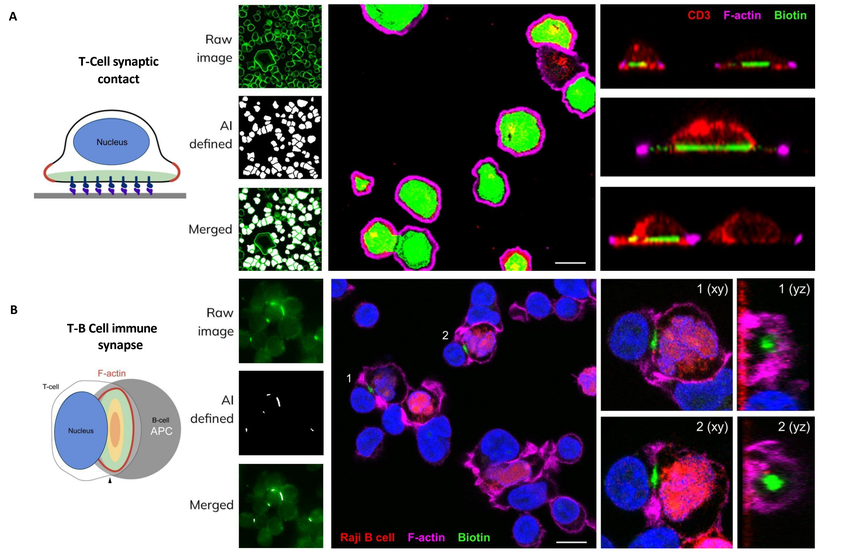
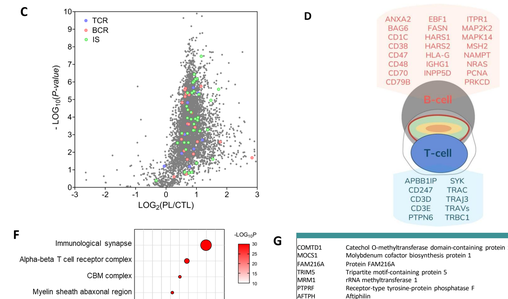
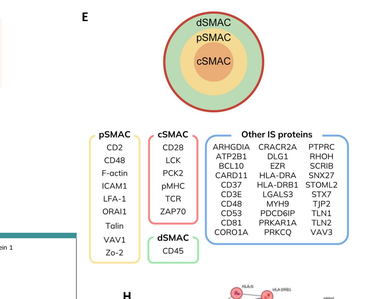


Fig. 4 | A, Images of primary cilia are processed by filtering and segmentation using traditional image processing (left), Confocal micrographs depicting precise and accurate photo-biotinylated primary cilia at lateral (xy)- and axial (z) directions (right). Red: GT335, Green: NeutrAvidin-488, Blue: DAPI. **B,** List of a few well-known ciliary proteins identified by Microscoop®. **C,** A distribution of overall protein abundances by the ratio of photolabeled (PL) sample to those in control (CTL) sample annotated as PL/CTL ratio. Ciliary proteins (red) are enriched in the PL group compared to the CTL sample. **D,** The top 100 enriched proteins were subjected to Gene ontology to reveal cilia related biological process. **E,** 427 enriched ciliary proteins were subjected to Reactome to reveal cilia related pathways. **F,** The list of the top 30 non-ciliary proteins (putative ciliary proteins) enriched by Microscoop®. **G,** The 30 putative ciliary proteins and 427 enriched ciliary proteins were subjected to STRING to reveal protein-protein interaction networks, where the 30 putative ciliary proteins (F) are indicated in red.

Mapping the immune synapse-localized proteome and immuno- oncologic protein interactions







COMTD1	Catechol O-methyltransferase domain-containing protein 1
MOCS1	Molybdenum cofactor biosynthesis protein 1
FAM216A	Protein FAM216A
TRIM5	Tripartite motif-containing protein 5
MRM1	rRNA methyltransferase 1
PTPRF	Receptor-type tyrosine-protein phosphatase F
AFTPH	Aftiphilin
SMAD5	Mothers against decapentaplegic homolog 5
SCD5	Stearoyl-CoA desaturase 5
MARCKSL1	MARCKS-related protein
GTF2H4	General transcription factor IIH subunit 4
ATG13	Autophagy-related protein 13
MICOS13	MICOS complex subunit MIC13
NDUFA5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5
HLA-DPB1	HLA class II histocompatibility antigen, DP beta 1 chain
CCNA2	Cyclin-A2
PEX16	Peroxisomal membrane protein PEX16
PDXK	Pyridoxal kinase
CD28P2	CD2 antigen cytoplasmic tail-binding protein 2
PPP2R5B	Ser/Thr-protein phosphatase 2A 56 kDa regulatory subunit beta isoform

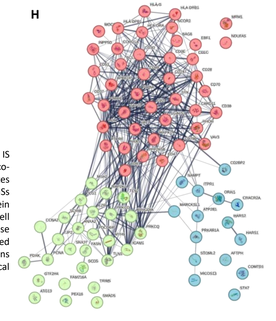


Fig. 5| A, T-cell synaptic contact model (left). Machine learning recognized the region of IS in the spreading assay shown in white (center). IS proteins in the spreading assay were photo-labeled (biotin: green), indicating a precise labeling on IS. The side view of labeling region is co-localized with CD3, a well-known marker of IS on the bottom of cells (right). **B,** T-B cell conjugation model (left). Machine learning indicates the region of IS in the T-B cell conjugation shown in white (center). AI-guided the recognition of IS in the T-B conjugation, and proteins on ISs were photo-labeled shown in green, localized within the actin ring, indicating the precise labeling on IS. **C,** Distribution of overall protein abundance according to the photolabeled/control (PL/CTL) ratio for immune synapse proteome study; TCR: T-cell receptor, BCR: B-cell receptor, IS: Immune synapse **D,** The TCR and BCR proteins identified by SYNCELL Microscoop®. **E,** The classical immunological synapse clusters (cSMAC, pSMAC, dSMAC) proteins and other immune synapse proteins identified by SYNCELL Microscoop®. F, 69 enriched immunological proteins were subjected to GeneOntology to reveal compartments. **G,** The list of the top 20 non-immune synapse proteins (putative immune synapse proteins) enriched by Microscoop®. **H,** The 20 putative immune synapse proteins and 69 enriched immunological proteins were subjected to STRING to reveal protein-protein interaction networks. Scale bar= 10 μm.

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

Summary

- **SYNCELL Microscoop®** : A novel platform for hypothesis-free subcellular spatial proteomic discovery
- **Integration of Technologies**: Combines microscopy, deep learning, photochemistry, two-photon/ illumination, and mechatronics
- **Purpose:** Facilitates high-content, image-guided photo-labeling at a nanoscale resolution
- **Capabilities:** Precisely labels spatially specific proteins from hundreds of thousands of individual cells, suitable for mass spectrometry analysis
- **Achievements:**
  - ➢ Discovered dozen of novel stress granule proteins and identified 608 known ciliary proteins, providing functional insights and listing putative proteins with high protein-protein interactions
  - ➢ Precision photo-biotinylation of millions of immune synapses, followed by LC-MS/MS analysis, resulted in the identification of a highly enriched set of immune synapse proteins. Notably, this approach also revealed potentially novel proteins with strong correlations to immunological functions