Total-sync ultra-content microscopic opto-biotinylation enables high-sensitivity hypothesis-free in situ subcellular protein discovery

Chih-Wei Chang[1,*], Yi-De Chen[1,*], Chantal Hoi Yin Cheung[1,*], Chia-Wen Chung[1], Hsiao-Jen Chang[1], Yong-Da Sie[1], You-Pi Liu[1], Yu-Chih Lin[1], Hsiang-Ju Kai[1], Weng Man Chong[1], Hsin-Yi Wu[2], Jung-Chi Liao[1,#],
[1]Syncell Inc, Taipei, TAIWAN, [2]National Taiwan University, Taipei, TAIWAN.
Corresponding author (Email: jcliao@syncell.com) * These authors contribute equally to this work

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Abstract

Localizing proteins at specific subcellular regions without using target-specific antibodies or fluorescent proteins is challenging. Laser capture microdissection is able to achieve hypothesis-free in-situ protein discovery, but the resolution cannot reach a subcellular level limited by the laser beam size, and the sensitivity is limited by the sensitivity of mass spectrometry, where only high copy-number proteins at a specific dissected region can be identified. Mass spec imaging with localized ionization can achieve a subcellular resolution, but the sensitivity is also highly limited. A major obstacle of in situ proteomic studies versus in situ transcriptomic studies is that proteins lack a PCR-equivalent amplification method to boost sensitivity. Here, in order to achieve hypothesis-free in situ subcellular protein discovery with high sensitivity and high specificity, we integrated methods of microscopy, artificial intelligence, photochemistry, and automation control to perform microscopy-guided targeted opto-biotinylation. Total-sync ultra-content biotinylation of proteins in thousands of fields of view (FOV) with similar morphological features was achieved by FPGA-based mechatronics. Convolutional neural networks-based deep learning is applied to the image to determine the regions of interest (ROIs) in real time. Multifunctional molecules that contain a photochemical warhead (such as $Ru(bpy)_3^{2+}$ or benzophenone) and a tagging molecule (such as biotin) were used for protein labeling. The three-step process of imaging-image processing-opto-biotinylation was repeated for thousands of FOVs automatically to enrich biotinylated proteins in similar ROIs enough for avidin bead purification and successive mass spectrometry analysis, effectively beating the limit of protein amplification. With this platform, we were able to validate the technology by showing a >90% specificity and a sensitivity of >1000 species for nuclear proteins. We were also able to identify novel protein players for specific biological problems, including EIF3CL, DDX17, PPIA, RPSA, and RPLP0 for stress granules positively validated by antibody staining. Together, our total-sync ultra-content microscopic opto-biotinylation method can be applicable to widely diverse cell biology problems to identify novel protein players in the ROIs, enabling hypothesis-free subcellular protein discovery with high sensitivity and specificity.

Microscoop™: hypothesis-free subcellular protein discovery platform

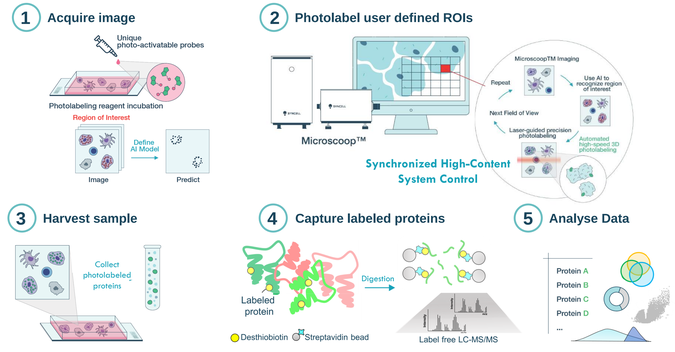

Fig. 1 | Schematic workflow of SYNCELL Microscoop™. A total-sync ultra-content microscopic platform that integrates image acquisition, photochemistry, microscopy, optics, and FPGA-based mechatronics enable high-content in situ photolabeling followed by mass spectrometry analysis.

Microscoop™: synchronized high-content system control to a high-speed and accurate image masking

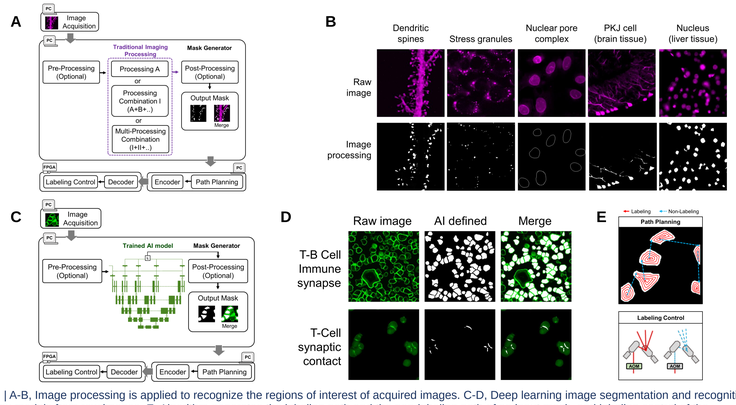

Fig. 2 | A-B, Image processing is applied to recognize the regions of interest of acquired images. C-D, Deep learning image segmentation and recognition of complex models from raw images. E, Algorithm generates the labeling path and the non-labeling path of an input mask, and labeling control of the galvanometer system and the AOM.

Photo-induced spatial biotinlyation in subcellular compartments at a nanoscale resolution

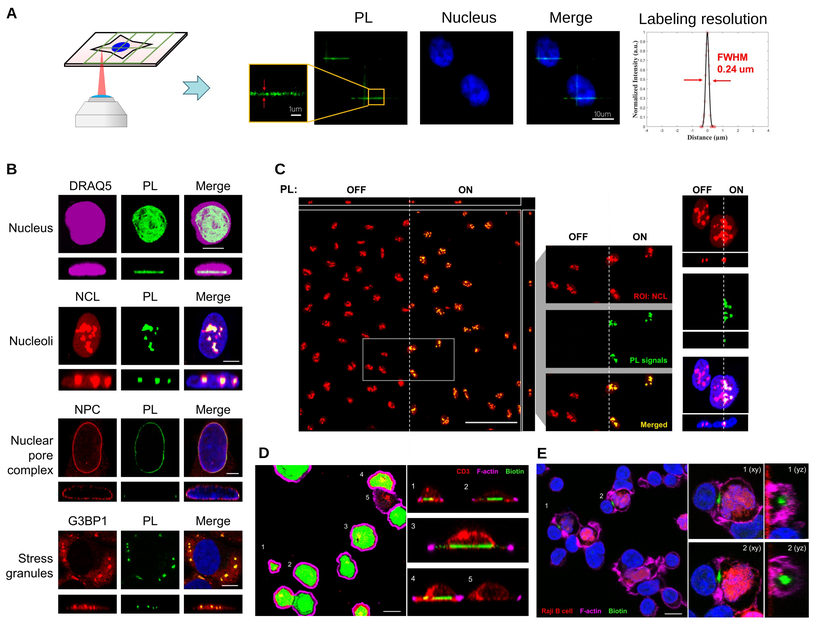

Fig. 3 | A, A thin "cross" pattern were photolabeled into PFA fixed U-2OS cells. Measurement of photolabeling resolution by super-resolution structured illumination microscopy. B, Top- (xy) and side- (z) views of photolabeled subcellular compartments. C, Photolabeled and non-labeled ROIs within nucleoli using antibody-based photoactive probe. D, Top- and side views of each labeled synapse (d_{1-4}) in spreading assay. The side view of photolabeling region is co-localized with CD3, immune synapse marker on the bottom of cells. No biotin signal is found in a non-photolabeled cell (d_5). E, Photolabeled regions of immune synapse of Jurket T cells and Raji B cells are shown in green as a precise and thin labeled layer. Scale bar: 10 μm. Green: Neutravidin-488 fluorescent, Red: anti-primary antibody-568, Blue: DAPI.

High sensitivity and specificity of photolabeled nuclear and nucleolar proteomes

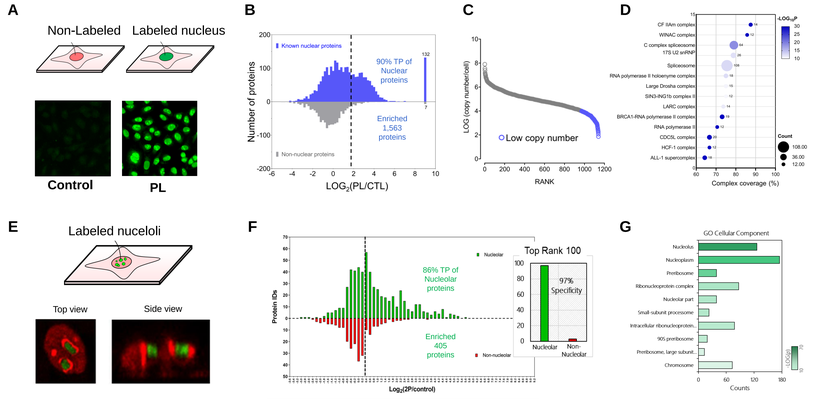

Fig. 4 | A, Photolabled nuclei were harvest for LC-MS/MS analysis. B, Protein distribution of true positive of 3 biological replicates of nuclear proteome. C, The distribution of protein copy number (Blue: < 10,000 protein copy number per cell). D, CORUM analyses of protein complexes. E, Photolabled nucleoli were harvest for LC-MS/MS analysis. F, Protein distribution of true positive of nucleolar proteome. Proteins are ranked by order of abundance, 97 out of the top 100 proteins were annotated as nucleolar proteins. G, Gene ontology analysis of nucleolar proteome.

Mapping the proteome of stress granules and discovery of novel biomarkers

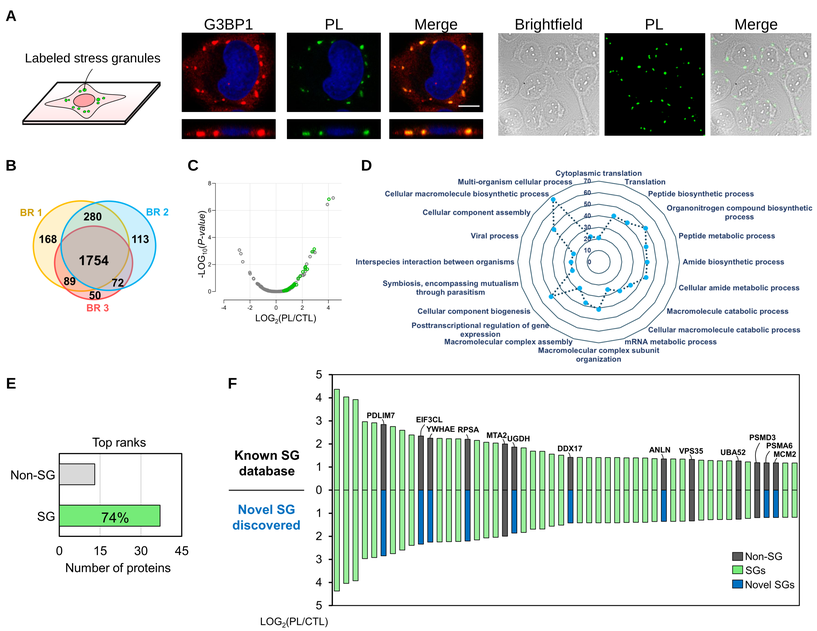

Fig. 5 | A, Photolabled stress granules were harvest for LC-MS/MS analysis. Confocal micrographs depicting precise and accurate photolabeling of SGs. B, Three biological replicates of stress granule proteomics. C, 124 enriched proteins were subjected to Gene ontology to reveal SG related biological process. D, Volcano plot of relative protein levels in photolabeled samples to control samples (PL/CTL ratio). 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. F, Proteins have no prior annotation as stress granule (gray) from the top 50 ranked proteins. Novel SG proteins (blue) discovered by Microscoop™. G, Confocal micrographs depicting SG formation of potential stress granule proteins in U-2OS cells with or without arsenite stress. Ten potential SG proteins (green) are highly co-localized with G3BP1 SG markers. lens: 63x oil. PL: photolabed (biotinlyated) proteins stained with NeutrAvidin-488, Red: G3BP1, Blue: DAPI.

Summary

- SYNCELL Microscoop™ is a novel platform to enable hypothesis-free subcellular spatial proteomic discovery
- The platform integrates microscopy, deep learning, two-photon illumination, and mechatronics to facilitate high-content image-guided photo-labeling
- Spatially specific proteins from hundreds of thousands of individual cells could be labeled precisely enough for mass spectrometry sensitivity
- Novel stress granule proteins were identified with Microscoop™