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WHITE PAPER

Microscoop® enables subcellular proteomic discovery of stress granules

Authors

Hsiao-Jen Chang, Chantal Hoi Yin Cheung, and Hsuan-Hsuan Lu

Introduction :

Stress granules (SGs) are dynamic, non-membrane bound organelles critical for cellular adaptation and survival during stress. They assemble essential proteins in response to conditions such as heat, malnutrition, and oxidative stress, acting as safeguards for cellular integrity[1]. When stress conditions ease, the detained proteins are subsequently released, facilitating the resumption of RNA translation and thereby promoting cellular recovery[2]. Discovering the protein composition of SGs has profound implications for our understanding of stress-related diseases and the development of innovative therapeutic strategies[3]. The study of SGs is challenging due to their small size (0.2 to 1µm), transient, and membrane-less nature[4]. Issues such as inadequate spatial resolution, incomplete proteome analysis, and difficulties in physically isolating SGs limit the investigation of SGs[5]. Moreover, the use of non-specific markers for SG identification often leads to false positive results, further complicating the accurate characterization of these structures.

To address these challenges, this white paper introduces an advanced platform developed by Syncell, termed Microscoop®, for spatial protein isolation with submicron precision. Microscoop® stands as a pioneering platform for automated, image-guided proteomic isolation. It precisely targets regions of interest (ROIs) as small as 0.24 µm, enabling accurate photo-biotinylation with femtosecond pulses for two-photon illumination, matching the scale of SGs. Furthermore, this method of fast and specific laser-induced biotinylation tags biotin within the SGs' region, effectively addressing the issue of non-specific markers and false positives. Our technology enables a comprehensive exploration of SGs' protein locations and interactions, offering hypothesis-free protein identification at subcellular level.

Microscopy-guided protein discovery platform for stress granules

Microscoop® is an advanced platform for protein isolation and identification within submicron cellular regions. It is equipped with a motorized epifluorescence microscope, a high-resolution sCMOS camera, and a two-photon light source, enhanced by a specially developed photochemical probe (Fig. 1). This configuration enables precisely targeting and isolating SGs for proteomic analysis. SGs are pre-stained with the well-known marker G3BP1 to facilitate visualization. Subsequently, images are analyzed in real-time to segment the SGs and eliminate non-specific signals from the cytosol. Image processing employs a combination of techniques, including thresholding, size exclusion, and morphological recognition. The platform efficiently segments each Field of View (FOV) image to identify stress granules, enabling effective path planning and labeling control. Following this, two-photon illumination triggers photochemical agents to biotinylate proteins within the SGs. The platform then sequentially photo-biotinylates individual SGs through mechatronic position control, processing millions of SGs to amass sufficient proteins for downstream proteomic analysis. The extracted proteins undergo streptavidin pull-down for hypothesis-free mass spectrometry (LC-MS/MS) analysis. The Microscoop® platform, with its integration of ultrahigh-content, high-speed microscopy and targeted photo-biotinylation, enables proteomics discovery by spatially isolating SGs.

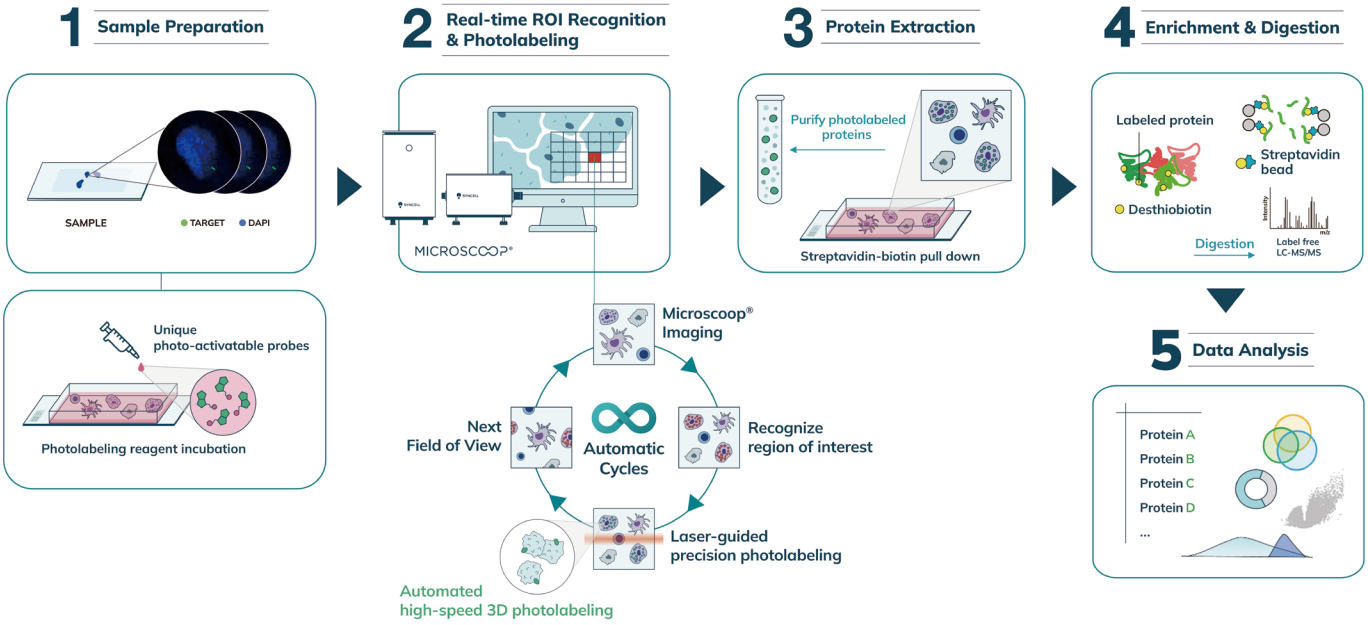

Fig. 1 | Schematic workflow for mapping the stress granules proteome. A microscopy-guided protein discovery platform integrates image acquisition, photochemistry, microscopy, optics, and mechatronics to enable ultrahigh-content *in situ* photolabeling followed by mass spectrometry analysis.

Synchronized ultrahigh-content system control to a high-speed and accurate image masking and labeling

To meet the protein quantity requirements for analysis, an ultrahigh-content, high-speed, real-time, targeted microscopy-based illumination system was developed for photolabeling at subcellular level ROIs such as SGs. This approach assumes that protein constituents of SGs are largely similar, identifiable by distinct morphological features and image contrasts under microscopy. The process, executed tens of thousands of times, unfolds in four key steps: 1) Microscopy imaging of the FOV to pinpoint and delineate SGs; 2) Instantaneous SG pattern recognition for an FOV image through image processing; 3) Targeted illumination within the SG for photochemical labeling; 4) Transitioning to the next FOV (Fig. 2). This methodical repetition ensures protein spatial isolation, key to amassing adequate proteins to overcome the issue of protein amplification. Remarkably, existing technologies lack the capability for such extensive and rapid repetition across locations and timespans. The speed for photo-biotinlyation averages about 70 SGs per second, making these enhancements in speed critical to obtaining enough proteins within a reasonable timeframe.

A software-firmware integrated program was required to control imaging, pattern generation calculation, photochemical illumination, and field change with tight coordination. The images were analyzed on the fly to segment the SGs using traditional image processing techniques. A set of steps, combining methods such as thresholding, filtering, size exclusion, and morphological recognition, was applied consistently across all FOVs (Fig. 3A). Pre-processing or post-processing was added to normalize image quality across different FOVs. The SG segmentation results are shown in Fig. 3B. This image processing step took between 0.1 to 1 second, depending on processing complexity and image quality. Coordinates of all grid points at the SGs were then obtained. An optimized planned path for photochemical activation was calculated and used to direct the galvanometers (galvos) to scan through these grid points. The galvos and the Acousto-

Optic Modulator (AOM) were synchronized at the ~100 μs scale to allow precise on and off labeling at the correct location with an exact illumination dwell time per spot, ensuring a uniform photochemical reaction duration. For multiple SG locations, the scanning path moved through one SG at a time, starting from a peripheral initiation point and spiraling clockwise toward the center before moving to the initiation point of an adjacent SG (Fig. 3C). This approach minimized traveling time and reduced motion jerk to protect the galvo from damage. The success of this intricate process within a reasonable timeframe hinges on speed optimization at every stage, seamless automation, and precise mechatronic control. These innovations have made *de novo* spatial proteomics a reality.

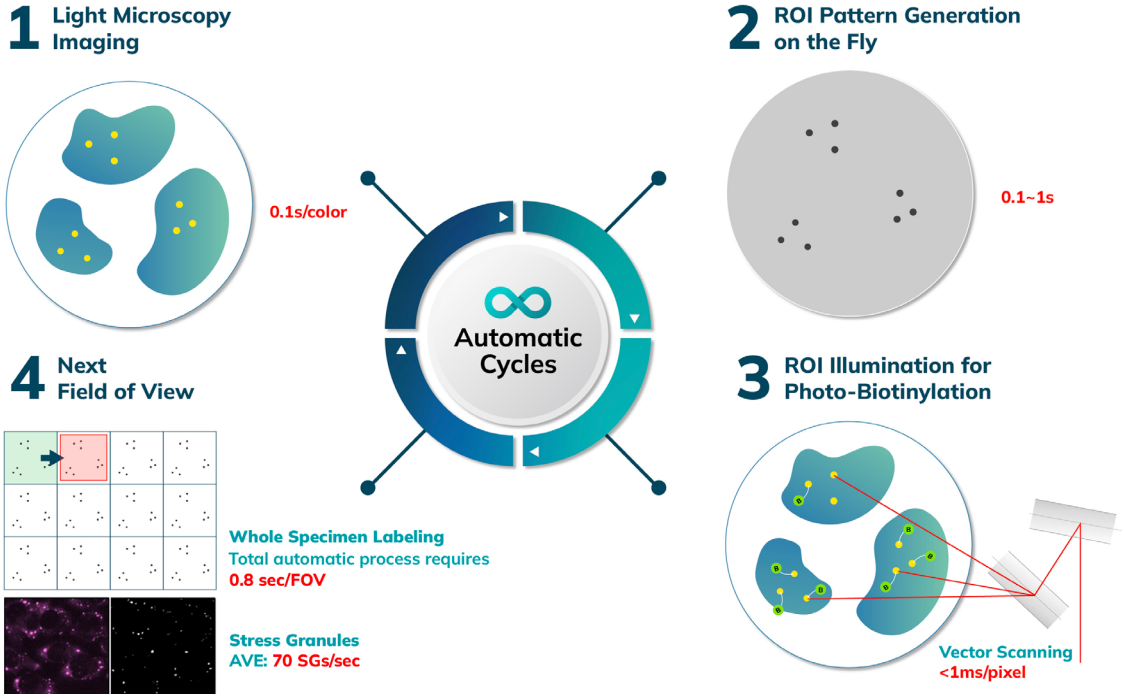

Fig. 2 | The process and design of the microscopy-guided protein isolation platform. Schematic workflow for ultrahigh-content targeted photo-biotinylation. The process includes: (1) light microscopy imaging of a FOV; (2) pattern generation of the SGs on the fly; (3) precision illumination on the selected region within SGs for protein photo-biotinylation; (4) stage movement to a next FOV; and (5) repeat of the steps 1-4 for each FOV until all FOVs of interest are processed.

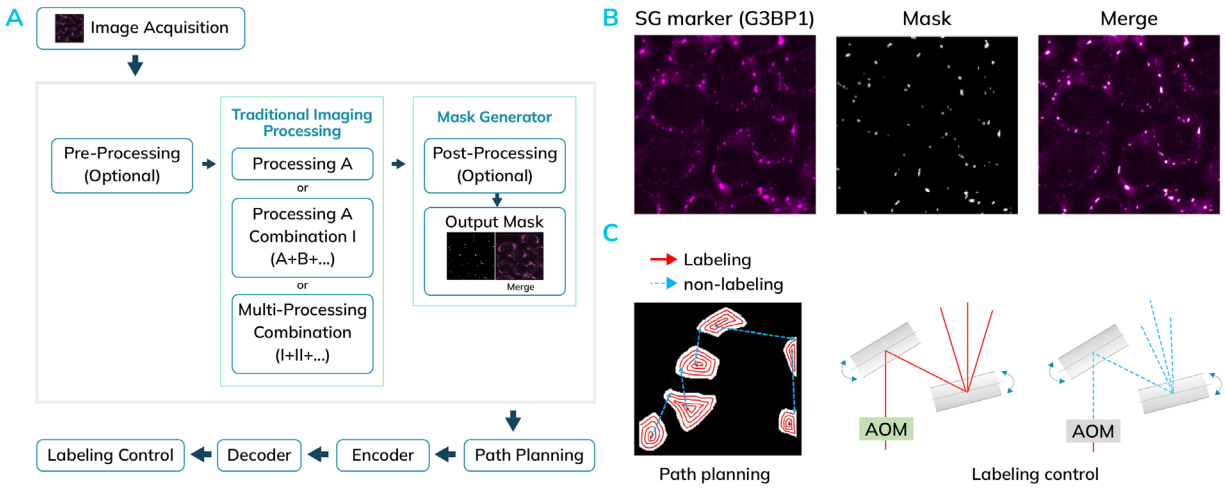

Fig. 3 | A, Image processing is applied to recognize the regions of interest of acquired images. B, SGs are processed by filtering and segmentation by image processing. C, The path planning algorithm generates the labeling path and the non-labeling path for an input mask, which can also apply for the labeling control on the galvanometer system and the AOM.

Precision photo-biotinylation within stress granules

The formation of SGs was induced in U-2 OS cells via arsenite exposure and stained with the SG marker G3BP1. Due to the diminutive size of SGs, measuring at approximately 200 nm for the smaller ones, we used an antibody-based photochemical probe to ensure precise photo-biotinylation. This method involved the utilization of antibody-conjugated $[Ru(bpy)_3]^{2+}$ molecules, which were hybridized to the G3BP1 primary antibody. This hybridization of photochemical probe on the selected regions of interest, facilitating targeted spatial photolabeling upon application of two-photon illumination. This approach allowed for the isolation of SG proteins with high specificity, as evidenced by the congruence between *in situ* biotinylated regions and SG fluorescence in both lateral (xy) and axial (z) directions (Fig. 4A-C). Additionally, merging the brightfield with photolabeled images, we gain insight into the exact location of stress granule signals within the cytoplasm (Fig. 4D).

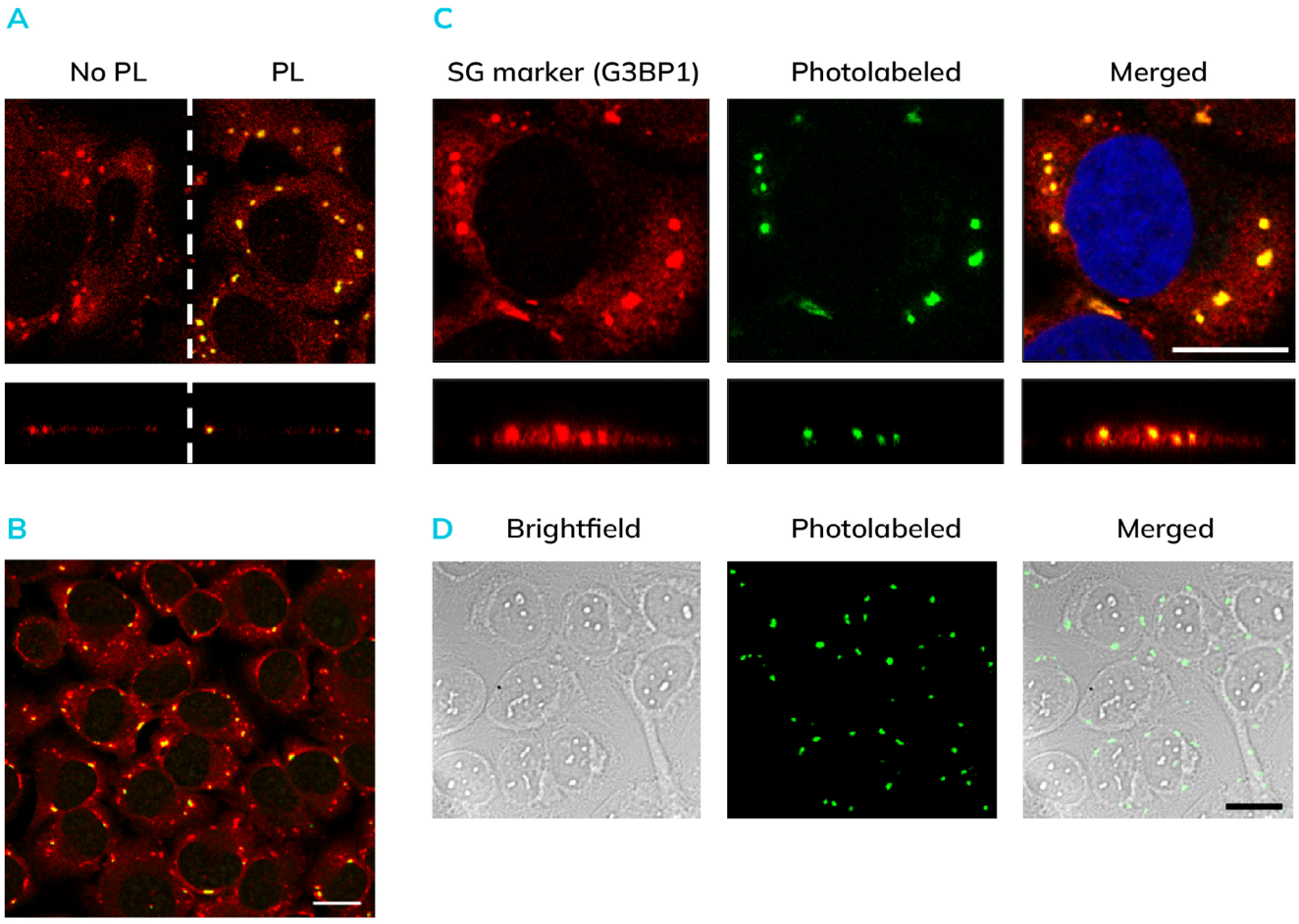

Fig. 4 | A, Confocal micrographs of non-photolabeled (No PL) and photolabeled (PL) at user defined stress granules. B, A field of view of photolabeled stress granules with Microscoop®. C-D, Confocal micrographs depicting precise and accurate photolabeled stress granules at lateral (xy)- and axial (z) directions. Red: G3BP1, Green: NeutrAvidin-488, Blue: DAPI. Scale bar: 10 µm.

Discovery of novel protein constituents of arsenite-induced stress granules

To verify if ultrahigh-content spatial biotinylation could isolate sufficient proteins for mass spectrometry (MS) sensitivity, we conducted targeted photolabeling and pulldown of SGs as a proof of principle. We

performed photolabeling on proteins of fixed U-2 OS cells at regions marked with G3BP1 for three biological replicates. After approximately 16 hours of targeted photolabeling per replicate, cells were scraped, harvested, and lysed to extract proteins. Biotinylated proteins were enriched by pulldown using streptavidin beads, tryptically digested on beads, and subjected to LC-MS/MS measurement.

A total of 2,785 proteins were identified with high confidence, with 1,754 proteins common across all replicates, indicating high reproducibility (Fig. 5A). The overlapping regions indicate a significant consensus among the replicates, underscoring the reliability of our method. Applying the $\log_2$ fold-change cutoff of 0.585, the number of unique peptides of 3, and the score sequest HT of 100 as the selection criteria, we retained 124 significantly enriched proteins (Fig. 5B).

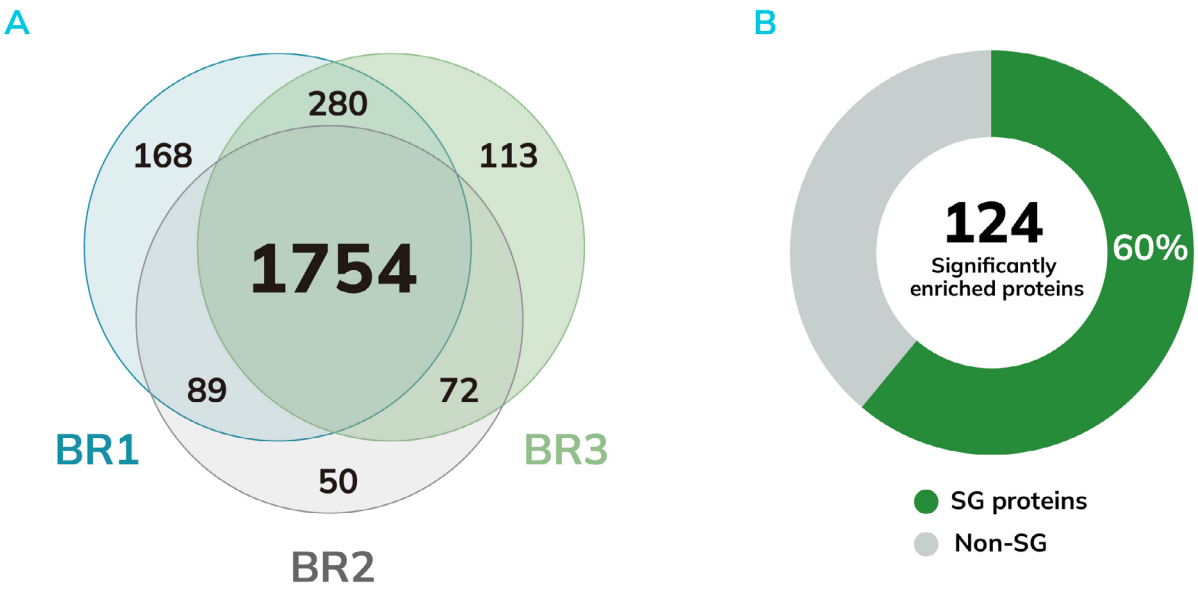

Fig. 5 | A, Venn diagram of three biological replicates of SG proteomics. B, The percentage of true positives (green) when comparing the significantly enriched proteins from spatial purification versus the SG proteins in the existing database.

Gene ontology enrichment analysis showed that the identified proteome was highly associated with cellular macromolecule biosynthetic processes, cellular component biogenesis and organization, RNA and nucleobase-containing compound metabolic processes, and cellular protein localization. Many well-known SG proteins including hnRNPs, eRF3a, PABP1, TADBP, FXR1, and eIF3s were enriched with high PL/CTL ratios (Fig. 6B). Surprisingly, 40% of these 124 proteins were absent in the existing SG proteome[2, 3, 5, 6] (Fig. 6C). These enriched proteins without prior annotation support as SG proteins exhibited high interaction relationship with the SG proteins.

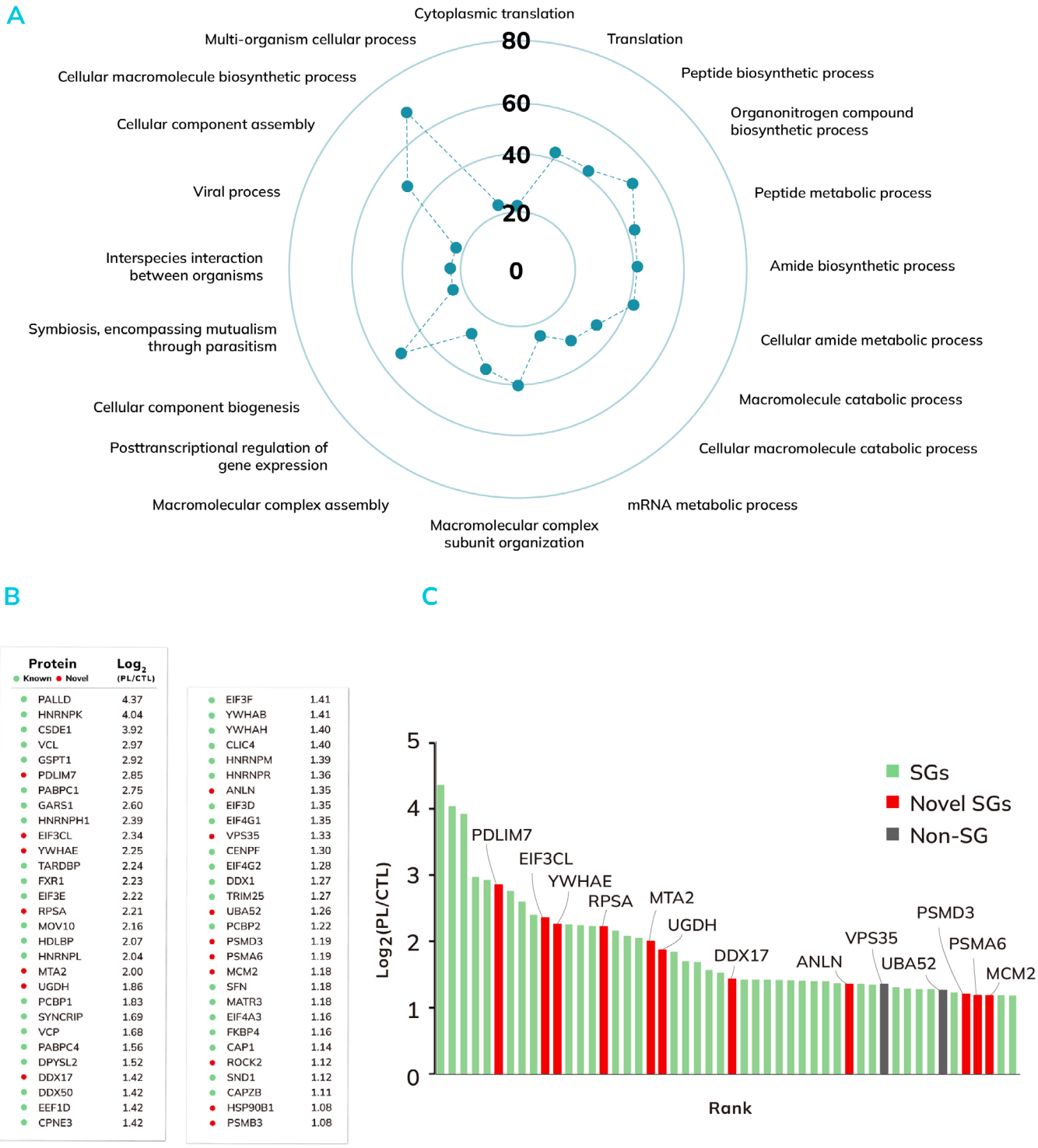

Fig. 6 | A, The 124 enriched proteins were subjected to Gene ontology to reveal SG related biological process. B, List of known (green) and novel (red) SG proteins discovered by Microscoop®. C, 74% of true positive SG proteins are found in the top 50 proteins ranked by PL/CTL ratio. Proteins have no prior annotation as SG proteins (gray and red), novel SG proteins (red), and known SG proteins (green), from the top 50 ranked proteins.

To assess if the proteomics results were specific enough to uncover new SG-associated proteins, we conducted immunostaining tests for colocalization of G3BP1 with proteins previously unannotated as SG components. Among 13 proteins tested, 11 of them were colocalized with G3BP1, i.e., PDLIM7, EIF3CL, YWHAE, RPSA, MTA2, UGDH, DDX17, ANLN, PSMD3, PSMA6, and MCM2 (Fig. 7A). Considering all known

SG proteins and these validated SG-localized proteins, the SG specificity of the obtained proteome reached 96% among the top 50 identified proteins, demonstrating the discovery power of Microscoop® with high specificity. Notably, the role of PDLIM7, DDX17, and PSMA6 in cell differentiation illustrated a potential crosstalk between stress response and cell development, as their arsenite-triggered cytosolic localization suggests a pivot from normal cellular activity to stress management, contributing to the SG formation.

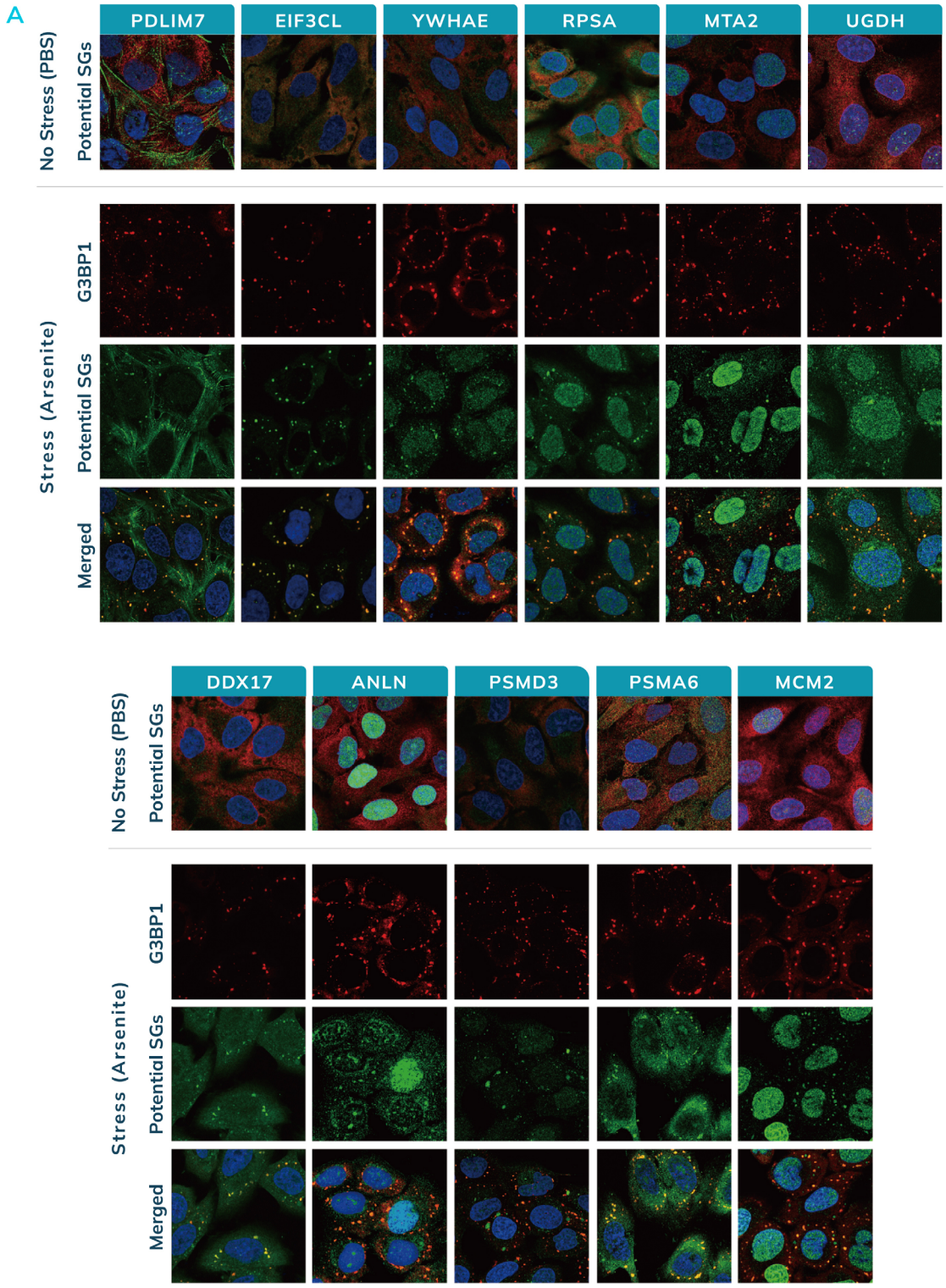

Fig. 7 | A, Confocal micrographs depicting SG formation of potential SGs proteins in U-2OS cells with or without arsenite stress. Eleven potential SG proteins (green) are highly co-localized with G3BP1 SG markers. lens: 63x oil. PL: photolabed (biotinlyated) proteins stained with NeutrAvidin-488, Red: G3BP1, Blue: DAPI.

Conclusion

The Microscoop® system is a revolutionary platform for conducting hypothesis-free subcellular spatial proteomics. It integrates advanced technologies, including real-time ROI recognition, microscopy-guided two-photon illumination, and mechatronics, to enable precise and high-throughput image-guided protein biotinylation. This enables the spatially specific labeling of proteins within individual cells at submicron precision. The approach has proven highly effective, allowing for the subsequent analysis of biotin-tagged proteins using mass spectrometry. In our exploration of subcellular structures, particularly stress granules, the system achieved 96% specificity in identifying SG proteins among the top-ranked proteins and discovered novel SGs not previously recorded in SG databases. The specificity and accuracy highlight the platform's capability in characterizing novel biomarkers for cellular components, enhancing our understanding of cellular stress mechanisms, and unveiling new therapeutic targets. It is not only advances disease research by elucidating stress granules' roles in disease mechanisms but also opens new avenues for the development of targeted therapies. Future research will focus on the functional roles of these proteins, the contribution of stress granule dynamics to disease pathogenesis, and the potential of specific proteins as therapeutic targets. Overall, Microscoop® is a transformative platform for cellular biology and disease research, providing novel insights and innovative treatments for stress-related diseases.

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SYNCELL Inc.

Info@syncell.com