

Live-Cell Monitoring and Omics Analysis of Liquid–Solid Transitions of Biomolecular Condensates

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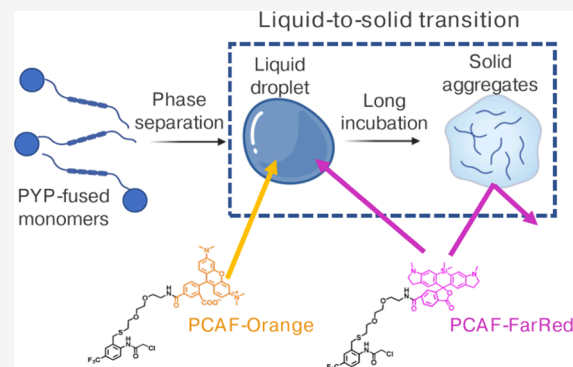


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ABSTRACT: Biomolecular condensates, or so-called membraneless organelles, transition from liquid into more solid-like states over time, contributing to the development of pathological conditions. The present study proposes a simple method using photoactive yellow protein (PYP) and its specific fluorescent covalent ligands to distinguish between the liquid and solid states of protein condensates in live cells. The method, compatible with fluorescence-activated cell sorting (FACS), correlates the stiffness of specific protein condensates with their accessibility to PYP ligands, enabling quantitative multicolor monitoring of condensate solidification. We applied this technique to 12 phase-separating proteins and their mutants, finding that TDP-43, particularly its A315T mutant linked to familial amyotrophic lateral sclerosis, most readily forms solid aggregates. Furthermore, this FACS-compatible strategy enabled the isolation of distinct cell populations based on condensate states, allowing for subsequent proteomic and transcriptomic analyses. Our findings demonstrate that condensate solidification is accompanied by the upregulated expression of extracellular matrix proteins, suggesting a previously unrecognized link between solid aggregate formation and extracellular matrix hardening.



INTRODUCTION

Cells organize their contents into organelles. Traditionally, organelles have been considered membrane-surrounded structures. However, a growing body of recent research has identified a new class of organelles known as biomolecular condensates, which confine biological reactions without a surrounding membrane. These membraneless organelles are generated and maintained through a physical process known as liquid–liquid phase separation (LLPS), where a solution of components segregates into two or more coexisting liquid phases. A substantial cohort of proteins has been shown to undergo LLPS, forming distinct liquid droplets within cells.^{1–3}

These liquid-like protein condensates occasionally transition into more solid-like states over time, contributing to the development of pathological conditions. For instance, reversible and functional condensates of fused in sarcoma (FUS)⁴ and transactive response DNA-binding protein of 43 kDa (TDP-43)⁵ have been reported to age and transition into nonfunctional solid aggregates, leading to the development of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD).^{6–8} The condensation of phase-separating proteins is typically visualized by fusing these proteins with a fluorescent protein tag, such as GFP, allowing the live-cell

observation of biomolecular condensates under a confocal microscope.⁴ However, this method cannot distinguish between condensates that are in a liquid state and those that have transitioned to a more solid-like state.

To assess the degree of solidification in fluorescently labeled biomolecular condensates, the fluorescence recovery after photobleaching (FRAP) assay has often been employed.^{9,10} This biophysical technique involves selectively bleaching a region of the fluorescent condensate and monitoring the recovery of fluorescence, providing insights into the dynamics and solidity of the condensate. Despite its usefulness, the FRAP assay is time-consuming and low-throughput, making it impractical for examining a large number of samples. There is a pressing need for a more robust methodology to monitor the transition of liquid–liquid phase separation into solid-like

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states in live cells. Such a method would facilitate high-throughput analysis of condensate dynamics, advancing our understanding of the factors involved in the liquid-to-solid transitions and consequent disease development.

The present work proposes a chemical technique that reports the status of protein condensates in live cells, utilizing photoactive yellow protein (PYP) and its specific fluorescent covalent ligands. The method, compatible with fluorescence-activated cell sorting (FACS), correlates the stiffness of specific protein condensates with their accessibility to PYP ligands. This advancement enables a quantitative evaluation of condensate solidification and an isolation of distinct cellular populations based on their condensate states.

RESULTS

Fluorescent Labeling of Phase-Separating Proteins.

Our fluorescence-based technique takes advantage of photoactive yellow protein (PYP), a small bacterial protein that covalently binds to 4-hydroxycinnamic acid and various unnatural fluorescent PYP ligands. The small size (14 kDa) of PYP and its efficient labeling kinetics would minimize potential interference with the LLPS properties of the PYP fusion proteins and permit quantitative and timely labeling of them, respectively.^{11,12} In particular, our previous studies demonstrated that a chloroacetyl PYP-specific fluorescent probe, PCAF-orange, achieves a higher labeling rate of PYP than traditional cinnamic acid-based PYP ligands.¹³ To demonstrate its utility, we conducted cell-based experiments with a PYP fusion protein of FUS(P525L), a pathologically relevant mutant of FUS that has been well documented to transition from the liquid to solid phase more rapidly than its wild-type counterpart.^{14,15} HEK293 cells were transfected with an expression vector encoding a PYP fusion protein of FUS(P525L) and a cotranslated eGFP marker and subsequently treated with various concentrations of PCAF-orange for 30 min. Separation of cell lysates on an SDS-PAGE gel and fluorescent scanning revealed that 0.5 μ M PCAF-orange achieved saturation labeling of PYP-FUS(P525L) (Figure S1), indicating that this condition is suited for complete labeling.

To confirm the ability of PYP fusions of phase-separating proteins to form condensates in cells, we constructed a PYP fusion plasmid library comprising 12 representative phase-separating proteins. Twenty-four hours after transfecting each plasmid into U2OS cells, PCAF-orange (0.5 μ M) was added to the culture medium and incubated for 30 min, as outlined in Figure S2A. The PYP fusions of the phase-separating proteins exhibited distinct condensates or punctate structures under a confocal microscope, whereas PYP alone was diffusively distributed in the cytosol (Figure S2B,D). Comparable results were obtained in HEK293 cells (Figure S2C,D). These results support our notion that fusing PYP has a minimal impact on the condensation behavior of these proteins.

Live-Cell Detection of Biomolecular Condensates' States. We hypothesized that solidified protein condensates exhibit greater resistance to covalent reactions with the PYP ligands than liquid condensates do. To monitor the labeling resistance of solidified condensates, we utilized two fluorescent PYP ligands with nonoverlapping emissions, PCAF-orange (Ex: 554 nm; Em: 570 nm) and PCAF-far-red (Ex: 700 nm; Em: 720 nm), at different time points. An expression vector encoding a PYP fusion of FUS(P525L) was transfected into the HEK293 cells. Twenty-four hours post-transfection, the

PYP condensates were first labeled with PCAF-orange. Following an extensive wash, the cells were incubated for an additional 48 h, an incubation time that has previously been reported to induce solidified aggregates of FUS(P525L).¹⁴ During this prolonged incubation, we anticipated the formation of three types of FUS(P525L) condensates: (i) liquid condensates of newly synthesized unlabeled proteins, (ii) pre-existing labeled liquid condensates merging with newly formed liquid condensates, and (iii) solid aggregates. These condensates were then subjected to labeling with PCAF-far-red prior to confocal microscopic observation. As expected, the resulting cells displayed three distinct patterns of protein labeling (Figure 1A,B). The first group (group 1) consisted of

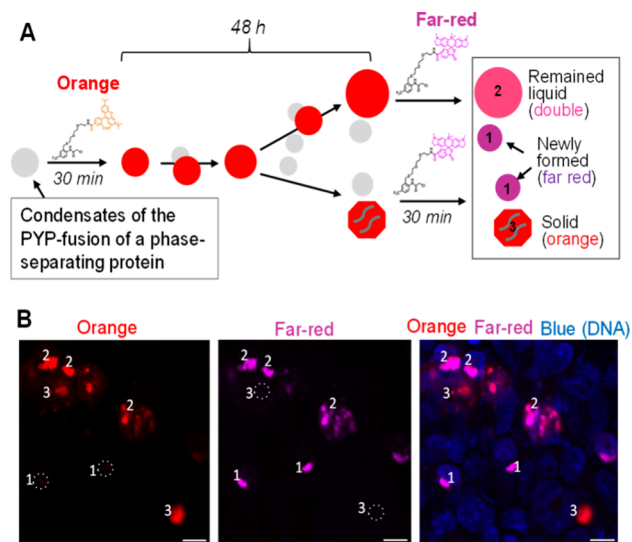


Figure 1. Live-cell detection of biomolecular condensates' states. (A) Schematic representation of the experimental workflow and the evolution of condensates. HEK293 cells were sequentially labeled with PCAF-orange and PCAF-far-red prior to observation under a confocal microscope. The classification into groups 1–3 is indicated. (B) Confocal microscopic images showcasing PYP-FUS(P525L) in different states. Three distinct types of protein condensates were identified: those labeled only with PCAF-far-red (group 1), those doubly labeled with both probes (group 2), and those exclusively labeled with PCAF-orange (group 3). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar: 10 μ m.

condensates marked exclusively by PCAF-far-red, indicating newly formed liquid protein condensates. The second group (group 2) included condensates doubly labeled with both PCAF-orange and PCAF-far-red, corresponding to condensates that remained liquid throughout the experiments. The third group (group 3) comprised condensates labeled solely by PCAF-orange, most likely representing solidified protein condensates.

To substantiate this interpretation, we monitored the doubly labeled condensates (group 2) for an additional 11 h. Time-lapse imaging revealed their fusion into larger droplets, a characteristic feature of liquid condensates (see Supporting Information, Video 1). In contrast, when a cell containing both a doubly labeled condensate (group 2) and a condensate labeled solely by PCAF-orange (group 3) was monitored, the two condensates did not fuse; instead, the solid condensate was eliminated through cell division and apoptosis (Supporting Video 2).

Our hypothesis was further validated using classical FRAP assays. PCAF-orange-labeled condensates of the FUS(P525L) protein were photobleached with a 559 nm laser. The fluorescent recovery of double-labeled condensates (group 2) and solidified condensates labeled only with PCAF-orange (group 3) revealed a correlation between the ratio of the far-red to orange fluorescence intensity and recovery rates. Specifically, a low $F_{\text{far-red}}/F_{\text{orange}}$ corresponded to the low recovery rates characteristic of solidified aggregates, while a high ratio was indicative of high recovery rates consistent with liquid condensates (Figure 2A, Figure S3A,B). This correlation

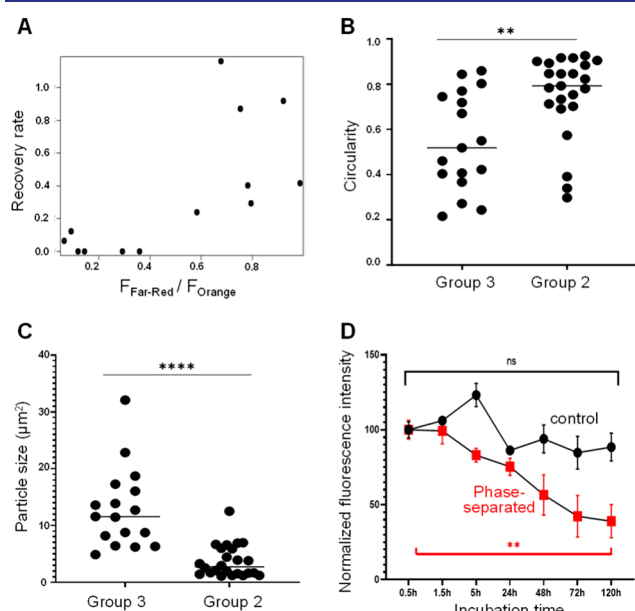


Figure 2. Validation experiments. (A) Proportional relationship between FRAP recovery rates and the ratios of the far-red to orange fluorescence intensity. FRAP analysis was performed on a total of 13 condensates of PYP-FUS(P525L) that were either double-labeled (group 2) or exclusively labeled with PCAF-orange (group 3). (B, C) Super-resolution imaging analysis of groups 2 and 3 condensates. Data are presented as median values, with individual points representing single condensates ($n = 17$ for group 3; $n = 23$ for group 2). (B) Circularity analysis of the condensates. Statistical significance was assessed using the Mann–Whitney U test; ** $p = 0.003$. (C) Particle size analysis of the condensates. Statistical significance was determined using the Mann–Whitney U test; **** $p < 0.0001$. (D) PYP labeling efficiency during the *in vitro* liquid-to-solid transition of PYP-FUS(P525L). The phase transition was induced by diluting a 1 M KCl solution of PYP-FUS(P525L) to a final concentration of 50 mM KCl, followed by incubation for varied durations and subsequent labeling with PCAF-orange. In control samples, the FUS protein stock was diluted in a high salt buffer (1 M KCl), which prevented phase separation. Normalized fluorescence intensity was determined by comparing the intensity of PCAF-orange labeling, measured using Typhoon FLA 900 imaging, to the protein content visualized on the SDS-PAGE gel stained with Coomassie blue. Data are presented as means $\pm$ standard deviations ($n = 3$). Statistical significance indicated ** $p = 0.0011$.

between the ratio of fluorescence intensities and recovery rates supports our hypothesis that more solid aggregates exhibit greater resistance to staining with PCAF-far-red.

Further support for our findings was obtained through super-resolution microscopy, visualizing distinct structural differences between double-labeled condensates (group 2) and solidified condensates labeled only with PCAF-orange

(group 3). We conducted an analysis of 16 fields for each type of condensate. Evaluation of circularity metrics indicated a significant decrease in circularity among the solid aggregates in group 3 relative to that of the double-labeled condensates in group 2 (Figure 2B). Furthermore, particle size analysis revealed a statistically significant increase in size for the solid aggregates (group 3) compared with the liquid condensates representing group 2 (Figure 2C). Interestingly, certain solid aggregates classified within group 3 exhibited a notable degree of rounded circularity. For instance, solid condensate O-13 in Figure S4A has a size and circularity comparable to those of double-labeled liquid condensate D-11 in Figure S4B; however, closer inspection of O-13 revealed a more heterogeneous and complex internal architecture. Overall, solidified condensates in group 3, labeled exclusively with PCAF-orange, displayed structural heterogeneity, including fibrillar-like features, whereas doubly labeled condensates from group 2 maintained a more uniform and rounded morphology.

To confirm the resistance of solid-like aggregates to PYP labeling *in vitro*, we conducted labeling experiments with a purified, recombinant PYP fusion of FUS(P525L). To control the protein condensation, we leveraged the method developed by Dormann and co-workers, in which maltose-binding protein (MBP) was fused to FUS(P525L) for increasing protein solubility.¹⁶ Following the removal of the MBP moiety by TEV protease (Figure S5C), the resulting PYP-FUS(P525L) was stored in 1 M KCl before initiating experiments. The *in vitro* phase transition was triggered by diluting the samples to a final concentration of 50 mM KCl, followed by incubation for varying durations to promote the solid phase formation, as outlined by Shen et al.¹⁷ In control samples, the FUS protein stock was diluted in a high salt buffer (1 M KCl), which prevented phase separation. Subsequent labeling with PCAF-orange revealed a statistically significant linear decrease in fluorescence intensity over time, particularly after 48 h of incubation, consistent with the reported timing of FUS-(P525L) solid transition *in vitro*. This reduction in labeling efficiency under extended incubation times contrasts with control experiments where the phase transition was not induced, showing no significant changes in labeling (Figure 2D, Figure S5A–C). These results confirmed the direct correlation between the protein's condensate state and its accessibility to a PYP ligand.

Quantitative Analysis by FACS. The overall results suggested the feasibility of monitoring the degree of solidification in protein condensates within live cells by assessing the ratios of PCAF-orange to PCAF-far-red labeling. To enhance the quantitative aspect of our analysis, we used the expression vector for PYP-FUS(P525L) with an IRES sequence linked to a green fluorescent protein (eGFP) at the 3' end as a transfection marker. Twenty-four hours post-transfection, the condensates were initially labeled with PCAF-orange, followed by further incubation and subsequent labeling with PCAF-far-red. We employed fluorescence-activated cell sorting (FACS) to analyze the resulting fluorescent cells, enabling the quantification of cell populations harboring condensates classified as groups 1, 2, and 3 (Figure 3A). In alignment with the reported time-dependent liquid-to-solid transitions, extending the incubation period from 24 to 72 h markedly increased the proportion of cells containing aggregates labeled exclusively with PCAF-orange (group 3) (Figure 3B and Figure S6A). Additionally, we evaluated the effect of sodium 4-phenylbutyrate (PBA), a chemical

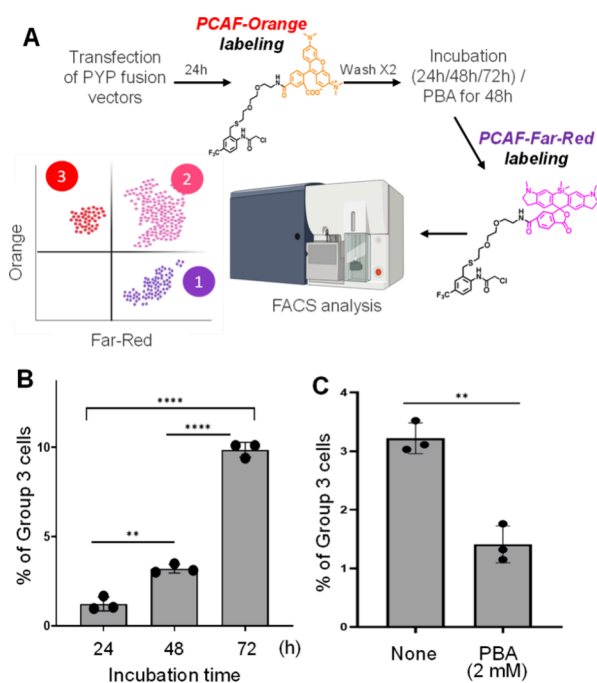


Figure 3. Quantification of cells harboring PYP-FUS(P525L) aggregates was done by FACS. (A) Workflow of the FACS analysis. The positions of cells containing condensates from groups 1–3 are indicated on the FACS chart. (B) Impact of incubation time on the proportion of group 3 cells. HEK293 cells, transfected with an expression vector encoding PYP-FUS(P525L), were labeled with PCAF-orange and subsequently incubated for 24, 48, and 72 h before labeling with PCAF-far-red. Statistical significance was assessed using an analysis of variance (ANOVA); $^{**}p = 0.0012$, $^{****}p < 0.0001$ ($n = 3$). (C) Influence of phenylbutyrate (PBA) treatment on the cell population in group 3. Cells were exposed to 2 mM PBA over a 48 h incubation period. Significance was determined using a Student's t test; $^{**}p = 0.0016$. Data are presented as mean $\pm$ standard deviation ($n = 3$).

chaperone known to mitigate misfolded protein aggregation.^{18,19} Treatment with PBA significantly decreased the proportion of cells exhibiting aggregates exclusively labeled by PCAF-orange (group 3) (Figure 3C and Figure S6B). These findings suggest that the proportion of cells containing group 3 condensates correlates with the degree of the liquid-to-solid transition of PYP fusion proteins.

We extended our investigation by constructing eGFP-marked expression vectors for PYP fusions of 11 additional phase-separating proteins and subsequently analyzed their condensation states after a 48 h incubation period by FACS. The proportions of cells containing group 3 condensates, indicative of a transition to solid aggregates, are summarized in Figure 4A and Figure S7A. In this ranking, FUS(P525L) and wild-type FUS were placed fifth and sixth, respectively. Repeated experiments with FUS(P525L), wild-type FUS, and its Prion-like domain (PrLD) demonstrated a trend in their propensity for liquid-to-solid transition consistent with previously documented observations^{16,20–22} (Figure 4B and Figure S8A): FUS(P525L) exhibited the greatest tendency to solidify, followed by wild-type FUS, with PrLD showing the least propensity.

Among the 12 phase-separating proteins tested, TDP-43 showed the highest propensity for forming solid aggregates (Figure 4A and Figure S7A). To further explore this behavior,

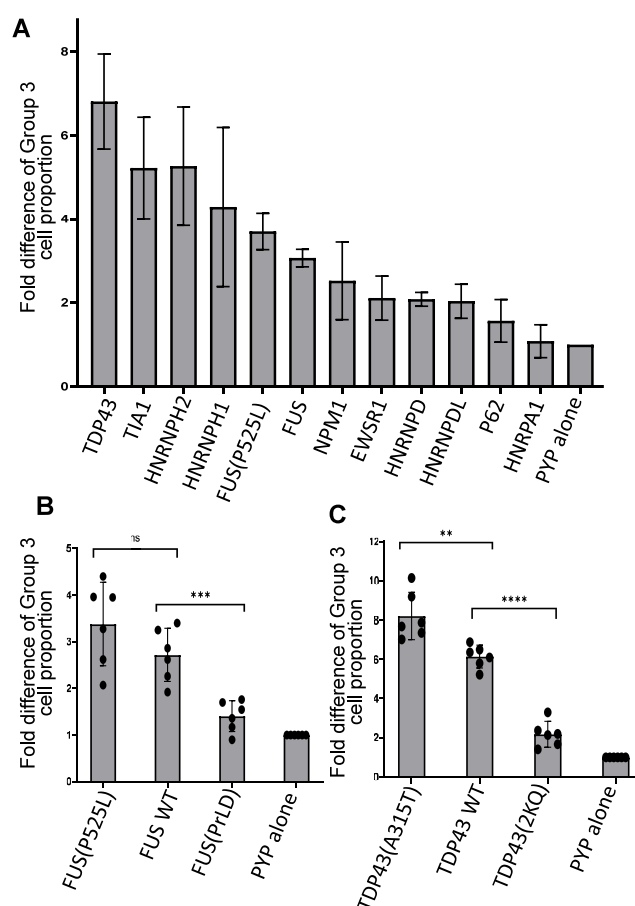


Figure 4. Solidification ranking of phase-separating proteins. (A) Proportions of cells containing group 3 condensates, indicative of a transition to solid aggregates. HEK293 cells were transfected with expression vectors encoding the PYP fusions of various phase-separating proteins and initially labeled with PCAF-orange. After an additional 48 h of incubation, the cells were labeled with PCAF-far-red. FACS was used to quantify the proportions of cells with group 3 condensates, highlighting the prevalence of solid aggregates. The fold change in the proportions of cells containing solid aggregates (group 3) is normalized relative to a control group expressing PYP alone. Data are presented as mean $\pm$ SEM ($n = 2$). (B) Ranking of the solidification propensity of FUS and its mutants; $^{***}p = 0.0006$. (C) Ranking of the solidification propensity of TDP-43 and its mutants. $^{**}p = 0.0036$, $^{****}p < 0.0001$. Significance was determined using a Student's t test. Data are presented as mean $\pm$ standard deviation ($n = 6$).

we investigated two TDP-43 mutants: TDP-43(A315T), associated with familial amyotrophic lateral sclerosis (ALS),²³ and TDP-43(2KQ), an acetylation-mimicking mutant with lysine-to-glutamine substitutions at RNA-binding sites, which enhance its liquid properties.²⁴ Our experiments on cells containing solid condensates demonstrated a clear hierarchy in their transition from the liquid to solid states. TDP-43(A315T) exhibited the highest solidification propensity, followed by wild-type TDP-43, while TDP-43(2KQ) showed the lowest propensity. These findings underscore the high solidification propensity of TDP-43 and the significant effects of specific mutations on its aggregation behavior (Figure 4C and Figure S8B).

The intracellular expression levels of phase-separating proteins could potentially influence the observed population of group 3. To address this possibility, we assessed the

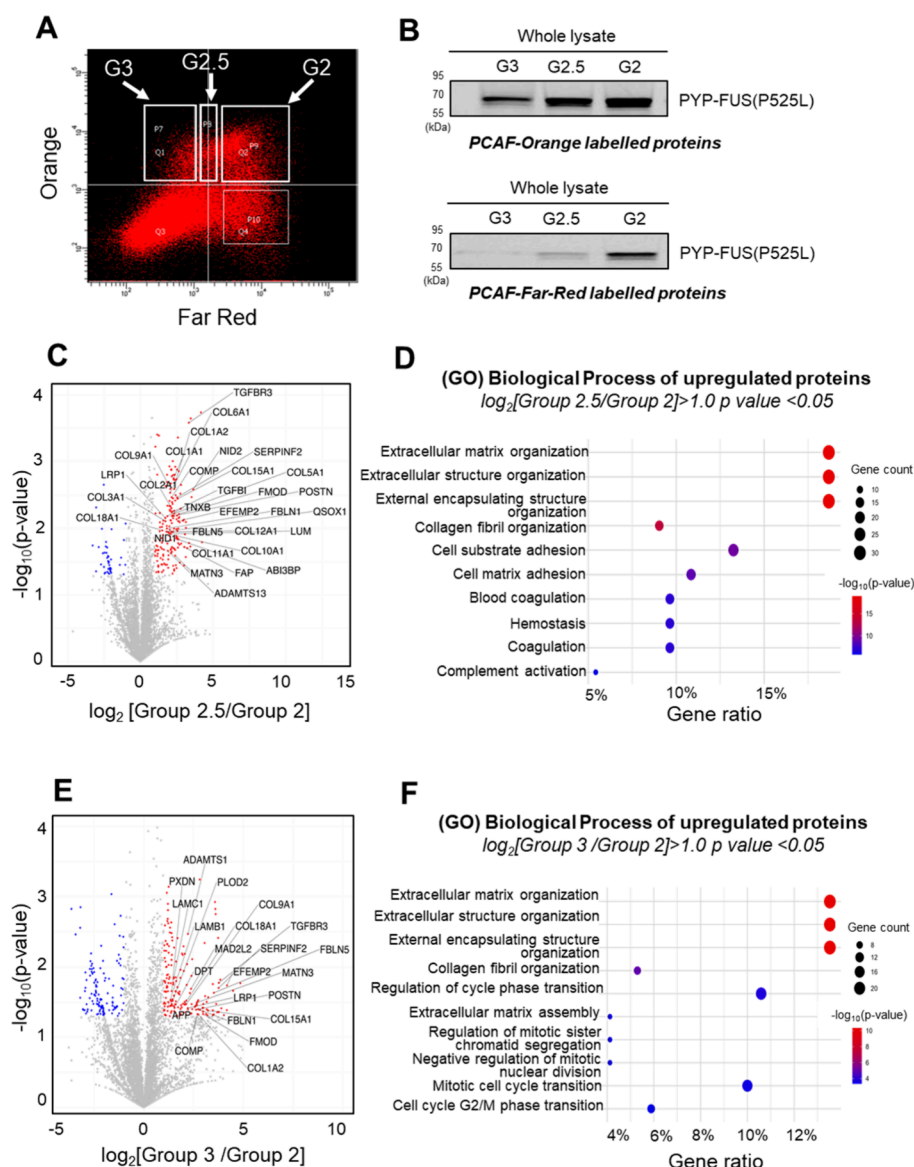


Figure 5. Protein fraction analysis and proteomic profiling of FACS-sorted cells expressing PYP-tagged FUS(P525L). (A) Flow cytometry profile of the sorted cell populations. Group 3 (G3) comprises cells labeled exclusively with PCAFA-orange probe; group 2.5 (G2.5) represents cells positioned at the interface between group 2 and group 3; group 2 (G2) consists of cells colabeled with both PCAFA-orange and PCAFA-far-red probes. The G2.5 gate was specifically introduced to isolate cells harboring condensates transitioning from liquid-like (G2) to solid-like (G3) states. The gating strategy was optimized to ensure comparable cell numbers, particularly between G2.5 and G3, to facilitate downstream analyses. (B) SDS-PAGE analysis of fluorescently labeled proteins from whole-cell lysates of sorted populations. Fluorescence detection was performed using the Typhoon FLA 9000 imaging system with Cy3 (PCAFA-orange) and Cy5 (PCAFA-far-red) filters. (C) Volcano plot illustrating differential protein expression between groups 2.5 and 2. Significantly upregulated or downregulated proteins ($\log_2[\text{group 2.5/group 2}] > 1$ or < -1 ; $p\text{-value} < 0.05$) are shown, with emphasis on proteins involved in extracellular matrix organization. (D) Gene ontology (biological process) enrichment analysis of proteins significantly upregulated in group 2.5 compared to group 2 ($\log_2[\text{group 2.5/group 2}] > 1$; $p\text{-value} < 0.05$). (E) Volcano plot comparing group 3 and group 2, highlighting significantly altered proteins ($\log_2[\text{group 3/group 2}] > 1$ or < -1 ; $p\text{-value} < 0.05$), with a focus on those related to extracellular matrix organization. (F) GO (biological process) enrichment analysis of proteins significantly upregulated in group 3 compared to group 2 ($\log_2[\text{group 3/group 2}] > 1$; $p\text{-value} < 0.05$).

expression levels of PYP fusion proteins by quantifying the cotranslated GFP expression (Figure S7B). The analysis revealed that expression levels were generally comparable across all of the tested constructs. We subsequently normalized the group 3 population by expression level to recalculate the solidification propensity rankings. Although minor differences were observed in the normalized rankings among the 12 proteins, the overall trend in solidification propensity remained consistent (Figure S7C).

Omics Analysis of FACS-Sorted Cells. Our methodological framework is compatible with FACS, enabling downstream comprehensive proteomic and transcriptomic profiling of sorted cell populations. We successfully sorted HEK293 cells expressing PYP-FUS(P525L) into three groups: (1) group 3, characterized by solid protein aggregates exclusively labeled with PCAFA-orange; (2) group 2, comprising cells with liquid condensates labeled with both PCAFA-orange and PCAFA-far-red; and (3) group 2.5, representing an intermediate state located between groups 2 and 3. The efficacy of our sorting

process was validated by fluorescent scanning of SDS-PAGE gels from whole-cell lysates (Figure S5A,B). As expected, PYP-FUS(P525L) from group 3 was labeled solely by PCAF-orange, while samples from group 2 showed dual labeling. Group 2.5 displayed an intermediate labeling pattern.

To investigate the cellular consequences of condensate solidification, the sorted cells were subjected to RNA-seq and proteomic analyses. RNA-seq revealed a subset of genes significantly up- or downregulated in group 3 and 2.5 cells relative to group 2 cells ($\log_2[\text{group 3 or 2.5/group 2}] > 1$ or < -1 and $p\text{-value} < 0.05$) (Figure S9A,C). Gene Ontology (GO) analysis of significantly downregulated genes (biological process) revealed enrichment of chromatin-related terms such as “nucleosome assembly,” “nucleosome organization,” and “protein-DNA complex organization” in groups 3 and 2.5 compared to group 2 (Figure S10). While the direct role of these processes in condensate solidification is not fully understood, the data suggest that chromatin architecture may influence or reflect transitions in the condensate state. Gene Ontology (GO) analysis of the upregulated genes (biological process) displayed little enrichment in particular biological processes except for the upregulation of “extracellular structure organization” in group 2.5 cells and “cell projection organization” in group 3 cells (Figures S9B,D and S10, and Supporting Excel Files 1 and 2).

On the other hand, the proteomic analysis revealed more distinct expression profiles for group 3 and 2.5 cells. The numbers of upregulated proteins ($\log_2[\text{group 3 or 2.5/group 2}] > 1$ and $p\text{-value} < 0.05$) were 178 and 173 for group 3 and 2.5 cells, respectively (Figure S5C,E). GO analysis of these upregulated proteins showed strong enrichment for “extracellular structure organization” in both groups (Figures S5D,F and Supporting Excel File 3). Among the upregulated extracellular matrix proteins, we selected collagen type I $\alpha 2$ chain (COL1A2) and laminin subunit γ -1 (LAMC1), two major components of the extracellular matrix, for further validation. Consistent with the proteome data, the expression levels of COL1A2 and LAMC1 increased progressively during condensate solidification (Figure S11). Collectively, these findings demonstrate that the condensate solidification of FUS(P525L) is accompanied by the upregulated expression of extracellular matrix proteins, suggesting a previously unrecognized link between solid aggregate formation and extracellular matrix hardening.

DISCUSSION

The present study introduces a simple method using PYP and its specific fluorescent covalent ligands to distinguish between liquid and solid states of protein condensates in live cells. During the course of our study, other techniques have emerged that also address the detection of protein aggregation states. Zhang and co-workers designed a series of molecular rotor-based fluorescent probes that distinguish the misfolded oligomers from insoluble aggregates of HaloTag fusion proteins in live cells.^{25,26} Shen and co-workers developed a microscopic technique that integrates spatial dynamic mapping and reflective confocal dynamic speckle microscopy, underscoring the interface of biomolecular condensates as a critical element in driving pathological protein aggregation.¹⁷ Xu and co-workers employed a set of multidimensional super-resolution microscopic approaches to investigate aging mechanisms in the FUS condensates.²⁷ Our PYP-based fluorescent method uniquely reports the history or progress

of the liquid-to-solid transition of biomolecular condensates without the need for specialized instruments. Our method complements these existing techniques, expanding the toolkit available for studying protein aggregation dynamics.

Condensates of TDP-43 and FUS are well-known to transition into nonfunctional solid aggregates, leading to the onset of neuronal diseases. In fact, implementing our FACS-compatible technique to 12 phase-separating proteins and their mutants identified TDP-43, particularly its A315T mutant, as the protein that most readily forms solid aggregates. Intriguingly, our study also highlighted TIA1 and HNRNPH1 and 2 as previously under-recognized proteins that transition into solid states at rates comparable to or exceeding that of FUS. These RNA-binding proteins exhibit intrinsically disordered structures that enable phase separation. TIA1, a stress granule protein, interacts with hyperphosphorylated tau, promoting toxic oligomeric tau formation and driving neurodegeneration.^{28–30} HNRNPH1 and 2, members of the heterogeneous nuclear ribonucleoprotein (HNRNP) family, are implicated in neurodevelopmental disorders due to their mislocalization to the cytoplasm and association with stress granules under stress conditions.^{31,32} Despite their critical functions, these proteins were previously underestimated for their capability to transition into solid states. Our findings suggest a broader spectrum of proteins potentially involved in disease mechanisms through solid-state transitions.

Our FACS-compatible strategy also enabled the isolation of distinct cell populations based on condensate states, thereby facilitating subsequent proteomic and transcriptomic analyses. The transition from dynamic condensates to more solid states—particularly evident in group 3 and the intermediate group 2.5—correlated with the upregulation of extracellular matrix proteins. These findings suggest that intracellular condensate states may actively influence the extracellular matrix composition and contribute to matrix stiffening. The solidification of intracellular biomolecular condensates is likely to alter the cytoplasmic fluidity and elasticity, potentially generating mechanical forces that promote the synthesis of extracellular matrix components. However, the precise underlying mechanism by which solid condensates induce the expression of extracellular matrix proteins remains unclear. One plausible pathway involves the mechanotransduction effectors YAP and TAZ, which translocate to the nucleus in response to matrix stiffening and subsequently activate genes involved in fibrosis and stress adaptation.^{33–36} Additionally, mechanical forces are known to modulate TGF- β signaling, a key pathway in extracellular matrix remodeling and fibrosis.^{37,38} It is therefore conceivable that intracellular condensate solidification may similarly influence TGF- β signaling.

We also considered the reciprocal possibility that extracellular matrix stiffening could promote the liquid-to-solid transition of intracellular condensates. To address this, we examined the influence of the extracellular mechanical environment on FUS(P525L) solidification using Cytosoft Rigidity plates (2 and 16 kPa), which mimic soft and stiffer extracellular environments.³⁹ Our quantification of cells harboring solid aggregates, exclusively labeled by PCAF-orange, revealed no significant difference in solid aggregate formation between cells grown on soft and stiff matrices (Figure S12A,B). These results suggest that extracellular matrix stiffness itself is unlikely to directly drive FUS(P525L) aggregation.

While this study focused on the cellular effects of FUS(P525L) aggregates, our omics approach is broadly applicable and can be extended to other phase-separating proteins. Preliminary investigation of the sorted cells expressing PYP-TDP-43 indicated a significant upregulation of LAMC1 in group 3 when compared to both group 2.5 and group 2 (Figure S13A,B). Further investigation is warranted to elucidate the molecular links between the intracellular condensate states and the extracellular matrix dynamics.

The biological consequences of the observed upregulation of extracellular matrix proteins remain to be fully elucidated. However, such upregulation is commonly associated with increased tissue stiffness and fibrosis.⁴⁰ The resulting altered microenvironment can impair cellular functions by obstructing cell migration and perturbing signal transduction pathways, ultimately compromising tissue homeostasis and promoting pathological conditions.³⁸ Although these possibilities require future experimental validation, the observed link between biomolecular condensate solidification and extracellular matrix upregulation suggests a previously unrecognized mechanistic pathway through which intracellular phase transitions may drive extracellular matrix remodeling and contribute to disease progression.

CONCLUSIONS

We acknowledge several potential limitations of our methodology. First, the use of transient transfection may lead to heterogeneous gene expression levels, introducing variability that can complicate direct comparisons among different phase-separating proteins. Additionally, transient transfection is not directly applicable to studying endogenous proteins. A potential solution to this limitation involves the use of CRISPR/Cas9-mediated knock-in strategies, which enable the precise tagging of endogenous loci and facilitate analysis at physiological expression levels. Another important consideration is the diffusion and accessibility of PYP ligands within liquid condensates. A recent work by Ambady Thody et al.⁴¹ demonstrates that the partitioning of small molecules into condensates is primarily governed by hydrophobicity and aqueous solubility, underscoring the importance of careful PYP ligand selection for the purpose of labeling condensates. Nevertheless, our FACS-compatible method may be suited for chemical screening of environmental factors and existing drugs and for genetic screening using a guide RNA library. Overall, the methodology introduced here is conceptually straightforward and readily implemented, offering a framework for understanding the transition of biomolecular condensates and, consequently, developing therapeutics to delay this process.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.5c07340>.

Experimental details including plasmid construction, cell culture, confocal imaging, flow cytometry analysis, fluorescence recovery after photobleaching, protein expression and purification, super-resolution microscopy visualization, FACS sorting, RNA-sequencing, proteomic analysis, and Western blotting. (PDF)

Supporting Excel file 1 (RNA-seq data) (XLSX)

Supporting Excel file 2 (RNA-seq data) (XLSX)

Supporting Excel file 3 (proteomic data) (XLSX)

Supporting Video 1 (MP4)

Supporting Video 2 (MP4)

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