

Protein Folding Stability Simultaneously Imaged in Stress Granules and the Cytoplasm of a Single Living Cell

Mailin Becker ^{1,2,3,*}, Nirnay Samanta ^{3,*}, Erik Tsvetaev ³, Miká Vollet ³,
Sara S. Ribeiro ^{1,2,3} and Simon Ebbinghaus^{1,2,3,†}

¹Chair of Biophysical Chemistry, *Ruhr University Bochum*, Bochum, Germany

²Research Center Chemical Sciences and Sustainability, *Research Alliance Ruhr Bochum*, Germany

³Institute of Physical and Theoretical Chemistry, *TU Braunschweig*, Braunschweig, Germany



(Received 9 September 2025; accepted 12 June 2026; published 16 July 2026)

Protein folding is governed by distinct subcellular environments with unique biochemical compositions. Unfavorable conditions can lead to unfolding and aggregation, and cells deploy spatially targeted protein quality control mechanisms to promote folding. Understanding how proteostasis is balanced across diverse cellular conditions requires novel techniques that measure protein folding with high spatiotemporal resolution in living cells. Here we introduce confocal fast relaxation imaging (cFReI), which eliminates wide-field signal averaging compared to previous fast relaxation imaging approaches and enables quantification of biomolecular folding stability with enhanced axial resolution in three dimensions. We apply cFReI to monitor the folding stability of a destabilized monomeric apo variant of SOD1 (SOD1_{bar}) inside stress granules (SGs), comparing the measurements simultaneously to the surrounding cytoplasmic fluid of a single living cell. Surprisingly, SOD1_{bar} is not destabilized in SGs relative to the cytoplasm, contrary to expectations from previous *in vitro* studies. The study demonstrates that cFReI is a pioneering technique for measuring folding kinetics and thermodynamics of fluorescently labeled biomolecules in different biomolecular condensates compared to their respective intracellular fluids.

DOI: [10.1103/dsw6-t4qf](https://doi.org/10.1103/dsw6-t4qf)

I. INTRODUCTION

The crowded, compartmentalized, and spatially heterogeneous intracellular environment can substantially alter protein-folding landscapes compared to dilute buffer conditions. Distinct subcellular environments are formed by membraneless or membrane-enclosed organelles that exhibit local variations in biomolecular content and conditions. Protective protein quality control (PQC) mechanisms are adapted to cope with proteostasis challenges arising from unfavorable folding conditions in different subcellular locations.

Phase separation is a process that leads to the formation of biocondensates and has attracted significant attention because it enables cells to dynamically organize and regulate biochemical reactions in space and time [1,2]. Biocondensates accelerate and inhibit chemical reactions [3,4], regulate transcription and translation processes [5,6], as well as synaptic signaling [7,8], and coordinate fast stress response [9,10]. An emergent body of work associates the formation of biocondensates with disease progression, since proteins involved in amyloid-associated pathologies, like nuclear transactive

response DNA-binding protein 43 (TDP-43), α -synuclein, huntingtin, or microtubule-associated protein tau, play a crucial role in determining the phase behavior [11–19]. These proteins contain marginally stable and disordered domains, which are crucial to respond to environmental changes, but these properties also make the proteins prone to misfolding and aggregation [12,13,17].

Stress granules (SGs) are biocondensates that help cells to cope with stress [20]. Under stress, they fulfill diverse functions, including the stimulation of the immune response, modulation of signaling pathways [21], or serving as PQC compartments, transiently sequestering mRNA and other cellular components [22]. Although SGs exhibit a density comparable to that of the cytoplasm [23], the mobility of many biomolecules is decreased, and their composition is different [24]. RNAs and specific proteins are enriched within SGs, while others are excluded [25,26]. Misfolded proteins have been identified to accumulate inside SGs. Examples are superoxide dismutase 1 (SOD1) [27–29] and a temperature-sensitive mutant form of the ubiquitin-conjugating enzyme 9 (Ubc9TS) [28]. However, it is unclear if SGs could act as protective protein quality control hubs, sequestering misfolded proteins, or, alternatively, as vulnerable sites susceptible to toxic aggregation and aberrant phase transitions.

To investigate how biocondensates modulate the protein-folding landscape, novel techniques are needed that can probe protein folding with high spatiotemporal resolution. Therefore, we develop confocal fast relaxation imaging (cFReI) as a method that allows the triggering of protein unfolding by small, tailored temperature jumps (T jumps) in SGs and the

*These authors contributed equally to this work.

†Contact author: simon.ebbinghaus@rub.de

Published by the American Physical Society under the terms of the [Creative Commons Attribution 4.0 International](https://creativecommons.org/licenses/by/4.0/) license. Further distribution of this work must maintain attribution to the author(s) and the published article's title, journal citation, and DOI.

cytoplasm, simultaneously. The T jumps are induced by an infrared (IR) laser, and subsequent unfolding relaxation kinetics can be measured by confocal fluorescence imaging and Förster resonance energy transfer (FRET). Compared to previous fast relaxation imaging (FReI) wide-field approaches, the confocal resolution allows to distinguish the signals obtained from SGs and the respective cytoplasmic regions, allowing for a quantitative comparison of the two distinct cellular environments. We use consecutive T jumps to measure the folding equilibrium of a destabilized monomeric apo variant of superoxide dismutase 1 (SOD1_{bar}) as a folding reporter [29–31] in SGs in comparison to the cytoplasm of the same HeLa cell. Our study reveals that cytoplasmic SOD1_{bar} is destabilized under cell stress, in agreement with previous studies. Surprisingly, we find that, in SGs of most cells, the protein is more stable compared to the cytoplasm. This is in contrast to previous *in vitro* [32] studies that cannot account for the different cellular factors contributing to folding stability in the respective intracellular environments.

II. RESULTS

A. Implementation of confocal fast relaxation imaging

FReI is a well-established technique that combines fast IR laser-induced heating with high-speed FRET imaging to conduct relaxation experiments with spatiotemporal resolution in living cells [33,34]. The technique was used in previous research to analyze equilibrium thermodynamics as well as folding and self-association kinetics on timescales ranging from a few milliseconds to minutes in living cells [35–37]. Previous FReI instruments were built on wide-field microscopes that allow fast image acquisition, but lack sensitivity and resolution for subcellular applications and were therefore mainly constrained to measuring the folding dynamics of proteins or nucleic acids in the cytoplasm [33,38]. A workaround is to target the protein of interest to subcellular environments, specifically membrane-encompassed organelles, by using localization tags. For example, phosphoglycerate kinase was investigated in the nucleus and endoplasmic reticulum [39], and SOD1_{bar} was investigated in the endoplasmic reticulum, mitochondria, and lysosomes [40]. This approach is, however, not applicable to membraneless biomolecular condensates. Furthermore, SGs are small in size (0.1–4 μm) [41] compared, e.g., to the nucleus (about 11.4 μm) [42] and wide-field FReI would collect cytoplasmic signals from above or below SGs that would falsify the assessment of thermodynamic and kinetic data between the two compartments. This is not the case for cFReI, where the signal is analyzed from confocal planes containing SGs (see Fig. S1 of the Supplemental Material [43] and Appendix D).

To build the cFReI technique, we integrate an IR laser (1550 nm) into a confocal laser scanning microscope (FV3000, Olympus; Fig. 1) using the same beam path as for confocal microscopy. The cells are imaged using a 60 $\times$ water objective lens (UPLSAPO 60XW, Olympus, Japan) with a numerical aperture of 1.2. The fluorescence intensities of the FRET donor (500–556 nm) and FRET acceptor (609–709 nm) are detected by high-sensitivity spectral detectors (Olympus). The IR diode laser was operated in

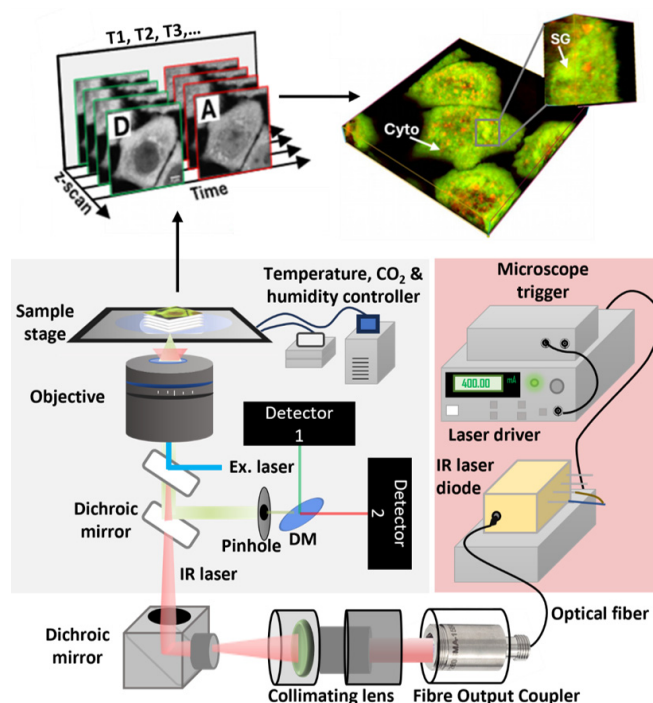


FIG. 1. Illustration of the cFReI technique. The cFReI setup combines a confocal laser scanning microscope (shaded gray) with IR laser heating (shaded red). The fiber-coupled IR laser (1550 nm) is collimated by a lens, reflected by a dichroic mirror, and implemented into the confocal imaging unit (shaded gray) using a dichroic mirror. The IR laser output power is controlled by a laser driver that is synchronized with confocal image acquisition, leading to confocal z stacks recorded for the different temperatures ($T_1, T_2, T_3, \dots$) in a cFReI experiment.

continuous-wave (cw) mode, and its output power is controlled by modulating the laser driver current. The IR laser output power is modulated with millisecond temporal resolution to achieve rectangular heating profiles that allow separation of temperature-dependent responses of the fluorophores from the observed folding kinetics [33]. The laser light is guided through an optical fiber and collimated on the back aperture of the microscope objective to defocus the IR laser onto the cells. This approach ensures broad and homogeneous heating of the cell to induce the same temperature change simultaneously inside the SGs and in the cytoplasm. Heterogeneous heating (e.g., spot heating by a focused IR laser beam) would lead to fluid dynamics in the cell [44] that would be detrimental for cFReI measurements. For IR laser alignment, the temperature is measured using Rhodamine B (RhB) as a molecular thermometer [45] using the temperature dependency of its quantum yield [46] (see Fig. S2 of the Supplemental Material [43]). To determine the laser intensity required for heating, we increase the laser intensity in 0.1% steps and calculate the resulting temperature, as described in Appendix E. To record folding over a wide range of temperatures, a stepwise heating profile is established of 14 consecutive T jumps with an amplitude of 2.3 K for each T jump [Figs. 2(a) and 2(b)], leading to unfolding of the FRET-labeled protein. As FRET decreases with unfolding, an increase in the donor (D) to acceptor (A)

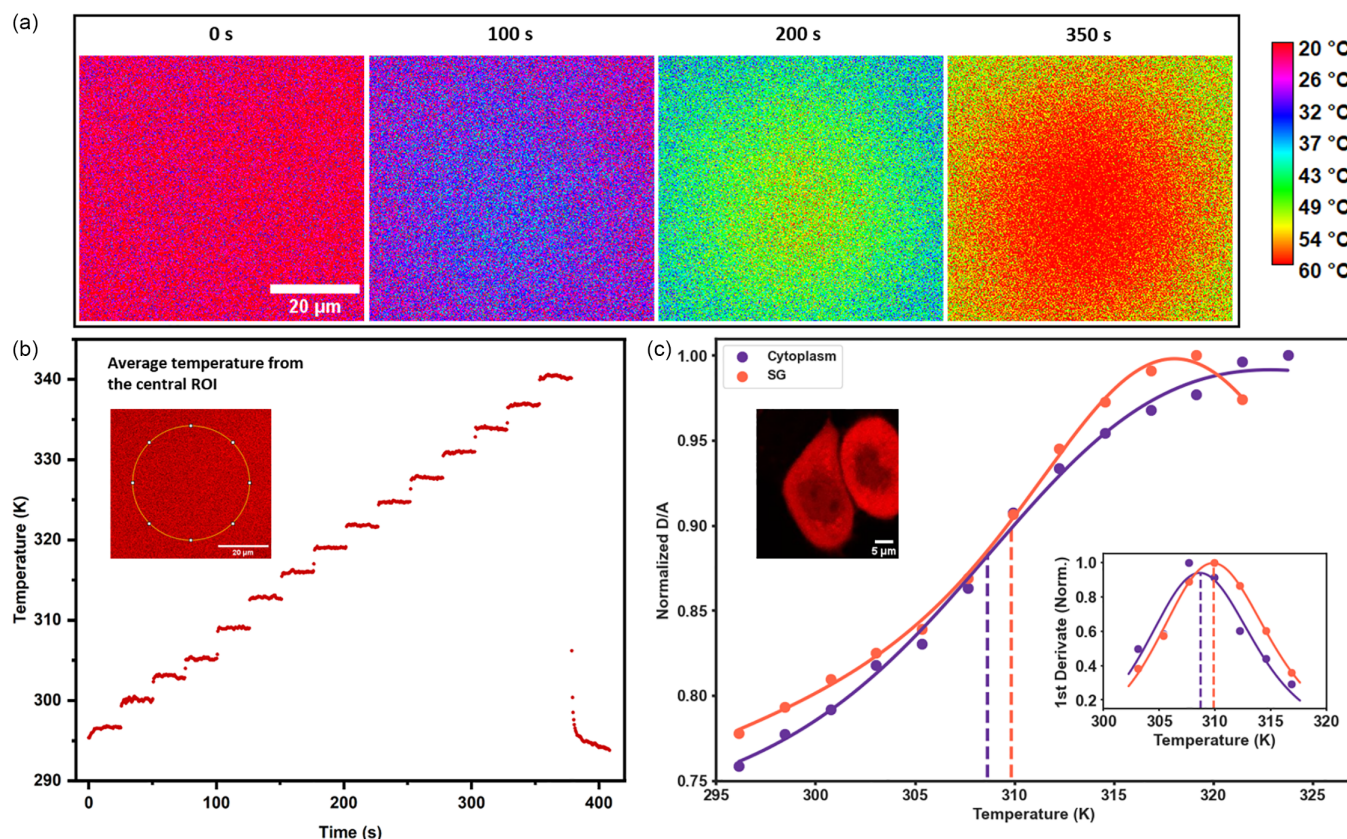


FIG. 2. Heating and heat-induced unfolding by cFReI. (a) Exemplary spatial temperature profiles at different temperatures. (b) Temperature change at different times of a T jump experiment. The temperature scale is generated by calibration using the temperature-dependent fluorescence of Rhodamine B. The graph shows the average temperature in the region of interest (inset). (c) Exemplary in-cell thermal unfolding curves and first derivatives (inset) in the SGs and the cytoplasm of the same HeLa cell. The respective melting temperatures T_m are marked by dashed lines. Additional details on SG visualization and data treatment are provided in Appendix F.

channel intensity was observed (see Fig. S3 [43]). Confocal point scanning is then used at each temperature to acquire D/A fluorescence intensity images for seven different focal planes with 1-μm resolution, recording 512×512 pixels for each plane with a dwell time of 2 μs/pixel (Appendix D). The thermal unfolding curves have the steepest slope at the melting point, so the first derivative can be used to determine the melting temperature (T_m) precisely [Fig. 2(c); see Appendixes F and G].

B. SOD1_{bar}-A4V folding stability under cell stress in stress granules compared to the cytoplasm

In this study, we measure the folding stability of the protein SOD1_{bar}-A4V, which was previously investigated by our laboratory in the cytoplasm, in the endoplasmic reticulum, and in the mitochondria of HeLa cells [29,40]. SOD1_{bar} is a monomeric apo variant of full-length SOD1, in which loop IV and loop VII are truncated and replaced by a Gly-Ala-Gly linker. The truncation disrupts the dimeric interface and prevents aggregation [30]. These modifications lead to loss of the metal-binding site of the protein. In comparison with the demetallized full-length SOD1, the truncation leads to an increase in thermodynamic stability, while causing only minor alterations of the β-barrel structure. The truncated protein folds reversibly inside cells, allowing for a thermodynamic

analysis [30,31]. In a previous study, we investigated the cytoplasmic folding stability [31] as well as the partition coefficient to SGs of different SOD1_{bar} mutants [29]. For the present study, we select the destabilized A4V mutant, as it shows the highest partitioning into SGs among the mutants examined [29]. In accordance with the earlier studies, we use AcGFP1 (FRET donor) and mCherry (FRET acceptor) labels at the N-terminus and C-terminus, respectively. The labels do not change the melting temperature of the protein, as previously demonstrated by FReI and NMR studies [31,47]. To identify the SGs, stable HeLa cells expressing human FUS (fused in sarcoma), labeled at the C-terminus with mCherry (HeLa^{FUS-mCh}), are used [48]. The cells are transiently transfected with AcGFP1-SOD1_{bar}-A4V-mCherry 48 h prior to measurement. The T_m of SOD1_{bar}-A4V, measured by cFReI in the cytoplasm, is 311.64 ± 0.64 K, which is in agreement with previous wide-field measurements (311.70 ± 0.31 K) [31], showing the successful establishment of the cFReI technique.

SGs are formed under different types of cellular stress, including heat stress (HS) [28], oxidative stress [49], endoplasmic reticulum (ER) stress [50], and osmotic stress [51]. We induce SG formation by exposing HeLa^{FUS-mCh} cells expressing SOD1_{bar}-A4V to HS for 120 min at 43 °C [28] or oxidative stress induced by arsenate (1 mM of Na₃AsO₄ at 37 °C for 120 min) [28,52,53]. SGs under HS have an average

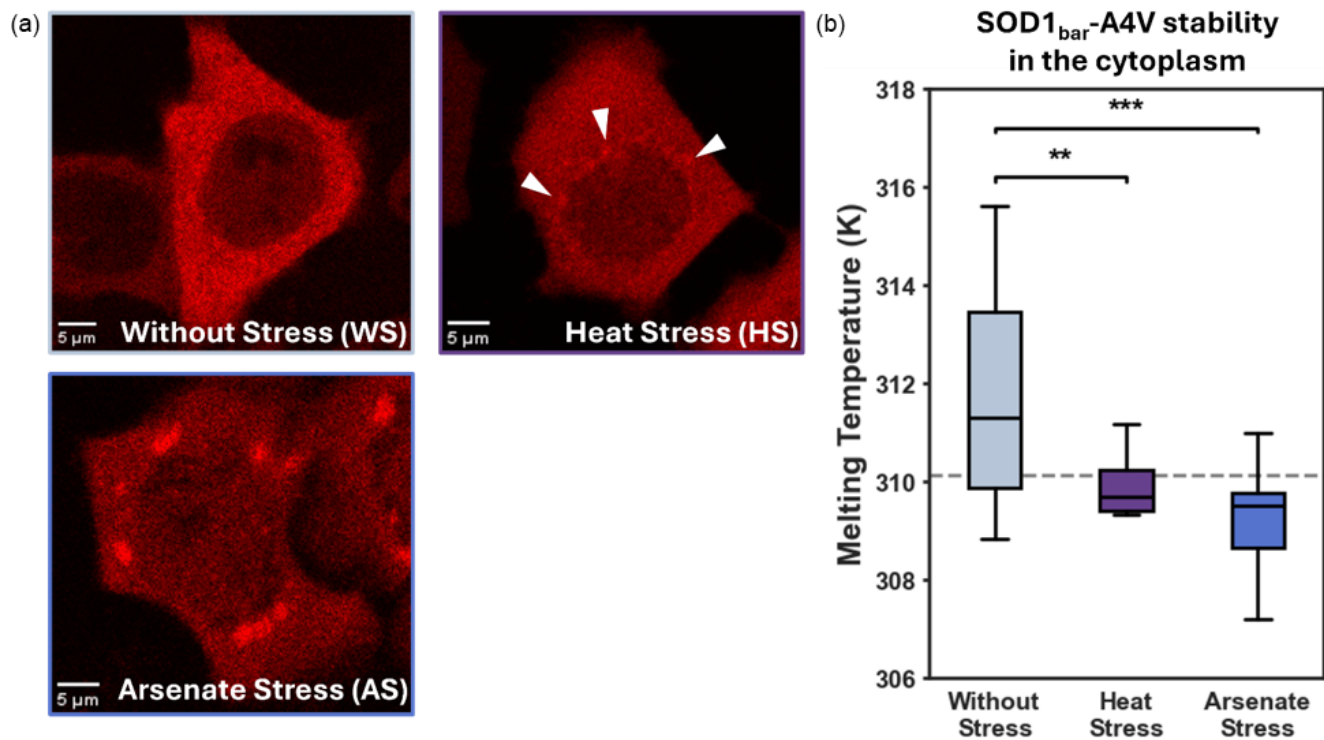


FIG. 3. Exemplary HeLa^{FUS-mCh} cells and comparison of the SOD1_{bar}-A4V folding stability (T_m) in the cytoplasm under HS and AS. (a) Exemplary images of HeLa^{FUS-mCh} cells transfected with SOD1_{bar}-A4V (mCherry channel). SOD1_{bar}-A4V is homogeneously distributed in the cytoplasm under physiological conditions. Distinct sizes of SGs are observed under HS and AS. (b) Boxplots of T_m in the cytoplasm for all three conditions [N = 19 for physiological conditions (Table SIII [43]), N = 15 for HS, N = 16 for AS]. The gray dashed line indicates physiological temperature. The boxplots show the first quartile, median, third quartile, and whiskers representing 1.5 times the interquartile range (IQR). Statistical analyses are performed using a two-sample *t*-test, with a 95% confidence interval. Asterisks indicate levels of significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Nonsignificant changes are indicated by “ns.”

size of $1.67 \pm 0.58 \mu\text{m}^2$ (more details and parameters are provided in Tables SI and SII of the Supplemental Material [43]). Under arsenate stress (AS), SGs have an increased size of $4.18 \pm 2.27 \mu\text{m}^2$. Former studies reported a diameter of 0.1–4 μm , depending on the type of stress [41]. Assuming a circular area of the SGs, this corresponds to an area up to $12.566 \mu\text{m}^2$, which is in agreement with our data. Both types of stress also affect the localization, folding, and aggregation of SOD1 in the cytosol itself [28,29]. We find that SOD1_{bar}-A4V is enriched in SGs, as shown by an increased partitioning coefficient [Fig. S4(a) [43]] in agreement with previous studies [29]. We then conduct cFREI experiments in cells forming SGs under HS and AS [Fig. 3(a)]. The cFREI experiments show that the folding reversibility is retained in the cytoplasm and SGs due to a recovery of the D/A values after the T jump (Tables SI and SII). Furthermore, protein aggregation, which is commonly observed by a decrease of the D/A ratio after the T jump [33], is not detected in the experiments. The observed melting temperatures in the cytoplasm and SGs are further independent of the partitioning coefficient (P) between SGs and cytoplasm, as well as the size of SGs [Figs. S4(b) and S5 [43]].

First, we analyze the folding stabilities in the cytoplasm, excluding the SGs. The T_m under both types of stress (HS, $309.72 \pm 0.87 \text{ K}$; AS, $309.38 \pm 1.28 \text{ K}$) shows a

significant decrease when compared to physiological conditions ($311.64 \pm 2.05 \text{ K}$) [Fig. 3(b)].

We then compare the stability of SOD1_{bar}-A4V in the SGs formed upon HS and AS with the stability of a nearby region in the cytoplasm (with the same size as the SG) of the same cell (Fig. 4, left). We plot the melting temperature in the SGs against the melting temperature in the cytoplasm (Fig. 4, right), with each point representing an individual HeLa cell. We find that, in most cells under HS, SOD1_{bar}-A4V is more stable in the SG environment compared to the cytoplasm. However, we observe that the differences in T_m (ΔT_m) are quite small, with a maximum difference of 3.91 K, and in total, the difference between SGs and the cytoplasm cell population is not significant (see Fig. S6 of the Supplemental Material [43]). A similar trend is observed for arsenate stress, where most cells exhibit a higher melting temperature within the SGs compared to the cytoplasm. For most cells, the differences in T_m between SG and cytoplasm are also quite small, with the exception of two HeLa cells, displaying a ΔT_m of more than 7 K.

III. DISCUSSION

Cell stress leads to the formation of SGs, constituting a novel environment within the “stressed” cytoplasm. To

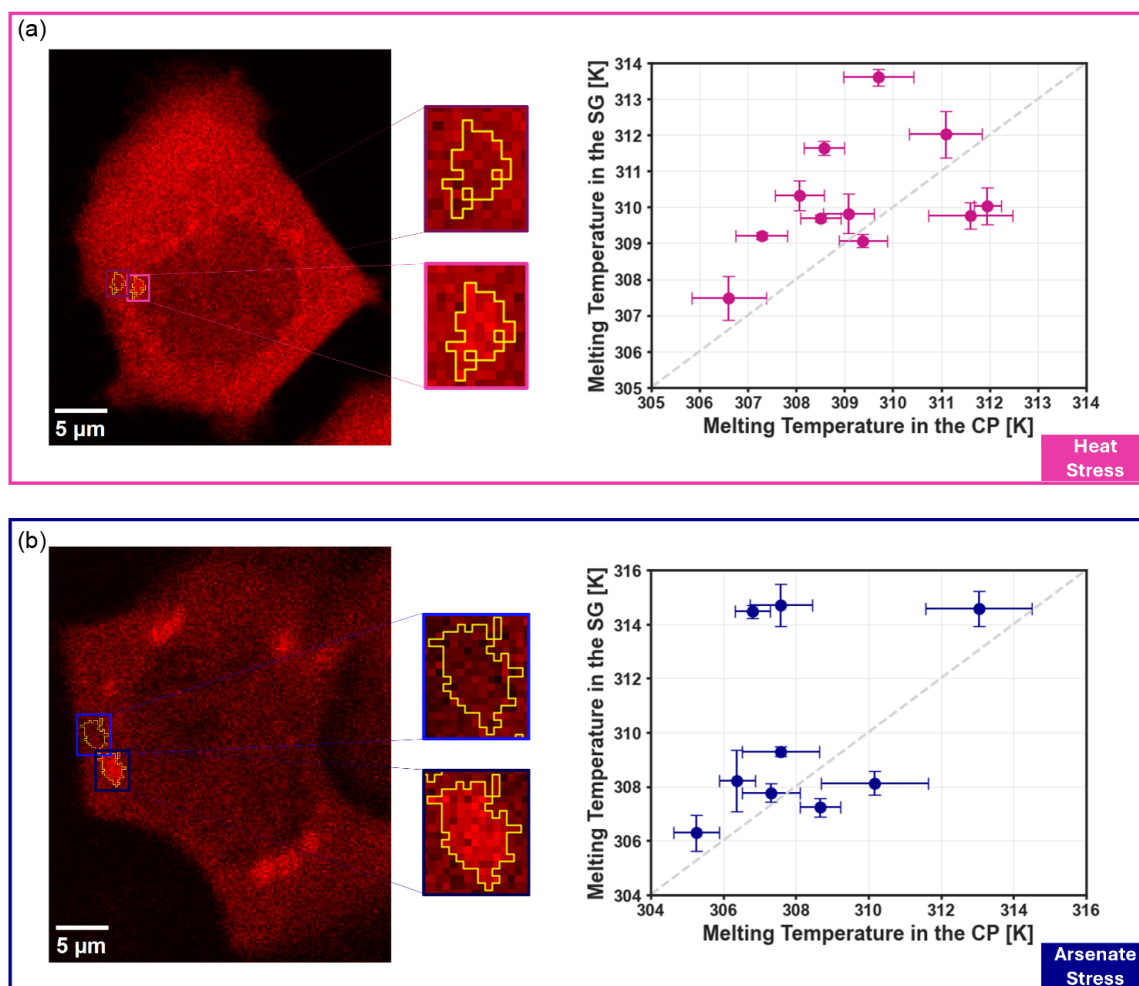


FIG. 4. Comparison of the T_m of SOD1_{bar}-A4V in SGs and the cytoplasm under HS and AS. Left: Exemplary images of HeLa^{FUS-mCh} cells, showing the analyzed regions of interest for the SGs and the corresponding cytoplasmic regions under (a) HS and (b) AS. Right: The graphs compare the measured T_m in SGs to the cytoplasm for each individual cell (data points).

clarify the role of phase separation in adjusting protein folding landscapes, their evolution in both environments must be considered. This motivates us to develop a technique that can resolve protein folding with high spatiotemporal resolution, simultaneously in SGs and the cytoplasm.

Similar to wide-field FReI, cFReI uses homogeneous heating of the entire cell and its vicinity by a defocused IR laser. In cFReI, we achieve this by using the same microscope objective as for confocal optical imaging. This configuration enables simultaneous bright-field imaging (e.g., differential interference contrast microscopy) that helps to identify cells and biomolecular condensates. The confocal scanning of the cFReI instrument is required to constrain the analysis to data obtained from SGs. The axial resolution is even more crucial if condensates are investigated that are smaller in dimension compared to SGs, e.g., nuclear speckles (0.8–1.8 μm) [54] or P-bodies (0.15–0.34 μm) [55].

In this study, we use cFReI to study the reversible folding-unfolding equilibrium of the folding reporter SOD1_{bar}. First, we observe that SOD1_{bar}-A4V is destabilized in the cytoplasm under cell stress for both HS and AS. This result is in agreement with a previous study showing a destabilization of

SOD1_{bar}-G41D in HeLa cells under proteostasis stress by inhibiting the 26S proteasome using MG132 [56]. One possible explanation could relate to the physicochemical changes of the cytoplasm of different cells under cell stress. Under both AS and HS, HeLa cells were reported to exhibit a decreased cell volume [57,58]. In *Escherichia coli* and yeast, reduced diffusivity was observed upon stress [59,60]. For yeast, this loss in mobility was reported to result from a pH drop that yielded a more solidlike state [60]. These observations point to an increase in cellular crowding upon HS and AS that could explain the destabilization of SOD1_{bar}-A4V [31].

Another explanation could be the preferential binding of upregulated Hsp70 [61,62] to SOD1_{bar}-A4V. Binding of the chaperone to unfolded protein and its function as a "holdase" could shift the folding equilibrium to the unfolded state, as observed *in vitro* [63] as well as in cells (HEK293) [64].

We then analyze the SOD1_{bar}-A4V folding stability in SGs. Our expectation is that SOD1_{bar}-A4V is destabilized inside SGs. This hypothesis is based on previous studies showing that SOD1 mutants with decreased folding stabilities preferentially associate with SGs [28,29,65]. The association is also shown to alter SGs' material properties and to promote

a detrimental phase transition [28,29]. Another study further showed that unfolded SOD1 is preferentially sequestered within reconstituted *in vitro* condensates formed by the RNA-binding cell-cycle-associated protein 1 (CAPRIN1) [32]. The NMR-spectroscopy results revealed preferential interactions of CAPRIN1 with unfolded SOD1, while the folded state was less affected. This leads to a destabilization of the protein, in particular for the less folded immature forms of SOD1 [32].

However, we found that in most cells, the protein was even stabilized in the SGs in comparison to the cytoplasm. Based on previous studies, we discuss two factors that could lead to a shift in the folding equilibrium to the native state, counteracting the destabilization expected from *in vitro* studies. One reason could be a preferential engagement of molecular chaperones with unfolded SOD1_{bar}-A4V in the cytoplasm [66], which is expected to cause relative destabilization of the protein compared to its state within SGs with less chaperone engagement [63,64]. Indeed, previous studies showed that Hsp70 preferentially interacted with unfolded and misfolded barnase protein in the dilute cytosolic phase [67]. This proposal is also consistent with prior reports of Hsp70 preventing SOD1 sequestration into SGs [28]. The second explanation for the minor changes in folding stability of SOD1_{bar}-A4V could rely on the abovementioned crowding effects. Since the changes in crowding densities inside SGs are small when compared to the surrounding cytoplasm [23,68], the stability of SOD1_{bar}-A4V should also not change much if crowding is the main factor determining the local folding stability.

Next to these two examples, further differences in the local environments of the cytoplasm and SGs that need to be considered include electrochemical gradients, reactive oxygen species, or pH [60,69,70]. The protein may also be subject to further processing, such as posttranslational modifications that may change its folding stability [71,72]. Although deciphering the different factors that could contribute to protein stability in the cytoplasm and SGs under cell stresses is beyond the scope of this study, our insights are important to reveal their net effect.

ACKNOWLEDGMENTS

We would like to acknowledge the support by the German Research Foundation DFG: SPP 2191 (Project No. 402723784) and FOR 5872 (Project No. 545039200). We acknowledge Dr. Aliaksandr Halavatyi (EMBL, Heidelberg) for the help with the software interface of cFReI. We would like to acknowledge Rachida Ussuale for experimental help.

DATA AVAILABILITY

The data that support the findings of this article are not publicly available. The data are available from the authors upon reasonable request.

APPENDIX A: CELL CULTURE AND PROTEIN EXPRESSION

Stable HeLa cells expressing human FUS labeled at the C-terminus with mCherry are used [48], which has no significant

effect on the T_m in comparison to HeLa without FUS-mCherry (see Fig. S7 of the Supplemental Material [43]). The cells are cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with GlutaMAX (Gibco), 4.5 g/L glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells are regularly maintained in a sterile T-25 flask and incubated at 37 °C in a humidified incubator with 10% CO₂.

APPENDIX B: TRANSIENT TRANSFECTION OF SOD1_{bar}-A4V

Transient transfection with AcGFP1-SOD1_{bar}-A4V-mCherry (the sequence of SOD1_{bar} is in Table SIV in the Supplemental Material [43]) is performed 48 h before measurement using a Lipofectamine 3000 transfection kit. Briefly, 4 µL Lipofectamine 3000 are mixed with 125 µL Opti-MEM and added to a mixture of 1 µg plasmid, 2 µL P3000 reagent, and 125 µL Opti-MEM. The mixture is incubated for 15 min and then transferred to a FluoroDish with HeLa^{FUS-mCh} cells in an antibiotics-free medium, seeded 24 h prior to transfection. The cells are incubated at 37 °C for 6 h. Cells are then washed with DPBS and maintained under regular conditions.

APPENDIX C: STRESS INDUCTION

For HS, the transfected cells are washed with DPBS buffer and equilibrated at 37 °C with growth medium for 5 min. To induce HS, the cells are transferred into another incubator with 5% CO₂ at 43 °C. After 120 min of incubation, the measurement is performed. For AS, cells are washed with DPBS buffer and incubated with 1 mM of sodium arsenate in the regular growth medium at 37 °C for 120 min before measurement.

APPENDIX D: cFReI MEASUREMENTS

For FRET measurements, AcGFP1 is excited by a 488-nm laser. Direct mCherry excitation at 561 nm is only used to localize SGs. Enrichment of SOD1_{bar}-A4V inside SGs is determined by its local partitioning coefficient (P) via AcGFP1 fluorescence emission:

$$P = \frac{I_{SG} - I_{background}}{I_{CP} - I_{background}}. \quad (D1)$$

Before the T-jump experiment, the sample stage is set to a fixed temperature of 23 °C ± 0.1 °C using a temperature controller (Okolab, Italy). Each of the 14 T jumps rapidly increases the temperature by about 2.3 K. Between each jump, there is a (un)folding equilibrium time of 25 s. For each measurement, images from seven planes (about 1 µm) are acquired (z-drift compensation is kept on during the time-series scan).

APPENDIX E: TEMPERATURE CALIBRATION

For calibration of the T jumps, 50 µM Rhodamine B (RhB) solution is used. From the average intensity, the temperature

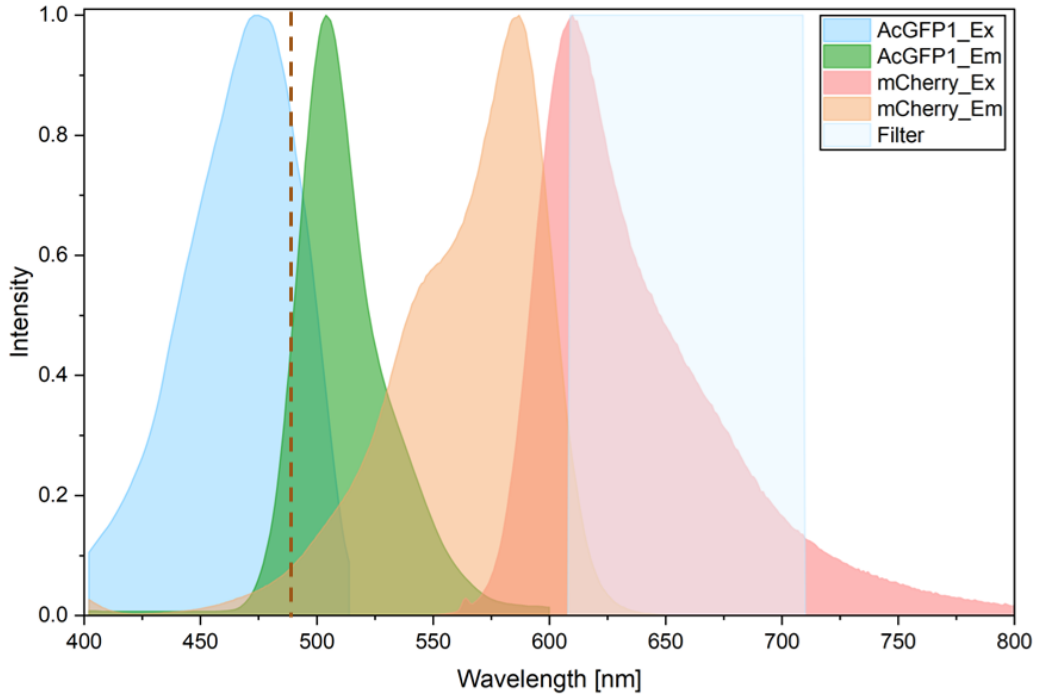


FIG. 5. Excitation and emission spectra of AcGFP1 (blue and green), excitation and emission spectra of mCherry (orange and red), excitation wavelength of the donor (dashed line). The light-blue shaded region represents the spectral detection window used for collecting the acceptor signal. Signal detection was performed using a volume-phase holographic diffraction grating.

could be acquired by using a third-order polynomial [73],

$$T(F) = 149.15 - 317.84F + 323.41F^2 - 131.84F^3, \quad (\text{E1})$$

where T is the temperature, and F is the RhB fluorescence intensity normalized by the intensity value at 23 °C. Prior to the T-jump experiment, the laser intensity I is increased linearly to measure the nonlinear temperature response $T(F[I])$. The corresponding curve is separated into linear and nonlinear response regimes and fitted to the reciprocal curve of the data with the functions $I(T) = mT + b$ (linear response) and $I(T) = T_0((T - a_1)/(a_2 - T))^{(1/p)}$ (nonlinear sigmoidal response).

To confirm the accuracy of the calibration, the laser intensity is increased so that there is a linear increase of the temperature T . The set temperature is then compared with the actual temperature of the sample. If the temperatures are matching, the corresponding laser intensities are used for the measurement.

APPENDIX F: DATA ANALYSIS

Image processing is done with ImageJ/Fiji [74]. Donor and acceptor intensities are acquired for the central plane and the two adjacent planes, with a 1- μm distance between each plane. To identify the SGs, the MaxEntropy global thresholding method is used for each cell [75]. A self-written PYTHON code is used to calculate the D/A ratio. The last two or three points of each jump are averaged and plotted against time to obtain the melting curve. As the two-state model for the folding process is valid for SOD1_{bar} [30,31], the van 't Hoff relationship is used to fit the first derivative of the melting

curve to Eq. (F1) [35,76]:

$$\frac{d\frac{D}{A}}{dT} = B \frac{e^{\frac{\Delta H_v}{R}(\frac{1}{T_m} - \frac{1}{T})}}{e^{\frac{\Delta H_v}{R}(\frac{1}{T_m} - \frac{1}{T})} + 1} \left(1 - \frac{e^{\frac{\Delta H_v}{R}(\frac{1}{T_m} - \frac{1}{T})}}{e^{\frac{\Delta H_v}{R}(\frac{1}{T_m} - \frac{1}{T})} + 1} \right) T^2. \quad (\text{F1})$$

There are three fitting parameters: the scaling factor B , the van 't Hoff enthalpy ΔH_v , and the melting temperature T_m [35,76]. Fitting is performed using OriginPro 2023, and graphs are created in PYTHON [77,78]. Statistical analyses are performed using a two-sample t -test, with a 95% confidence interval. Asterisks indicate levels of significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Nonsignificant changes are indicated with “ns.”

APPENDIX G: QUANTIFICATION OF DONOR BLEEDTHROUGH AND DIRECT ACCEPTOR EXCITATION IN cFREi

For quantification of the donor bleedthrough and direct acceptor excitation, we transfect HeLa-FUS-mCherry cells as described in Appendix B, using a SOD1_{bar} plasmid, only containing AcGFP1 as a fluorescent protein.

First, we determine the donor bleedthrough correction factor α . The donor bleedthrough is the donor emission that is detected by the acceptor channel (Fig. 5), which would contribute to the acceptor fluorescence intensity.

To determine α [Eq. (G1)], we analyze cell images that do not show any emission in the acceptor channel when the

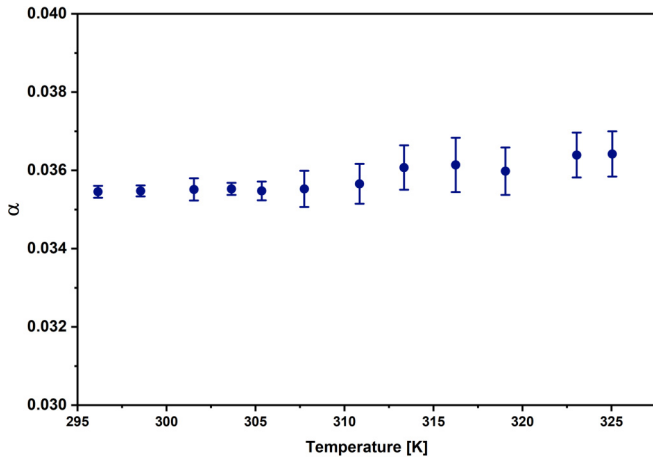


FIG. 6. Donor bleedthrough correction factor α at different temperatures in a cFReI experiment.

acceptor is excited:

$$\alpha = \frac{E_{DO}}{1 - E_{DO}} = \frac{\frac{I_{Aem/Dex}}{I_{Aem/Dex} + I_{Dem/Dex}}}{1 - \frac{I_{Aem/Dex}}{I_{Aem/Dex} + I_{Dem/Dex}}} = \frac{I_{Aem/Dex}}{I_{Dem/Dex}}. \quad (G1)$$

In Eq. (G1), E_{DO} is the FRET efficiency when only the donor is present, $I_{Aem/Dex}$ is the acceptor fluorescence intensity when the donor is excited, and $I_{Dem/Dex}$ is the donor fluorescence intensity when the donor is excited [79]. As described in the Results, the acceptor is detected from 609 to 709 nm, which should have almost no overlap with the emission spectrum of the donor (Fig. 5). As expected, the values for α are rather small (Fig. 6). The figure also reveals that α shows only minor changes with temperature.

We also determine the correction factor for direct acceptor excitation, δ [Eq. (G2)], which occurs because the excitation wavelength of the donor overlaps with the excitation spectrum of the acceptor (Fig. 5), so that the acceptor is directly excited (without FRET). This emission also contributes to the acceptor fluorescence intensity.

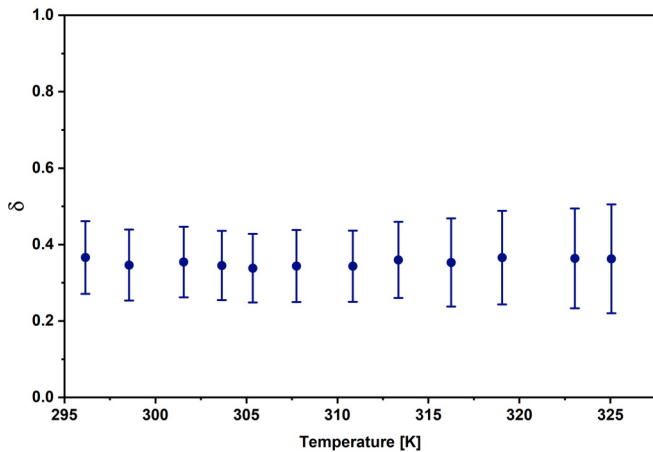


FIG. 7. Direct acceptor excitation correction factor δ at different temperatures in a cFReI experiment.

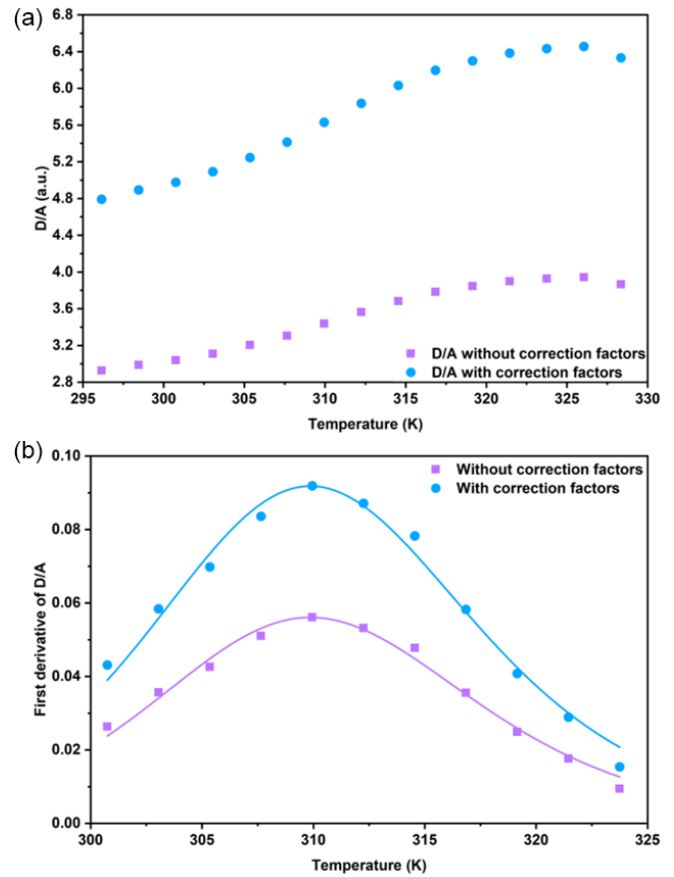


FIG. 8. (a) Representative melting curves of SOD1_{bar}-A4V in HeLa-FUS-mCherry cells at physiological conditions. The blue curve is corrected for donor bleedthrough and direct excitation. (b) First derivative (points) and fitted melting temperatures (lines) of the melting curves from (a).

To quantify δ , we use images of cells that do not show any donor emission when the donor is excited:

$$\begin{aligned} \delta &= \frac{S_{AO}}{1 - S_{AO}} = \frac{\frac{I_{Aem/Dex} + I_{Dem/Dex}}{I_{Aem/Aex} + I_{Aem/Dex} + I_{Dem/Dex}}}{1 - \frac{I_{Aem/Dex} + I_{Dem/Dex}}{I_{Aem/Aex} + I_{Aem/Dex} + I_{Dem/Dex}}} \\ &= \frac{I_{Aem/Dex} + I_{Dem/Dex}}{I_{Aem/Aex}}. \end{aligned} \quad (G2)$$

In Eq. (G2), S_{AO} is the stoichiometry when only the acceptor is present, $I_{Aem/Dex}$ is the acceptor fluorescence intensity when the donor is excited, $I_{Dem/Dex}$ is the donor fluorescence intensity when the donor is excited, and $I_{Aem/Aex}$ is the acceptor fluorescence intensity when the acceptor is excited [79]. The mean value of δ is determined to be 0.3536 ± 0.0936 (Fig. 7) and no temperature dependence is observed in the cFReI experiment.

To evaluate how the correction factors influence the cFReI data analysis, we compare the melting curves and the first derivative of corrected D/A values with uncorrected D/A values for a representative dataset. We use α and δ to obtain the corrected FRET acceptor fluorescence intensity $I_{Aem/Dex,corr}$

[Eq. (G3)]:

$$I_{\text{Aem/Dex,corr}} = I_{\text{Aem/Dex}} - \alpha I_{\text{Aem/Dex}} - \delta I_{\text{Aem/Dex}}. \quad (\text{G3})$$

In Eq. (G3), $I_{\text{Aem/Dex}}$ is the acceptor fluorescence intensity in the cFRET experiment. The obtained melting curves [Fig. 8(a)] and the first-derivative curves [Fig. 8(b)] are

comparable in shape (with different amplitudes, which do not change the determination of T_m). Fitting with Eq. (F1) yields $T_m = 309.57 \pm 0.20$ K for the corrected as well as the uncorrected dataset, showing that both donor bleedthrough and direct acceptor excitation have no influence on the determination of T_m .

- [1] C. P. Brangwynne, C. R. Eckmann, D. S. Courson, A. Rybarska, C. Hoege, J. Gharakhani, F. Jülicher, and A. A. Hyman, Germline P granules are liquid droplets that localize by controlled dissolution/condensation, *Science* **324**, 1729 (2009).
- [2] S. F. Banani, H. O. Lee, A. A. Hyman, and M. K. Rosen, Biomolecular condensates: Organizers of cellular biochemistry, *Nat. Rev. Mol. Cell Biol.* **18**, 285 (2017).
- [3] W. Peeples and M. K. Rosen, Mechanistic dissection of increased enzymatic rate in a phase-separated compartment, *Nat. Chem. Biol.* **17**, 693 (2021).
- [4] F. Stoffel, M. Papp, M. Gil-Garcia, A. M. Küffner, A. I. Benítez-Mateos, R. P. B. Jacquat, N. Galvanetto, L. Faltova, and P. Arosio, Enhancement of enzymatic activity by biomolecular condensates through pH buffering, *Nat. Commun.* **16**, 6368 (2025).
- [5] G. Pei, H. Lyons, P. Li, and B. R. Sabari, Transcription regulation by biomolecular condensates, *Nat. Rev. Mol. Cell Biol.* **26**, 213 (2025).
- [6] C. Iserman *et al.*, Condensation of Ded1p promotes a translational switch from housekeeping to stress protein production, *Cell* **181**, 818 (2020).
- [7] X. Chen, X. Wu, H. Wu, and M. Zhang, Phase separation at the synapse, *Nat. Neurosci.* **23**, 301 (2020).
- [8] J. Lützkendorf and S. J. Sigrist, Condensate dynamics at the synapse: Phase separation tunes presynaptic function, *PLoS Biol.* **23**, e3003201 (2025).
- [9] T. J. Nott *et al.*, Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles, *Mol. Cell* **57**, 936 (2015).
- [10] J. A. Riback, C. D. Katanski, J. L. Kear-Scott, E. V. Pilipenko, A. E. Rojek, T. R. Sosnick, and D. A. Drummond, Stress-triggered phase separation is an adaptive, evolutionarily tuned response, *Cell* **168**, 1028 (2017).
- [11] T. R. Peskett, F. Rau, J. O'Driscoll, R. Patani, A. R. Lowe, and H. R. Saibil, A liquid to solid phase transition underlying pathological huntingtin exon1 aggregation, *Mol. Cell* **70**, 588 (2018).
- [12] J. Wen, L. Hong, G. Krainer, Q.-Q. Yao, T. P. J. Knowles, S. Wu, and S. Perrett, Conformational expansion of tau in condensates promotes irreversible aggregation, *J. Am. Chem. Soc.* **143**, 13056 (2021).
- [13] X. Yan *et al.*, Intra-condensate demixing of TDP-43 inside stress granules generates pathological aggregates, *Cell* **188**, 4123 (2025).
- [14] N. M. Scherer *et al.*, RNA-binding properties orchestrate TDP-43 homeostasis through condensate formation in vivo, *Nucleic Acids Res.* **52**, 5301 (2024).
- [15] S. T. Dada, M. C. Hardenberg, Z. Toprakcioglu, L. K. Mrugalla, M. P. Cali, M. O. McKeon, E. Klimont, T. C. T. Michaels, T. P. J. Knowles, and M. Vendruscolo, Spontaneous nucleation and fast aggregate-dependent proliferation of α -synuclein aggregates within liquid condensates at neutral pH, *Proc. Natl. Acad. Sci. USA* **120**, e2208792120 (2023).
- [16] A. Röntgen, Z. Toprakcioglu, S. T. Dada, O. M. Morris, T. P. J. Knowles, and M. Vendruscolo, Aggregation of α -synuclein splice isoforms through a phase separation pathway, *Sci. Adv.* **11**, eadq5396 (2025).
- [17] Y. Shen *et al.*, The liquid-to-solid transition of FUS is promoted by the condensate surface, *Proc. Natl. Acad. Sci. USA* **120**, e2301366120 (2023).
- [18] W. P. Lipiński, B. S. Visser, I. Robu, M. A. A. Fakhree, S. Lindhoud, M. M. A. E. Claessens, and E. Spruijt, Biomolecular condensates can both accelerate and suppress aggregation of α -synuclein, *Sci. Adv.* **8**, eabq6495 (2022).
- [19] E. E. Boczek *et al.*, HspB8 prevents aberrant phase transitions of FUS by chaperoning its folded RNA-binding domain, *eLife* **10**, e69377 (2021).
- [20] P. Gallardo, S. Salas-Pino, and R. R. Daga, Reversible protein aggregation as cytoprotective mechanism against heat stress, *Curr. Genet.* **67**, 849 (2021).
- [21] D. S. W. Protter and R. Parker, Principles and properties of stress granules, *Trends Cell Biol.* **26**, 668 (2016).
- [22] S. Alberti and A. A. Hyman, Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing, *Nat. Rev. Mol. Cell Biol.* **22**, 196 (2021).
- [23] T. Kim, J. Yoo, S. Do, D. S. Hwang, Y. Park, and Y. Shin, RNA-mediated demixing transition of low-density condensates, *Nat. Commun.* **14**, 2425 (2023).
- [24] L. Streit, T. Kuhn, T. Vomhof, V. Bopp, A. C. Ludolph, J. H. Weishaupt, J. C. M. Gebhardt, J. Michaelis, and K. M. Danzer, Stress induced TDP-43 mobility loss independent of stress granules, *Nat. Commun.* **13**, 5480 (2022).
- [25] N. Curdy *et al.*, The proteome and transcriptome of stress granules and P bodies during human T lymphocyte activation, *Cell Rep.* **42**, 112211 (2023).
- [26] A. Khong, T. Matheny, S. Jain, S. F. Mitchell, J. R. Wheeler, and R. Parker, The stress granule transcriptome reveals principles of mRNA accumulation in stress granules, *Mol. Cell* **68**, 808 (2017).
- [27] J. Gal, L. Kuang, K. R. Barnett, B. Z. Zhu, S. C. Shissler, K. V. Korotkov, L. J. Hayward, E. J. Kasarskis, and H. Zhu, ALS mutant SOD1 interacts with G3BP1 and affects stress granule dynamics, *Acta Neuropathol.* **132**, 563 (2016).
- [28] D. Mateju, T. M. Franzmann, A. Patel, A. Kopach, E. E. Boczek, S. Maharana, H. O. Lee, S. Carra, A. A. Hyman, and S. Alberti, An aberrant phase transition of stress granules triggered by misfolded protein and prevented by chaperone function, *EMBO J.* **36**, 1669 (2017).

- [29] N. Samanta, S. S. Ribeiro, M. Becker, E. Laborie, R. Pollak, S. Timr, F. Sterpone, and S. Ebbinghaus, Sequestration of proteins in stress granules relies on the in-cell but not the in vitro folding stability, *J. Am. Chem. Soc.* **143**, 19909 (2021).
- [30] J. Danielsson, M. Kurnik, L. Lang, and M. Oliveberg, Cutting off functional loops from homodimeric enzyme superoxide dismutase 1 (SOD1) leaves monomeric β -barrels, *J. Biol. Chem.* **286**, 33070 (2011).
- [31] D. Gnutt, S. Timr, J. Ahlers, B. König, E. Manderfeld, M. Heyden, F. Sterpone, and S. Ebbinghaus, Stability effect of quinary interactions reversed by single point mutations, *J. Am. Chem. Soc.* **141**, 4660 (2019).
- [32] R. Ahmed, M. Liang, R. P. Hudson, A. K. Rangadurai, S. K. Huang, J. D. Forman-Kay, and L. E. Kay, Atomic resolution map of the solvent interactions driving SOD1 unfolding in CAPRIN1 condensates, *Proc. Natl. Acad. Sci. USA* **121**, e2408554121 (2024).
- [33] S. Ebbinghaus, A. Dhar, J. D. McDonald, and M. Gruebele, Protein folding stability and dynamics imaged in a living cell, *Nat. Methods* **7**, 319 (2010).
- [34] A. Dhar and M. Gruebele, Fast relaxation imaging in living cells, *Curr. Protoc. Protein Sci.* **65**, 28.1.1 (2011).
- [35] R. Feng, M. Gruebele, and C. M. Davis, Quantifying protein dynamics and stability in a living organism, *Nat. Commun.* **10**, 1179 (2019).
- [36] H. Yoo and C. M. Davis, An in vitro cytomimetic of in-cell RNA folding, *ChemBioChem* **23**, e202200406 (2022).
- [37] L. Kisley, K. A. Serrano, D. Guin, X. Kong, M. Gruebele, and D. E. Leckband, Direct imaging of protein stability and folding kinetics in hydrogels, *ACS Appl. Mater. Interfaces* **9**, 21606 (2017).
- [38] M. Gao, D. Gnutt, A. Orban, B. Appel, F. Righetti, R. Winter, F. Narberhaus, S. Müller, and S. Ebbinghaus, RNA hairpin folding in the crowded cell, *Angew. Chem. Int. Ed. Engl.* **55**, 3224 (2016).
- [39] A. Dhar, K. Girdhar, D. Singh, H. Gelman, S. Ebbinghaus, and M. Gruebele, Protein stability and folding kinetics in the nucleus and endoplasmic reticulum of eucaryotic cells, *Biophys. J.* **101**, 421 (2011).
- [40] S. S. Ribeiro *et al.*, Intracellular spatially-targeted chemical chaperones increase native state stability of mutant SOD1 barrel, *Biol. Chem.* **404**, 909 (2023).
- [41] H. Mahboubi and U. Stochaj, Cytoplasmic stress granules: Dynamic modulators of cell signaling and disease, *Biochim. Biophys. Acta* **1863**, 884 (2017).
- [42] M. Lherbette, Á. Dos Santos, Y. Hari-Gupta, N. Fili, C. P. Toseland, and I. A. T. Schaap, Atomic force microscopy micro-rheology reveals large structural inhomogeneities in single cell-nuclei, *Sci. Rep.* **7**, 8116 (2017).
- [43] See Supplemental Material at <http://link.aps.org/supplemental/10.1103/dsw6-t4qf> for Figs. S1–S7 and Tables SI–SIV.
- [44] G. Rapp, C. Van Ingen, E. Erben, N. Maghelli, M. Kreysing, and F. Huhn, Focused light induced cytoplasmic streaming (FLUCS): Non-invasive microscopic flow fields and their applications, *Biophys. J.* **121**, 426a (2022).
- [45] L. Sistemich and S. Ebbinghaus, Heat application in live cell imaging, *FEBS Open Bio* **14**, 1940 (2024).
- [46] S. Büning, A. Sharma, S. Vachharajani, E. Newcombe, A. Ormsby, M. Gao, D. Gnutt, T. Vöpel, D. M. Hatters, and S. Ebbinghaus, Conformational dynamics and self-association of intrinsically disordered huntingtin exon 1 in cells, *Phys. Chem. Chem. Phys.* **19**, 10738 (2017).
- [47] J. Danielsson *et al.*, Thermodynamics of protein destabilization in live cells, *Proc. Natl. Acad. Sci. USA* **112**, 12402 (2015).
- [48] I. Poser *et al.*, BAC TransgeneOmics: A high-throughput method for exploration of protein function in mammals, *Nat. Methods* **5**, 409 (2008).
- [49] T. Q.-T. Do, A. Gaudreau-Lapierre, C. G. Palii, V. M. F. Resende, D. Campuzano, C. S. Aeschmann, M. Brand, and L. Trinkle-Mulcahy, A nuclear stress pathway that parallels cytoplasmic stress granule formation, *iScience* **23**, 101664 (2020).
- [50] K. Fujimura, F. Kano, and M. Murata, Identification of PCBP2, a facilitator of IRES-mediated translation, as a novel constituent of stress granules and processing bodies, *RNA* **14**, 425 (2008).
- [51] W. Zeng, C. Lu, Y. Shi, C. Wu, X. Chen, C. Li, and J. Yao, Initiation of stress granule assembly by rapid clustering of IGF2BP proteins upon osmotic shock, *Biochim. Biophys. Acta* **1867**, 118795 (2020).
- [52] P. A. Ivanov, E. M. Chudinova, and E. S. Nadezhdina, Disruption of microtubules inhibits cytoplasmic ribonucleo-protein stress granule formation, *Exp. Cell. Res.* **290**, 227 (2003).
- [53] H. Uechi *et al.*, Small-molecule dissolution of stress granules by redox modulation benefits ALS models, *Nat. Chem. Biol.* **21**, 1577 (2025).
- [54] Y. S. Mao, B. Zhang, and D. L. Spector, Biogenesis and function of nuclear bodies, *Trends Genet.* **27**, 295 (2011).
- [55] N. Cougot, A. Cavalier, D. Thomas, and R. Gillet, The dual organization of P-bodies revealed by immunoelectron microscopy and electron tomography, *J. Mol. Biol.* **420**, 17 (2012).
- [56] D. Gnutt, L. Sistemich, and S. Ebbinghaus, Protein folding modulation in cells subject to differentiation and stress, *Front. Mol. Biosci.* **6**, 38 (2019).
- [57] V. Giliberti, L. Baldassarre, A. Rosa, V. De Turris, M. Ortolani, P. Calvani, and A. Nucara, Protein clustering in chemically stressed HeLa cells studied by infrared nanospectroscopy, *Nanoscale* **8**, 17560 (2016).
- [58] D. N. Wheatley, C. Kerr, and D. W. Gregory, Heat-induced damage to HeLa-S3 cells: Correlation of viability, permeability, osmosensitivity, phase-contrast light-, scanning electron- and transmission electron-microscopical findings, *Int. J. Hyperthermia* **5**, 145 (1989).
- [59] D. Di Bari *et al.*, Diffusive dynamics of bacterial proteome as a proxy of cell death, *ACS Cent. Sci.* **9**, 93 (2023).
- [60] M. C. Munder *et al.*, A pH-driven transition of the cytoplasm from a fluid- to a solid-like state promotes entry into dormancy, *eLife* **5**, e09347 (2016).
- [61] R. Oehler, E. Pusch, M. Zellner, P. Dangel, N. Hergovics, M. Homoncik, M. M. Eliassen, M. Brabec, and E. Roth, Cell type-specific variations in the induction of hsp70 in human leukocytes by feverlike whole body hyperthermia, *Cell Stress Chaperones* **6**, 306 (2001).
- [62] L. Bhagat, V. P. Singh, R. K. Dawra, and A. K. Saluja, Sodium arsenite induces heat shock protein 70 expression and protects against secretagogue-induced trypsinogen and NF- κ B activation, *J. Cell Physiol.* **215**, 37 (2008).

- [63] G. Molinari *et al.*, Multiple chaperone DnaK–FliC flagellin interactions are required for *Pseudomonas aeruginosa* flagellum assembly and indicate a new function for DnaK, *Microb. Biotechnol.* **18**, e70096 (2025).
- [64] R. J. Wood *et al.*, A biosensor-based framework to measure latent proteostasis capacity, *Nat. Commun.* **9**, 287 (2018).
- [65] N. E. Farrarwell, I. A. Lambert-Smith, S. T. Warraich, I. P. Blair, D. N. Saunders, D. M. Hatters, and J. J. Yerbury, Distinct partitioning of ALS associated TDP-43, FUS and SOD1 mutants into cellular inclusions, *Sci. Rep.* **5**, 13416 (2015).
- [66] F. Claes *et al.*, Exposure of a cryptic Hsp70 binding site determines the cytotoxicity of the ALS-associated SOD1-mutant A4V, *Protein Eng., Des. Sel.* **32**, 443 (2019).
- [67] K. M. Ruff, Y. H. Choi, D. Cox, A. R. Ormsby, Y. Myung, D. B. Ascher, S. E. Radford, R. V. Pappu, and D. M. Hatters, Sequence grammar underlying the unfolding and phase separation of globular proteins, *Mol. Cell* **82**, 3193 (2022).
- [68] R. Schlüßler *et al.*, Correlative all-optical quantification of mass density and mechanics of subcellular compartments with fluorescence specificity, *eLife* **11**, e68490 (2022).
- [69] M. C. Owen, D. Gnut, M. Gao, S. K. T. S. Wärmländer, J. Jarvet, A. Gräslund, R. Winter, S. Ebbinghaus, and B. Strodel, Effects of *in vivo* conditions on amyloid aggregation, *Chem. Soc. Rev.* **48**, 3946 (2019).
- [70] H. Gu, C. Gu, A. Du Toit, W. Yu, M. W. Chen, H. L. Struckman, J. R. Silva, Y. Dai, and A. G. Ewing, Single-entity resolution single-cell nanosensor reveals reactive oxygen species at stress granules are formed by interfacial redox chemistry, *J. Am. Chem. Soc.* **147**, 27020 (2025).
- [71] C. J. Banks and J. L. Andersen, Mechanisms of SOD1 regulation by post-translational modifications, *Redox. Biol.* **26**, 101270 (2019).
- [72] J. M. Fay, C. Zhu, E. A. Proctor, Y. Tao, W. Cui, H. Ke, and N. V. Dokholyan, A phosphomimetic mutation stabilizes SOD1 and rescues cell viability in the context of an ALS-associated mutation, *Structure* **24**, 1898 (2016).
- [73] R. Fu, B. Xu, and D. Li, Study of the temperature field in microchannels of a PDMS chip with embedded local heater using temperature-dependent fluorescent dye, *Int. J. Therm. Sci.* **45**, 841 (2006).
- [74] J. Schindelin *et al.*, Fiji: An open-source platform for biological-image analysis, *Nat. Methods* **9**, 676 (2012).
- [75] J. N. Kapur, P. K. Sahoo, and A. K. C. Wong, A new method for gray-level picture thresholding using the entropy of the histogram, *Comput. Vision, Graphics, Image Process.* **29**, 273 (1985).
- [76] D. M. John and K. M. Weeks, Van 't Hoff enthalpies without baselines, *Protein Sci.* **9**, 1416 (2000).
- [77] M. Waskom, Seaborn: Statistical data visualization, *J. Open Source Software* **6**, 3021 (2021).
- [78] J. D. Hunter, Matplotlib: A 2D graphics environment, *Comput. Sci. Eng.* **9**, 90 (2007).
- [79] B. Hellenkamp *et al.*, Precision and accuracy of single-molecule FRET measurements—a multi-laboratory benchmark study, *Nat. Methods* **15**, 669 (2018).