DropFit: Determination of the Critical Concentration for Protein Liquid–Liquid Phase Separation ☆

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Abstract

In the liquid–liquid phase separation of proteins, dense liquid droplets often form within the dilute phase below the critical concentration. The resulting size distribution of these precritical droplets can be described by a scale-invariant distribution, which is characterized by an increasing average as the concentration approaches from below the critical value. This phenomenon can be leveraged for the quantitative estimation of the critical concentration. Here, to facilitate applications of this approach, we present the DropFit web server (https://www-cohsoftware.ch.cam.ac.uk/index.php/dropfit). DropFit can be used to estimate the critical concentration using experimental data on the length, area, or volume of the precritical droplets, which are taken away from the critical concentration and thus be more accurate and reproducible. We anticipate that the accurate value of the critical concentration under different experimental conditions will help understand the contributions from different macromolecules to the formation of protein condensates, and to investigate the perturbations that lead to pathological processes through the disruption of membraneless organelles.

Introduction

The discovery that many proteins can undergo liquid–liquid phase separation to form dense, droplet-like condensates has considerably changed our understanding of cellular organization [1–10]. These biomolecular condensates, which lack enclosing lipid membranes, are now known to play key roles in a variety of physiological processes, including gene regulation, stress response, and intracellular signaling [1–10]. The underlying mechanism of liquid–liquid phase separation enables cells to spatially and temporally compartmentalize biochemical activities without the need for membrane-bound organelles [1–11].

The phase behavior of the proteins forming these condensates is tightly controlled under physiological conditions, and its dysregulation has been associated with numerous pathologies, including neurodegenerative diseases [1,12,13]. This realization has sparked intense interest in identifying the molecular principles governing protein phase separation and developing methods to quantify the conditions under which it occurs [1–10].

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Classical thermodynamic models, such as the Flory-Huggins theory, describe phase separation as a first-order transition, where a homogenous solution separates into dense and dilute phases above the critical concentration [8–10]. However, emerging experimental evidence has begun to challenge this framework [14–16]. Several studies have reported the presence of protein droplets even below the concentration typically associated with phase separation [14–16]. Furthermore, these pre-critical droplets, also known as nanoclusters, may be associated with specific biological behaviors [16]. This observation is not easily reconciled with classical nucleation theory, which predicts that such droplets should be small and unstable in subsaturated conditions [8–10,14–17].

The apparent formation of droplets in the pre-critical regime has prompted a reassessment of the underlying physical principles of protein condensation [14–16]. In these studies, the droplet size distributions of condensates formed by different proteins have been analyzed [15]. The droplet size distribution was found to exhibit a scale-invariant behavior that can be modeled by a log-normal distribution, suggesting a universal behavior of the phase separation process in biological systems, which may exhibit at least certain features characteristic of critical phenomena [15]. Furthermore, this behavior can be exploited for the quantitative estimation of the critical concentration [15].

In this context, further advances can be facilitated by introducing tools that can account for the presence of droplets below the transition point and provide accurate estimations of critical concentration. To address this need, we introduce DropFit – a web server designed to estimate the critical concentration of phase separation from experimental droplet size distributions. By providing a framework rooted in statistical physics, DropFit enables researchers to extract quantitative estimates of the critical concentration from measurements away from the critical concentration itself, where statistical fluctuations and experimental variability are small, thus offering a valuable resource for studying protein self-assembly in both health and disease.

Theoretical background of phase separation

The theoretical understanding of phase separation in protein systems has traditionally relied on concepts from polymer physics and thermodynamics [5,8,15]. One of the earliest and most influential frameworks is the Flory-Huggins theory, which describes phase separation as a first-order transition in homopolymer solutions [5,8,15]. This model has provided a useful starting point for describing how proteins separate into dilute and condensed phases. According to this theory, phase separation occurs when the free energy of mixing becomes unfavorable due to interactions between molecules, leading to the coexistence of two distinct phases [5,8,15]. The critical concentration marks the threshold at which these two phases begin to form. Extensions of the Flory-Huggins model, such as the Flory-Stockmayer theory, incorporate features of gelation and network formation that are particularly relevant for multivalent proteins and protein-RNA mixtures [5,8,15].

Despite their utility, these classical models face limitations in explaining recent experimental observations, in particular the formation of protein droplets in subsaturated solutions, at concentrations below the critical concentration [14,15]. These observations challenge the idea that droplet formation is governed by nucleation in a metastable regime. Classical nucleation theory implies that in such subsaturated conditions, the formation of large droplets is exponentially suppressed due to the high free energy cost of creating an interface between the droplet and its surrounding medium [5,8,15].

Yet, experimental studies have shown that sizeable droplets can form even under these conditions, suggesting the presence of possible alternative mechanisms [14,15]. This deviation from classical behavior implies that protein phase separation may not always conform to first-order transitions but may instead involve processes akin to critical phenomena [15,18]. In such transitions, a continuous increase in density fluctuations and a diverging correlation length lead to scale-invariant behavior – a hallmark of critical systems [15].

This scale invariance implies that the statistical properties of the system do not depend on microscopic details but instead follow universal laws [15]. In the context of protein condensation, it means that the droplet size distribution reflects an underlying universality that transcends the specific sequence or structure of the protein [15]. This idea is supported by the observation that droplet size distributions in subsaturated systems are well described by log-normal functions with scaling behavior [15].

Furthermore, the presence of such scale-invariant distributions indicates that the system may exhibit features of finite-size scaling, where the correlation length, which is related to the degree of collective behavior in the system, becomes large in biological systems [15]. This behavior mirrors that observed near the spinodal line in phase diagrams, where the dilute phase becomes unstable and fluctuations grow without bound [15]. Although the spinodal decomposition is typically associated with first-order transitions, its proximity to the critical point can create a regime where scale-invariant characteristics dominate the behavior of the system [15]. We note that scale-

invariance is characteristic of a wide range of biological systems describing phenomena related to hierarchical organization or multiplicative growth processes [19].

In this framework, droplet formation is no longer interpreted solely as a result of nucleation overcoming a free energy barrier, but instead as an emergent property of a system approaching criticality [15]. This conceptual shift has significant practical implications, as it suggests that the position of a protein on its phase diagram – and thus its physiological state – can be inferred from the statistical properties of its droplet size distribution.

DropFit is built upon this theoretical foundation by leveraging the scale-invariant nature of droplet formation in subsaturated systems. By analyzing the scaling properties of droplet size distributions, DropFit offers a robust means to estimate the critical concentration of phase separation and thus provides insights into the phase behavior of proteins under physiological conditions.

The DropFit approach and the scaling ansatz

DropFit is based on a scaling ansatz that characterizes droplet size distributions as scale-invariant and with a log-normal distribution [15]. The survival distribution function $P_>(s|\rho)$, representing the probability of droplets larger than size s at concentration ρ, follows

$$P_{>(s|\rho)} = s^{-\alpha} f(s/s_c) \quad (1)$$

Here, $s_c \sim |(\rho - \rho_c)|^{-\varphi}$ is a characteristic size diverging near the critical concentration. The parameters α and φ are non-negative critical exponents, while f is a universal scaling function. This framework allows for the estimation of ρ_c by analyzing the scaling behavior of droplet size moments.

By plotting the ratios of successive moments of the droplet size distribution against $1/|(\rho - \rho_c)|$, linear relationships emerge, allowing extraction of critical exponents and determination of ρ_c [15].

The DropFit method uses the scaling relationship of the form:

$$< s^{k+1} > / < s^k > \sim s_c \sim |(\rho - \rho_c)|^{-\varphi} \quad (2)$$

where *k* indicates the moments of the droplet sizes. Using various *k* values, the method plots the moment ratios. Then it performs a linear regression, where ρ_c can be estimated as the *x*-intercept (Figure 1). This approach provides consistent results across independent measurements and proteins [15].

DropFit workflow

The input for DropFit are experimental droplet dimensions (length, area, or volume) measured at minimum 2 protein concentrations in replicates and the *k* values (Figure 1A–C). In the first part, the program computes the critical concentration from the moments of the droplet sizes (steps i–vi) (Figure 1D–G), in the second part, it validates the log-normal behavior (steps vii–ix) (Figure 1H). Afterwards the user can validate the critical concentration computed with the droplet sizes at selected concentration values as well as determine the critical exponents using the selected ρ_c, which provide information about the underlying mechanism and possible deviations from criticality (steps (x–xi) (Figure 1I–K).

I. ***Computation of the critical concentration***

(i) Computation of the moments of the droplet sizes (s^k) for each replicate at each concentration using *k* values in 0.25–2.25 range. The *k* values can be automatically generated, or the user can provide these values as an optional input (Figure 1D).

(ii) Determination of the mean and standard deviation of the moments of the droplet sizes as $\langle s^k \rangle^{-1/k}$ for each concentration. The standard deviation is estimated from the replicates (Figure 1E).

(iii) Plot of the $\langle s^k \rangle^{-1/k}$ values against the concentration values (ρ).

(iv) Calculation of a weighted linear regression fit on the data point, where the weights are derived from the standard deviations. When there are no replicates for the given concentration but there are concentrations with replicates present, the standard deviation is derived from the standard deviations of concentrations with replicates. In case no replicates are provided, the standard deviation is set to 0.001 (Figure 1F).

(v) Determination of the values of the *x* cross-sections for each line, computed with different *k* values (Figure 1F).

(vi) Determination of the critical concentration as the mean of the cross-sections, and its standard deviation (Figure 1G).

Steps i–vi can be repeated using different concentrations and *k* values.

II. ***Validation of the log-normal behavior data collapse to a universal distribution***

Additionally, DropFit confirms that σ, the variance of the log-normal distribution, remains approximately constant across concentrations. This result reinforces the validity of the scale-invariant model and allows generalized application across different experimental conditions.

(vii) Determination of the droplet size distributions $P(s)$ using kernel density estimates for each concentration by merging all the replicates.

(viii) Determination of the mean (s_0) and standard deviation (σ) from the data of the droplet size distributions for each concentration using Kernel density estimates.

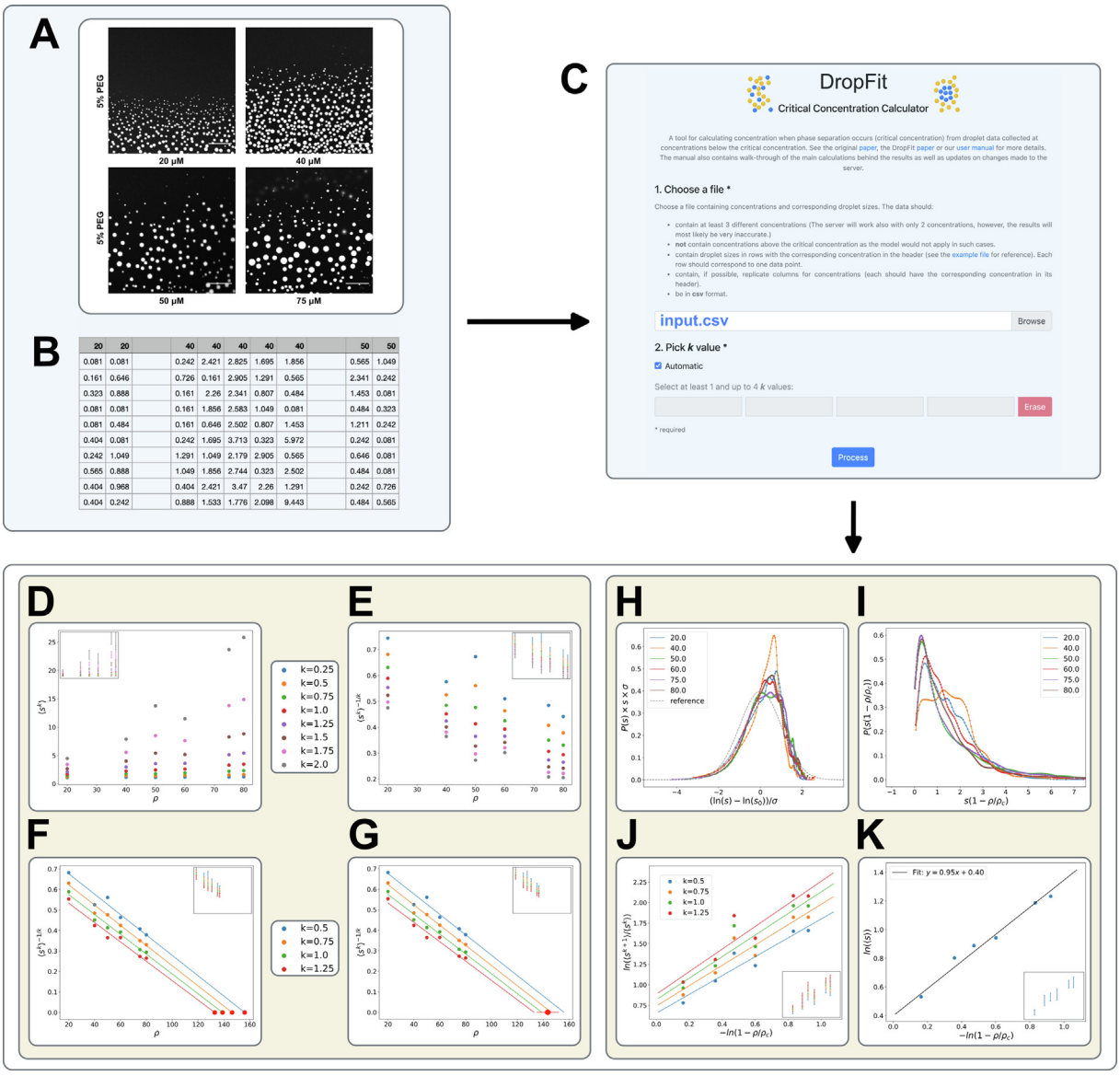


Figure 1. Illustration of the DropFit workflow. (A) Experimental data from imaging following the droplet size dimensions at different protein concentrations. **(B)** The input must be given in.csv; here a file is shown for different α-synuclein concentrations. **(C)** The user also needs to define the *k* values for the calculation. **(D)** Calculation of the moments of the droplet sizes (step 1). **(E)** Calculation of the average and standard deviation of the moments (step 2). **(F)** Plot of the mean of the moments against the concentration (step 3), regression using a weighted linear fit with selected concentrations (Step 4), and determination of the values of the *x*-axis cross-sections (step 5). **(G)** Determination of the average of the cross-sections and its standard deviation to provide the critical concentration and its standard deviation (step 6). **(H)** Calculation of the droplet size distributions (step 7), determination of the mean and standard deviation of the droplet size distributions (step 8), scaling of the distributions and plotting the collapse step 9). **(I)** Scaling the droplet size distributions using the values at the critical concentration. **(J)** Weighted linear fits of the droplet size moments to determine the critical exponent φ. **(K)** Linear weighted fit of the logarithm of the average droplet sizes to determine the critical exponent α.

(ix) Plot of $P(s)\, s\, \sigma$ against $((\ln s) - (\ln s_0))/\sigma$, where s_0 is the mean of the droplet size distributions and σ is the deviation determined in point (viii) (Figure 1H).

Steps vii–ix can be repeated using different concentrations.

III. ***Validation of the critical concentration***

(x) Plots of $P(s)$ against s, and of $P(s)$ against $s(1 - \rho/\rho_c)$. These plots show the collapse of the scaled size distributions onto a universal distribution (Figure 1I).

(xi) Determination of the critical exponents φ (Figure 1J) and α (Figure 1K) using the selected ρ_c and comparison with those of scaling model and percolation.

Steps x–xi can be repeated using different concentrations.

Application of DropFit to experimental data

DropFit can be applied to experimental data from fluorescence microscopy, nanoparticle tracking analysis, or other imaging methods, as long as the droplet size distribution is stationary and well-sampled. However, in practice nanoparticle tracking analysis (NTA) is preferred when the goal is to analyze freely diffusing droplets in bulk solution, especially for small condensates below the optical diffraction limit. Fluorescence microscopy can be advantageous for imaging larger droplets or for systems where spatial localization is of interest, but care must be taken to minimize artifacts from surface interactions or droplet fusion. Although the optical diffraction limit can make it difficult to distinguish between pre-critical and post-critical droplets in microscopy, particularly below 300 nm, the analysis of size distribution can inform about them (see validation of log-normal behavior and collapse). In addition, we recommended combining techniques, where possible, or performing control experiments, for example with sedimentation assays or fluorescence recovery after photobleaching (FRAP), to confirm the nature of the assemblies. We expect that despite the difference in average size, the size distributions follow similar behaviors and collapse at the comparable critical concentrations. The output of the program is the estimated critical concentration and its standard deviation. In addition, DropFit provides a plot to validate the applied theoretical model.

Input data

DropFit requires experimental droplet dimensions (length, area, or volume) measured at a minimum of 2 protein concentrations. We suggest using at least 3 concentration values, and for error estimation of the critical concentration providing 3 replicates for each concentration. The DropFit analysis assumes that the system has reached a quasi-stationary state in which the droplet size distribution does not change significantly over time. Therefore, the method is best applied to data acquired after an initial transient period, for example a time delay of 5 min post-onset of phase separation once the distribution has stabilized. The data must be provided in.csv format.

In addition, the user may provide 4 k values in the range of 0.25–2.25 used for the calculations. If the user does not provide these values, the program will automatically select the values based on the data provided. The *k* values will influence the distance between the moments, i.e. the linear fits used for estimation of the critical concentration. The user can modify these later, in case the variation/error of the critical concentration is large.

Data processing

DropFit processes the input file, and removes invalid data, including non-numeric or missing droplet size values, or measurements from concentrations lacking replicates, when error estimates are needed. The program also returns an error if the number of concentration values is less than 2. DropFit groups the replicates by concentrations. In case replicates are not presented for some of the concentration values, to provide a balanced weighting the standard deviation is estimated based on the mean to standard deviation ratios of the droplet sized at concentrations with replicates. DropFit requires at least 50 data points for each replicate at each concentration. The program performs quality control checks and gives warnings (Figure 2C), in case it finds inconsistencies in the data, regarding the number of data points for different concentrations or replicates, or large deviation in the mean droplet size in between replicates. This can be due to a non-stationary measurements, for example following an irreversible maturation or aggregation process. In the complete absence of replicates, the program automatically assigns a small standard deviation value to compute the fits for the critical concentrations. In this case, however, the information on data variability is lacking and the relative importance of the different concentrations in estimating the critical concentration cannot be determined.

The program performs the calculations as described in the workflow steps i-vi. The user can repeat these calculations using different concentrations and *k* values. This may be helpful if the droplet sizes at a given concentration exhibit a systematic problem/error, for example were measured at later time point than in other concentrations. These anomalous behaviors can be identified from the collapse plot (steps vii–ix).

Output – Estimation of critical concentration and validity of the model

The key output of DropFit is the estimation of the critical concentration ρ_c (Figure 2A). This parameter can be computed using the experimental data (droplet dimensions) at different concentrations. In case replicates are also provided, the weighted linear regression will provide the error for estimating ρ_c.

The use of log-normal distributions is central to the DropFit approach. Log-normal distributions are

A

Critical Concentration

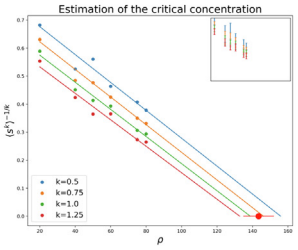
143 ± 9

Choose concentrations to **leave out** (Optional)

☐ 20.0 ☐ 40.0 ☐ 50.0
☐ 60.0 ☐ 75.0 ☐ 80.0

Regenerate

Determination of Critical Concentration



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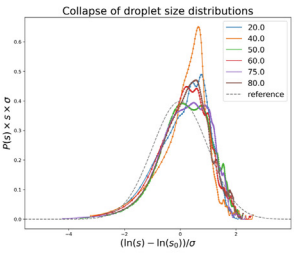
Model Validation

Collapse of distributions at various concentrations



Download PNG

B

Model Validation

Choose critical concentration to use to see if your data fits the model*

The critical concentration to use should be larger than the concentrations in the data.

◉ Automatic (DropFit-calculated) ○ Other: Analyse

* required

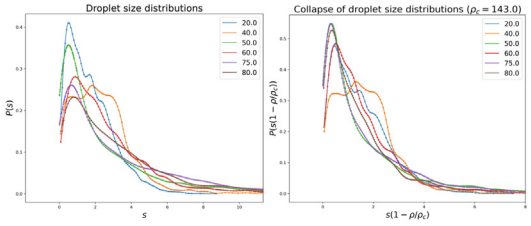
C

Warnings

- Data contained zero or negative values that have been removed (could have resulted in removal of some concentrations)
- There is variation in number of datapoints across replicates for concentrations: 40.0, 60.0, 80.0

D

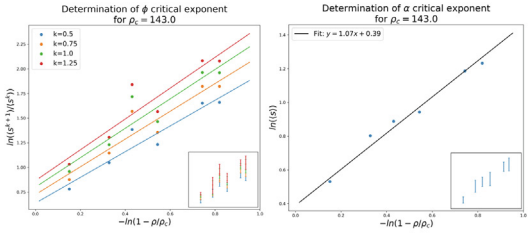
1. Collapse Verification



Please remove the concentrations which do not collapse well or exhibit multimodal distribution and recompute the critical concentration by repeating the procedure above.

E

2. Critical Exponents



ϕ = 1.4 ± 0.1
α = 0.24 ± 0.07

characterized by their mean and variance in log space. The characteristic size s_c can be computed from experimental data, and rescaled size distributions collapse onto a single universal curve when plotted using normalized log variables. This collapse is a strong indicator of scale invariance in the system (Figure 2A).

In addition to the log-normal plot, DropFit validates the critical concentration computed with the selected concentration values. ρ_c can be automatically derived from the DropFit calculation, or define another ρ_c value obtained by other approaches (Figure 2B). DropFit scales the droplet size distributions with the values at the critical concentration, which should fall onto a universal distribution at the value of ρ_c (step x) (Figure 2D). The web server shows both the original and scaled distributions, and the user can observe the collapse or possible problems with distributions at given concentration values. In case the distributions obtained at a few (usually one) concentrations do not collapse, they might be beyond the validity of the model or have systematic experimental errors.

Another way to validate the model is to compute the values of the critical exponents φ and α (Figure 2E). The fits (φ left, α right) inform on the robustness on the model, the right choice of the *k* values and possible deviations at given concentrations. The values and standard deviations of φ and α are displayed below the fits. These values can be compared with φ = 2 and α = 1.5 of percolation [20]. This provides some insights into the mechanism of the phase separation for the given system.

Monitoring possible problems with experimental data

The quality of experimental data may vary depending on concentration and time. In case the droplet sizes of the replicates are recorded consecutively, the time-dependence of condensation and possible aggregation, can cause a systematic error. If the droplet-size distributions exhibit time-dependent changes, the system is not in the stationary regime required for the scaling analysis, and DropFit may not be applied. In these cases, deviations from scale-invariant behavior (e.g., inconsistent scaling collapse or variable critical exponent fits) can serve as diagnostics of non-equilibrium effects, helping to flag problematic datasets. Another problem may derive from redefining the masks for image analysis of the droplets and collect foci with different intensity/background ratio. In addition, some concentrations, where droplet sizes are measured may be above the critical concentration.

Some concentration values may systematically deviate from linear fits of the moments of the droplet sizes (Figure 2E). This may indicate that those concentrations are beyond the validity of the scaling model, either too far below the phase boundary, or they are above the critical concentration. Large standard deviation of the critical concentration may also indicate (Figure 2A) problems the data. In this case, it is useful to eliminate one or a few concentrations and recompute the critical concentration. The imperfect collapse of the scaled droplet size distributions may also signal problems with the measurements performed at given concentration values (Figure 2D).

Further problems can be identified using the collapse variation and determination of the universal exponents (Figure 2E). The data should be examined of the scaled droplet size distribution does not collapse at given concentrations or the standard deviations for the fit obtaining the φ and α values are high. These might indicate problems with the measurements, e.g. large fluctuations, which are unexpected at this regime, or

Figure 2. Example of a DropFit web server output. (A) The DropFit output displays the critical concentration and its standard deviation, computed from the moments of the droplet sizes, and given in the units provided by the user (*top left*). The fits from the weighted linear regression at different *k* values are displayed below the value of the estimated ρ_c. The variability of the moments, estimated from the replicates are shown in the inset of the plot. The concentration values of the input data, offering an opportunity to regenerate the plots with excluding some values, which may be above or far below the critical concentration (*top right*). The collapse plot of the droplet sizes is scaled by the mean and variance of the distribution. The log-normal distribution is a signature of scale invariance in the system. **(B)** The user can define the value of the critical concentration in the model validation panel or it can be automatically derived from the ρ_c value computed by the server. **(C)** Warnings regarding the data are shown below with concentrations specified. We suggest fixing numerical problems, or remove the actual concentration and repeat the calculation. **(D)** The ρ_c value is used to scale the size distributions, which should collapse using the critical concentration. The left panel shows the non-scaled size distributions, the right panel displays the values scaled by the critical concentration. The quality of the collapse validates the model or indicates concentrations to be used. **(E)** The fits to determine the values of the φ and α critical exponents. On the bottom of the page the critical exponents and their standard deviations are displayed, which are computed with the given value of the critical concentration. The critical exponents are also displayed as a reference.

concentration values above or far below the critical concentration.

Step-by-step guide to applying DropFit

(1) Titrate the protein concentration across a broad range of values, ideally starting well below and extending above the expected critical concentration.
(2) Acquire droplet size distributions at each concentration, ensuring imaging is done after an initial equilibration time to reach a stationary distribution. Measure at least 3–4 replicates at each concentration values.
(3) Input the resulting data into DropFit, initially using all the concentration values. DropFit will warn about problems with the data, in case of non-numeric values, lack of replicates or replicates with too few data points. The user should correct these before running the analysis.
(4) DropFit will provide the critical concentration (ρ_c) (Figure 2A). The user is advised not to use measurements above this concentration, as these may reflect characteristics of an already phase-separated system. The user should repeat the analysis without these concentration values.
(5) The user should observe log-normal size distributions (displayed below the critical concentration, Figure 2A). Large deviations from log-normality at given concentration values may inform about concentration regimes beyond the applicability of the model. The user is advised to repeat the analysis without using these concentrations.
(6) The user should also compare the standard deviations between the experimental replicates, which are displayed on the graph of log-normal size distributions (Figure 2A). One must be careful with inconsistent replicates, about which the program will provide warning. These can inform about technical/experimental problems.
(7) Below the estimate for the critical concentration, the user can also check the collapse of the scaled droplet size distributions (Figure 2D). In case some concentrations exhibit poor collapse, the use should investigate these concentrations, as they may be below or above the applicability of the model.
(8) The user is advised to repeat the analysis without using the concentrations, identified in points 4–7. The use can specify the included concentration values in the upper panel (Figure 2A).
(9) The user can also check the collapse of the droplet size distributions at the saturation concentration (c_{sat}), which refers to the concentration at which phase transition is macroscopically observed (Figure 2C). We note that the critical concentration marks the point when the transitions between microscopic and macroscopic phase transition occurs, which can deviate from (c_{sat}) in either direction (see 'Comparison of the DropFit approach with the Flory-Huggins theory' section). The user can compare with the collapse at the estimated critical concentration and at the estimated saturation concentration. In this case, the user can provide the estimated saturation concentration using the parameter ρ_c and re-compute the collapse (Figure 2D).
(10) The user computes and analyze the critical exponents φ and α and their standard deviations (Figure 2E). It is advised to carefully check the standard deviations at the different k values and modify these values accordingly.

Case studies: FUS and α-synuclein

We describe the application of DropFit to FUS and α-synuclein to illustrate the use of DropFit. Both proteins exhibit droplet formation below ρ_c, and their droplet size distributions are well described by log-normal statistics [15].

The critical exponents derived from the scaling fits are consistent across experiments. In the case of FUS, droplet size distributions were analyzed using dynamic light scattering (DSL) using a buffer of 20 mM Tris, pH 7.4, and 100 mM KCl at ~25 °C in the concentration range of 0.25–2 μM, approaching the critical concentration from below [14,15]. In addition, nanoparticle tracking analysis (NTA) was applied using a concentration range 0.125–2 μM. The DropFit analysis found that 0.125 μM was far below the critical concentration and beyond the applicability of the scaling model. For the droplet sized measured at 0.25–2 μM FUS concentrations, we derived $\varphi \approx 1$ and $\alpha \approx 0$, indicating a divergence of s_c without power-law scaling in the tail of the distribution [15]. For FUS, we observed consistent scaling behavior in droplet distributions across concentrations below the transition point [15]. The estimated critical concentration was 5.0 ± 0.2 μM for untagged FUS and 5.4 ± 0.4 μM for SNAP-tagged FUS, higher than transition concentrations determined by conventional spin-down assays [14,15]. We also note that the c_{sat} = 4-μM, which was determined by microfluidic confocal spectroscopy (MCS) was higher than the values derived from DLS and NTA [14,15].

In the case of α-synuclein, the wild-type protein was mixed with an A90C variant labeled with Alexa-647 at a 100:1 M ratio in 50 mM Tris-HCl and 5% polyethylene glycol 10,000 (PEG), and the mixture was pipetted onto a 35-mm glass-bottom dish and immediately imaged on Leica Stellaris Will inverted stage scanning confocal microscope using a 40×/1.3 HC PL Apo CS oil objective (Leica Microsystems) at room temperature. The excitation wavelength was 633 nm for all experiments. For liquid droplet size characterization, images were captured 10 min post-liquid droplet formation. To analyze the possible time dependence of the liquid droplet size distribution, phase separation was induced, and 5 min post onset of phase separation, the glass-

bottom dish containing the experiment was sealed and closed to maintain a stable and controlled environment. Subsequently, images were captured at each designated time point. The results were analyzed for five replicates, carried out by ImageJ. Images were analyzed by applying a threshold function in ImageJ that excluded the background of the image and identified the liquid droplets as having a circularity of 0.8–1 [15]. The resulting size distributions followed a log-normal profile and exhibited scale-invariant behavior, confirming the applicability of DropFit. For α-synuclein, we estimated ρ_c in the range of 125–137 μM depending on the method of analysis. These case studies highlight the general applicability of DropFit across diverse protein systems and experimental techniques.

Advantages and practical use of DropFit

The practical value of DropFit lies in its ability to extract from complex experimental data quantitative estimation of the critical concentration of phase separation. One of its most convenient features is that it allows researchers to estimate the critical concentration of phase separation before the system visibly undergoes phase transition. This capability is useful in systems where conventional methods are affected by noise, variability, or limited experimental resolution. By analyzing droplet size distributions for concentrations relatively far from the critical value, DropFit can be used with reproducible measurements.

Researchers can input droplet size data through a web interface, initiate the fitting process with a few clicks, and obtain downloadable outputs containing critical concentration estimates and model parameters. Moreover, the compatibility of DropFit with different measurement techniques, including fluorescence microscopy, nanoparticle tracking analysis, and light scattering, makes it a versatile tool adaptable to diverse laboratory setups. This flexibility enables broad adoption and utility across various research domains interested in phase separation phenomena.

Comparison of the critical (ρ_c) and saturation (c_{sat}) concentrations

The critical concentration (ρ_c) estimated via DropFit corresponds to the point at which the correlation length of density fluctuations diverges and scale-invariant behavior emerges. This marks a transition from a regime where droplet formation is driven by microscopic fluctuations to one where macroscopic phase separation occurs. The saturation concentration (c_{sat}), which is commonly used to characterize phase transitions, typically refers to the concentration above which phase separation is macroscopically observed, such as the formation of visible condensates or a plateau in turbidity. It is often associated with the binodal or coexistence curve in phase diagrams derived from first-order thermodynamics, which can be derived from Flory-Huggins theory. Importantly, ρ_c may lie above or below the empirically observed c_{sat} depending on the detection method and the proximity of the system to equilibrium. Thus, while c_{sat} is an operationally-defined threshold for visible phase separation, ρ_c is a theoretical parameter that provides a quantitative and model-independent indicator of the proximity of the system to criticality. DropFit can detect scaling behavior in the precritical regime, even before visible condensates appear or turbidity changes are detectable. Estimating both concentrations is valuable, as c_{sat} offers a practical guide for experimental design, while ρ_c offers insight into the underlying physical mechanisms and the potential tunability of the system.

Generality and limitations of the DropFit model

Future studies should address the question of whether the scaling behavior underlying DropFit can be observed for multi-component condensates, as well as for condensate formation under cellular conditions. In the latter case, deviations from log-normality or absence of consistent scaling collapse can be observed when droplets are measured under non-equilibrium conditions. More generally, deviations from log-normality and lack of scaling collapse are observed for systems far from equilibrium, such as those undergoing aging, coalescence, or irreversible aggregation, where the droplet size distribution evolves in time and the stationary distribution has not been reached. Deviations from log-normality are also expected when measurements are performed too far from the transition, where the droplets are small or sparse, leading to poor statistics and weak signal-to-noise ratios in the size distribution data. These observations help delineate the physical regimes in which the assumptions of scale invariance and statistical stationarity, which underlie the DropFit framework, hold. They also provide insight into the mechanisms that may dominate in more complex or out-of-equilibrium systems, pointing to opportunities for extending or refining theoretical models beyond the current scaling ansatz. Most importantly, however, these will inform the researcher on the phase diagram of the system and indicates that the system is far from the phase boundary under the given conditions.

Broader implications and future directions

We anticipate that the use DropFit can have broad implications for the study of biomolecular condensates, as it provides a quantitative framework for evaluating proximity to phase boundaries. This tool can thus be instrumental for facilitating an understanding of the regulatory mechanisms of condensate formation. For example, the study of the effects of molecular chaperones on the critical concentration can be studied quantitatively. Future developments include expanding DropFit for application of the method to in vivo data from imaging studies in cells and tissues. Further, DropFit could aid drug discovery efforts by quantifying shifts in ρ_c upon treatment with small molecules, facilitating rational modulation of phase separation in disease contexts.

Conclusions

We introduced the DropFit web server as a convenient tool for the quantitative analysis of protein phase separation. By exploiting the scale-invariant nature of droplet size distributions below the critical concentration for phase separation, it offers a robust, accessible, and generalizable method to estimate the critical concentration. Its foundation in statistical physics and applicability across protein systems make it a valuable tool for dissecting the principles underlying biomolecular condensates and their physiological roles. The DropFit server enables researchers to connect experimental droplet observations to theoretical models, advancing our understanding of protein self-assembly in health and disease.

Code availability.

The DropFit code is available at https://github.com/MichaelaBrezinova/DropFit/. The manual, which contains all the equations used by the server, can be downloaded from the server website following the pointer.

CRediT authorship contribution statement

Michaela Brezinova: Visualization, Software, Investigation, Data curation. **Samuel Toluwanimi Dada:** Investigation, Visualization. **Monika Fuxreiter:** Writing – review & editing, Writing – original draft, Validation, Methodology, Funding acquisition, Conceptualization. **Michele Vendruscolo:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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