Measuring material properties of phase separated protein droplets with dual-trap optical tweezers

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Abstract

Measuring material properties of phase separated protein liquids can tell us about their internal dynamics, about their shape stability and about molecular interaction energies that mediate liquid-liquid phase separation. We present here an approach that uses optical tweezers to displace and mechanically perturb protein droplets with forces in ~pN range.

Introduction

Membrane-free cellular compartments have been suggested to be formed by liquid-liquid phase separation out of the cytoplasm. Liquid-liquid phase separation of proteins in aqueous buffer constitutes thus a new paradigm for the organization of complex molecular reactions inside the cell in terms of phase-separated reaction centers that provide locally a different biochemical environment than the bulk of the cytoplasm [1–3]. Membrane-free cellular compartments are for instance the nucleolus, Cajal bodies, stress and germline granules. Membrane-free cellular compartments often comprise intrinsically disordered proteins that have been suggested to scaffold them and to mediate phase separation through electrostatic interactions. Reconstituted condensates of such proteins show many characteristics of liquid droplets and serve as *in vitro* model.

To answer the question how membrane-free organelles form inside the cell, we developed methods to study the material properties of protein condensates (such as surface tension, viscosity and/or an elastic modulus) in different biochemical conditions. Material properties of protein condensates do not only inform us about the shape stability of droplets in the presence of acting forces but can also be used to infer internal molecular dynamics inside protein condensates or molecular interaction energies.

A way to measure these material properties with smallest amounts of purified proteins is to use dual-trap optical tweezers (Fig. 2 and 3).

1) As a first method, we will perform droplet coalescence experiments of two phase separated protein droplets to determine the droplets' ratio between viscosity η and surface tension γ. Droplet surface tension drives droplet coalescence, since the overall surface of the droplet material becomes smaller through coalescence. Internal viscous friction, however, slows the process down. Thus, it is intuitive that the time τ that it takes two equal-sized droplets to coalesce is $\tau \approx \eta R/\gamma$, where R is the radius of the two droplets. Thus $\eta/\gamma \approx \tau/R$. To achieve coalescence, two phase separated protein droplets can be trapped with the optical tweezer and brought into contact (Fig. 2A-D). The relaxation behavior of droplet shape is monitored over time and a size-dependent relaxation time is extracted (Fig. 2D, E).

2) Additionally, in a second set of experiments we will perform oscillatory deformations of protein droplets through immersed polystyrene microspheres that are trapped and translocated by the optical tweezer (Fig.2 a,b). Capturing a phase-separated protein droplet between two microspheres, we carefully move one of the optical traps back and forth at various oscillation frequencies (f=0.01 Hz, 0.1 Hz, 1Hz, 10 Hz) while measuring the forces exerted by the optical tweezers (Fig.2 a). These forces contain information about the droplet's surface tension, the droplets viscosity and the droplets elastic modulus.

Reagents and Materials

Parafilm, double-sided sticky tape, razor blade, glass cover slip (#1.5, thickness ~175 μm, possibly passivated), reagents to freshly prepare solution of phase separated protein droplets, pipette,

2nd method, in addition: 1μm polystyrene beads

Equipment

1. You need a dual-trap optical tweezer microscope with at least one movable optical trap. Ideally your trapping laser wavelength is in the near infrared (e.g. 1064 nm to avoid heating artefacts. For direct droplet coalescence experiments, relatively low laser intensities are sufficient to manipulate the droplets, so trapping lasers with output power of 500 mW should work.

2. The microscope should be equipped with an imaging solution that allows for the measurement of the droplet sizes before fusion. A bright-field imaging system or a low resolution fluorescence based imaging system are sufficient.

Procedure, 1st method

Sample preparation:

1. Cut piece of parafilm three fingers wide
2. Cut 2 layers of double-sided sticky tape and place on Parafilm. Leave protective coat on last layer.
3. Use razor blade to cut rectangular opening.
4. Glue this onto a clean or passivated coverslip (#1.5, 175 um thick).
5. Carefully remove parafilm
6. Protein solution: Prepare solution of phase separated protein droplets and pipette 10ul into the sample chamber.
7. Carefully seal sample chamber with a second clean or passivated coverslip.

Tweezer coalescence measurements:

1. Use relatively low light intensities (< 200mW) as compared to tweezer measurements that involve microspheres as the light directly interacts with the phase separated material. CRITICAL!
2. Separate the two optical traps - each should have similar intensity - and trap two droplets leaving at least 1um between the droplet surfaces.
3. Start data and image recording.
4. Slowly approach the two droplets by stepping one movable trap (e.g. at 50 nm/s). Stop the movement as soon as the surfaces touch. Coalescence should proceed spontaneously. If not, gently push droplets further into each other at very low stepping speeds (e.g. 10 nm/s).
5. As fusion between the two droplets proceeds, material flows from the two trap centers to the region between the traps. This material flow results in a deviation of the laser signals.
6. Wait until laser signal does not change anymore and stop the recording.
7. Discard droplets and start again. Aim to get at least 20 independent droplet pairs to fuse. To get size independent material properties, we will normalize coalescence times by characteristic droplet size. Averaging this procedure over droplets of different sizes gives us more confidence in the extracted material properties.

Data analysis:

1. Select region of interest in recorded tweezer data. Avoid trials that had additional droplets fused during recording.
2. Combine laser signals from both optical traps along the axis connecting the two droplets (say along the vertical). $S = (S_1+S_2)/2$.
3. Fit exponential growth curve or stretched exponential growth curve to the combined laser signal. The first model gives you one dynamic parameter, the relaxation time τ, the second one two, again a relaxation time τ and β, the stretch exponent. However, for stretched exponential decay or growth processes, it is informative to calculate the mean relaxation time $< t >= (\tau/\beta) \cdot \Gamma(1/\beta)$. For accurate fitting, these models require additional parameters related to the measurement like offset, plateau value and onset time of fusion.
4. Extract droplet radii from recorded images before fusion (e.g. with FIJI)
5. Calculate characteristic droplet size for each droplet pair as the geometric mean $R = \sqrt{r_1 r_2}$. Calculate normalised time constant as τ/R. This should be a material constant with units s/um.
6. Plot coalescence times versus characterisic droplet size and extract ratio of viscosity to surface tension from the slope of a linear fit.

Procedure, 2nd method

Sample preparation:

1. Prepare a solution of dilute beads (1um polystrene) with 1:1000 dilution into your buffer of choice. (Do this in two dilution steps)
2. Prepare a solution of your protein with phase separation with a small fraction of dilute beads Fill in exact dilution amounts and bead fraction!
3. Construct a sample holder from double stick tape on a **passivated** glass surface, see Fig. 1.

The following steps should be done relatively quickly to avoid drying.

4. Add 1-2ul of "naked" beads to the cut-out region on the sample
5. Quickly add 1-2ul of the droplet + beads to the cut-out region on the sample (careful to not touch the "naked" bead droplet
6. Quickly add a top passivated cover slip (We will flip the sample over and this will eventually be the side that is down).

Tweezer Measurement:

1. Set up the optical trap and load the sample. Adjust laser powers such that the spring constants of the optical trap are about equal.
2. In the "naked" bead region, trap two beads (one in each trap). Calibrate the trap stiffness using these beads.
3. Move to the bead+droplet region
4. Trap a bead in each trap. Each bead can have an associated droplet. If there is no droplet, trap a droplet with the bead as well.
5. Move beads and droplets slow up (in z) into approximately the center of the chamber (so approximately 50um from the coverslip
6. Slowly bring the traps together until droplet wets both beads as shown in fig. 3 b
7. Stretch the droplet slightly past spherical.
8. While recording the force perform an oscillation series with 100-200nm amplitude. Do frequencies f=0.01 Hz, 0.1 Hz, 1Hz, 10 Hz, 20Hz, 40Hz. It is best if you do the series from highest to lowest frequency since the fastest ones take the least amount of time. You should have at least 5 oscillations for each frequency.

Data Analysis:

From oscillatory measurements, we obtain for each frequency f measured an output of position oscillations of the mobile trap x(t) and an output of trap forces $F_1(t)$ and $F_2(t)$. Forces are roughly equal and opposite. Calculate now an averaged force output $F(t)=(F_1(t)- F_2(t))/2$. Use x(t) and F(t) to determine an effective spring constant of uniaxial droplet oscillation $\chi^*_{sys} = \hat{F}/\hat{x} \cdot e^{i\varphi}$ (complex number!), where $\hat{F}$ and $\hat{x}$ are the oscillation amplitudes of signals x and F and φ is the phase shift between them.

The real part of the effective spring constant χ^*_{sys} is $\chi'_{sys} = \hat{F}/\hat{x} \cdot \cos(\varphi)$ and the imaginary part $\chi'_{sys} = \hat{F}/\hat{x} \cdot \sin(\varphi)$.
Assuming that trap stiffnesses were adjusted to be approximately of equal stiffness k, we can now determine the effective spring constant of periodic droplet elongation $\chi^*(f) \approx \chi^*_{sys} k/(k - 2\chi^*_{sys})$, i.e.

$$\chi'(f) \approx (k^2\chi'_{sys} - 2k\chi'^2_{sys})/((k - 2\chi'_{sys})^2 + 4\chi''^2_{sys})$$

and

$$\chi''(f) \approx (k^2\chi''_{sys} - 2k\chi''^2_{sys})/((k - 2\chi'_{sys})^2 + 4\chi''^2_{sys})$$

For a high frequency regime (your module supervisor can help you to identify that), we can determine the elastic modulus of droplet material as[4]

$$G^*(f) \approx \frac{\chi^*(f) - (1.75 + 6.31\,\theta_0^2)\gamma}{\mathrm{R}\,(0.58 + 3.42\,\theta_0^2)}$$

For slow droplet deformations (small f), we can use the droplet spring constant to determine surface tension as[4]

$$\gamma \approx \frac{\chi'(f)}{(1.25 + 4.36\,\theta_0^2)}$$

where $\theta_0 = r_{bead}/R$ (should be much smaller than 1). Theoretical assumptions that lead to the derivation of the above formulae are explained in detail in Reference[4].

Figures

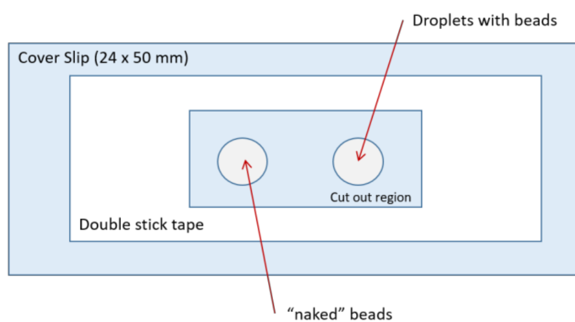

Figure 1: Schematic of flow cell for oscillation experiments

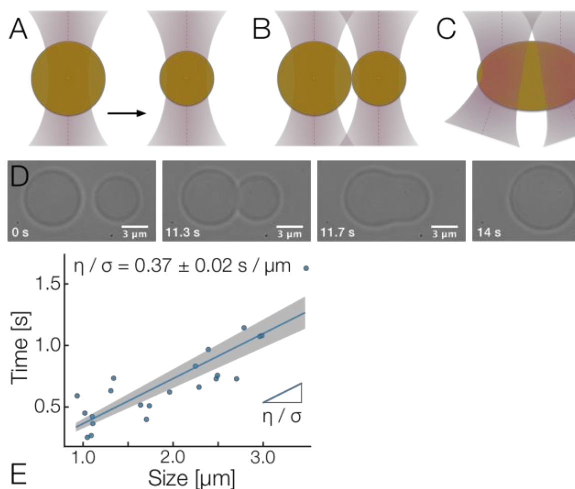

Figure 2: Measuring material properties with femtolitres of protein Liquids. (A-D) Controlled coalescence of protein droplets using two optical traps (schematic [A-C] and images from experiments with P-granule protein PGL-3 [D]). (E) The relaxation time from coalescence events as a function of characteristic droplet size gives the ratio of viscosity to surface tension.

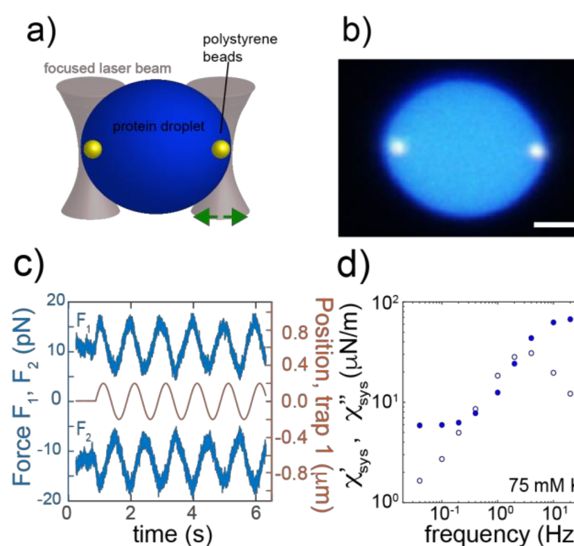

Figure 3: Microrheology setup based on a dual optical trap.

a) Schematic. b) Fluorescent micrograph of protein droplet during the measurement. Scale bar: 5 um. c) Exemplary experimental output during oscillatory droplet deformation: position of optical trap 1 (red) and forces associated to optical traps 1 and 2 (blue) at frequency 1 Hz and 75 mM KCl. d) Exemplary system spring constants χ'_{sys} (solid circles) and χ''_{sys} (open circles) from the measurement of a protein droplet with a diameter of ~12 um at a salt concentration of 75 mM KCl. Figure taken from Reference 4.

Anticipated results

1st set of experiments (droplet coalescence):

Anticipated results are shown in Fig. 2E.

2nd set of experiments (droplet oscillation):

Anticipated results for χ^*_{sys} are shown in Fig. 3d and Fig. 3 in Reference[4].

Discussion and Conclusions

Tweezer Fusion or Coalescence Experiments

+ Works with unlabeled WT and fluorescently tagged proteins.

+ Fast. Easy sample preparation. First data can be obtained in less than 2 min after inducing phase separation.

+ Requires very little material.

+ Experiments are performed free in solution. No surface interactions and surface effects need to be accounted for.

- Coalescence of small droplets (r<500nm) and very large droplets (r>5um) cannot be accurately measured.

- Material state of very stiff gel-like condensates can not be assessed if they do not fuse.

- This procedure assumes the droplets are simple liquids; therefore, it is unclear how to treat droplets with signficant elastic (or gel-like) contributions.

- Quantitative theoretical description of material flow during fusion process and the associated change in laser signal is missing. Thus, only order of magnitude values can be assessed.

- Only the ratio of surface tension and viscosity can be assessed. A second method is required to find either of them independently.

Tweezer Oscillation Experiments

+ Detailed frequency-dependent description of material properties from single droplets

+ Works with unlabeled WT and fluorescently tagged proteins

+ Experiments require little material.

+ Material state of fluid-like and of very stiff gel-like condensates can be investigated.

- Surface interactions between microspheres and protein droplet may affect results

- Relatively high laser intensities required to oscillate microspheres quickly on stiff samples could lead to heating effects.

- The force from the optical tweezers are not high enough to use this technique on droplets with large surface tensions. Therefore, this technique is ideal for samples with small uN/m surface tensions

References

1. Brangwynne, C. P. *et al.* Germline P Granules Are Liquid Droplets That Localize by Controlled Dissolution/Condensation. *Science* **324**, 1729–1732 (2009).

2. Hyman, A. A. & Brangwynne, C. P. Beyond Stereospecificity: Liquids and Mesoscale Organization of Cytoplasm. *Dev. Cell* **21**, 14–16 (2011).

3. Hyman, A. A., Weber, C. A. & Jülicher, F. Liquid-Liquid Phase Separation in Biology. *Annu. Rev. Cell Dev. Biol.* **30**, 39–58 (2014).

4. Jawerth, L. M. *et al.* Salt-dependent rheology and surface tension of protein condensates using optical traps. Phys. Rev. Lett. **121** (2018).