Characterization of protein-based phase separation parameters using temperature-controlled microscopy

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Abstract

Since the first description of membraneless condensates in cells as liquid-like, phase separated entities, various techniques to characterize their behavior have been described. In this module we use temperature as a means to dynamically trigger phase separation. Using confocal fluorescence microscopy, we determine pairs of solute and condensate concentrations at different temperatures and determine the binodal of a specific protein solution. We show that in the right buffer conditions a temperature shift can switch a protein system from a one-phase dissolved state to a two-phase condensate state. This enables us to study the various forms of coarsening of the protein condensates, e.g. fusion and Oswald's ripening.

Introduction

The dynamic nature of intracellular phase separated condensates can serve many different purposes. For example, they can act as storage compartments during stress, or as concentrating centers in the assembly of centrosomes. Cell have various ways of controlling the formation and dissolution of biomolecular condensates. These include a change in concentration, post-translational modifications or modulating the local environment, e.g. pH, ionic conditions or crowding. However, in everyday life we think most often of temperature as a variable that controls different phases of matter. When using a boiling kettle, water evaporates as steam or during winter days ice crystals form on the outside of window glass. In most cases cells cannot use temperature as a means to actively control phase separation. However, temperature is easily accessible to control the phase state in both an *in vitro* and *in vivo* situation and thus can help to understand the function of a given condensate. A common representation of phase transitions are phase diagrams. These give a graphical readout of the distinct phases, like for example a solute single-phase mix and a two-phase coexistence of condensates and solute (see figure 1). The two phases are separated by a line that is called binodal. The binodal can be measured using quantitative fluorescence microscopy to determine pairs of solute and condensate concentrations at different temperatures. Another approach that does not require quantitative fluorescence microscopy is to record the temperature at which phase transition occurs for various protein concentrations. Measuring a phase diagram of a specific protein solution allows further to use physical models like the Flory-Huggins (polymer-based phase separation model) to study protein interaction parameters. This allows to draw conclusions about the entropic and enthalpic contributions in the phase transition.

Various approaches can be used to control the temperature of a sample while simultaneously imaging it. The simplest method is based around a sample chamber that can be heated and cooled from an external temperature stage (A homebuilt version and a commercially available solution will be used in the practical). A more advanced concept uses absorption of IR light in aqueous media. Here, we use a scanning IR laser beam to "write" a temperature pattern of choice. This allows for extremely fast, precise, and local manipulation of the temperature and thus opens the door for new interesting research in the field of phase separation. This device might be used during the chaos days of the workshop.

Reagents and Materials

- Buffer conditions as previously determined from the salt and pH titrations. To be able to switch between solute and condensed state a protein concentration close to the saturation concentration has to be used. This is because we only have a defined window of temperatures before denaturation of the protein.

- labeled and unlabeled protein solutions

- glass slides

- pipettes and tips

- Pegylated glass cover slides
- Scalpel to cut parafilm stripes
- Parafilm stripes as spacer
- Tweezers
- Two-component silicone to seal sample chambers

Equipment

A spinning disc confocal microscope with a dedicated stage allowing for fast and accurate temperature control of protein solutions. Here, we use both a custom-made and a commercially available temperature-controlled stage. The custom-made stage is based on computer-controlled Peltier elements that are connected to a transparent, highly heat conducting material. While the commercial system uses water circulation in a microfluidic system and mixing of two water baths to control the temperature.

Procedure

Preparation of protein solutions:

1. Prepare protein solutions as determined in the previous lectures.
2. Important: Mix a solution of labelled and unlabeled protein to make sure we are able to measure useful concentrations. This is necessary as the high concentrations of fluorophore inside the condensates can quench the emission yield. By a low amount of labelled species (spike in) this effect can be minimized.

Preparation of sample chambers and loading of the protein solution:

1. Cut stripes of parafilm on a glass slide using a scalpel and a glass slide as ruler (approx 3x25 mm)
2. Place stripes parallel to each other with a gap of approx 3 mm between the stripes on the sapphire slide of the temperature stage.
3. Mount pegylated cover slide on top of the parafilm stripes and heat the stage to 50 °C.
4. Use the backside of a tweezers to bond the cover slide to the parafilm.
5. Cool the stage to the desired temperature for mounting of the protein solution.
6. Prepare two-component silicone by mixing them in a 1:1 ratio by the use of a pipette tip in a small weighing dish.
7. Add protein solution to one of the chambers and seal the chamber with a small drop of the silicone.
8. Mount stage on the confocal microscope.

Calibration of fluorescence intensities vs. protein concentration:

1. Use high salt solutions of at least 3 protein concentrations to get an idea of the relationship between fluorescence intensities and protein concentrations. We use high salt to make sure there are no condensates present in the solution.
2. Acquire z-stack for each protein concentration using fixed camera settings and laser intensities.

- Use image analysis tools to get a calibration curve for further evaluation of concentrations for the binodal

Determining the binodal of the protein solution. Repeat protocol for successive temperatures in the range between 10 °C – 50 °C:

1. Set desired temperature and wait until the device has reached the new temperature.
2. Wait for 5 min to equilibrate the solution to the new temperature.
3. Acquire z-stack of a multiple regions with sufficient numbers and large enough condensates for later segmentation and analysis. We use the same camera settings and laser intensities as for the calibration measurements.

- Use image analysis tools to segment the solute and condensate intensities. Via the calibration curve we can estimate the concentrations of the solute and the condensates for the different temperatures and draw a phase diagram for the protein mixture.

Measurement of the time dependent coarsening of the protein solution:

1. Use a protein concentration close to the saturation concentration.
2. Start by increasing the temperature above a point where there are no condensates left.

3. Keep this temperature for approx. 5 min to make sure all condensates are dissolved.
4. Now we quench the solution with one temperature step into the condensed region of the binodal and start a time-lapse recoding.

- After segmentation of the condensates we can plot their number and size as time evolution. The resulting curves can be compared to the scaling predicted from theory.

Figures

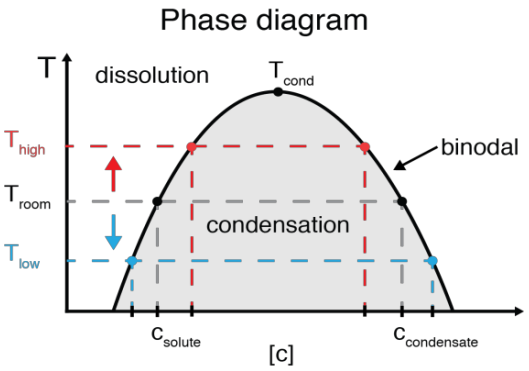

Figure 1: Schematic of a phase diagram. Below the condensation temperature T_{cond} a protein solution phase separates into a condensed and a solute fraction. For a given temperature below T_{cond} we find a pair of concentrations for c_{solute} and $c_{condensate}$. This pair of concentrations describes the binodal of protein solution. Other parameters to control the phase behavior of a protein solution are for example pH or salt concentration.

Anticipated results

- Condensation temperature as a function of a variation in protein concentration (and buffer formulation)
- Quantitative estimate of solute and condensate concentrations over temperature for a given protein
- Phase diagram of a protein solution (binodal).
- Extra: Fitting parameters of a Flory-Huggins like model (enthalpic and entropic contributions of the transition)
- Extra: Growth dynamics of condensates (Oswald's ripening vs. fusion)
- Extra: Local control of phase separation via IR laser-based temperature perturbations

Discussion

There are several steps that are critical to be able to perform quantitative fluorescence microscopy using temperature as a variable.

A first step is to think about the calibration of the fluorescence versus concentration of the protein of interest. It is necessary to make sure to have condensate free solutions with known concentration. The simplest way to perform quantitative measurements is to use the same laser and imaging settings for both calibration and measurements.

A further essential part is the knowledge about shortcomings of the imaging system used for your experiments. For example, a spinning disc system will facilitate rapid imaging of confocal 3D images. However, pinhole crosstalk has an influence on the measurement of the solute concentration. By using a bigger pinhole spacing this influence can be reduced or avoided entirely by using a point-scanning device. However, especially for suspended samples a point-scanner might show smeared out condensates due to their fast motion.

The imaging system also introduces another limitation regarding the size of the condensates. We expect (assuming a simple phase-separating system) that the protein concentration in small and large condensates should be the same. However, from experiments we know, that the fluorescence intensity inside the condensate is a function of size that saturates above a specific size. This stems mostly from the resolution limits of the microscope used.

A change of the temperature of a sample also introduces a change of the yield of a fluorophore. For most dyes that means a decrease in intensity upon increasing the temperature. This will of course influence our quantitative analysis of the concentrations within a condensate. Thus, we need to know the function of intensity versus temperature for the specific dye that is used. This allows to calculate the change in concentrations of the solute and condensate for various temperatures and draw a phase diagram (see Figure 1).

Literature

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