Determining the in vivo saturation concentration using quantitative fluorescence microscopy

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Abstract

A key parameter of a protein capable of undergoing phase separation is its saturation concentration, which defines the critical level above which the protein demixes from solution and forms condensates. Here we present a simple protocol for determining the saturation concentration of a fluorescently tagged protein *in vivo*. Through transient transfection we generate a population of *HeLa* cells with a stochastic and broad range of protein concentrations. Using fluorescence microscopy, we determine both, condensates and the protein concentration inside of cells. Correlating the protein expression level with the presence of dense protein condensates allows estimation of the protein saturation concentration. Modifying culture conditions, such as osmolarity, temperature or pH, allows generation of accurate complex and multidimensional phase diagrams.

Introduction

The formation of membranelles compartments through liquid-liquid phase separation is dependent on concentration of the scaffold protein nucleating the compartment. Once the concentration reaches a high enough value, the phase-separating protein will demix from the uniform bulk phase and form a dense condensate. That parameter is called saturation concentration and it is specific to every phase-separating protein. The saturation concentration is determined primarily by the amino-acid composition of the protein (Wang et. al. 2018) but can be affected by post-translational modifications such as phosphorylation (Zachary et al. 2017), acetylation (Ferreon et al. 2017) or methylation (Nott et al. 2015). Addition of these groups often increases the saturation concentration by shielding the charged amino-acids that underpin the phase separation process. The saturation concentration is also highly dependent on environmental conditions, such as temperature, osmolarity and pH (Nott et al. 2015). Importantly, the presence of other small or complex molecules, such as ATP (Patel et al. 2017) or RNA (Maharana et al. 2018), can either increase saturation concentration (and thus solubilize the protein) or it can decrease the saturation concentration promoting the nucleation of condensates.

Phase diagrams have extensively been used in physics to depict how a given compound changes its state as a function of physical parameters, such as temperature, pressure or concentration. For proteins undergoing liquid-liquid phase separation, the protein concentration is a key parameter determining its phase behavior. In a simple one-dimensional phase diagram, a binary classifier depicting either the presence (1) or the absence (0) of a dense phase is plotted as a function of the protein concentration. A more quantitative readout is obtained by plotting relative amount of fluorescence intensity emanating from phase separated objects as a fraction of total intensity in the field of view. The saturation concentration can then be extracted from such diagram by identifying the minimum concentration at which the dense phase forms (Figure1).

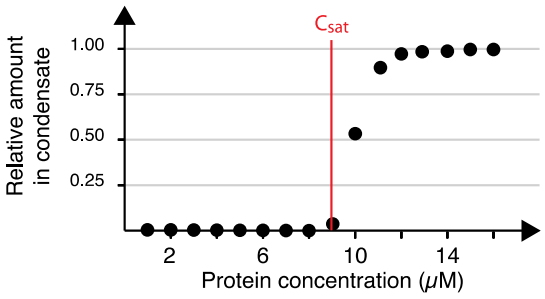

Figure 1. One-dimensional phase diagram. Saturation concentration (c_{sat}) is given by the minimum protein concentration at which condensates form,

Further variables such as pH, temperature or salt concentration can also be plotted against each other giving rise to two-or multi- dimensional phase diagrams (Figure 2).

The phase diagram can be constructed ***in vitro*** using purified protein and performing appropriate assays over a range of concentrations. A range of assays can be used to determine the presence of phase-separated condensates in solution, including light scattering measurements, as well as bright-field or fluorescence microscopy (Wang et. al. 2018).

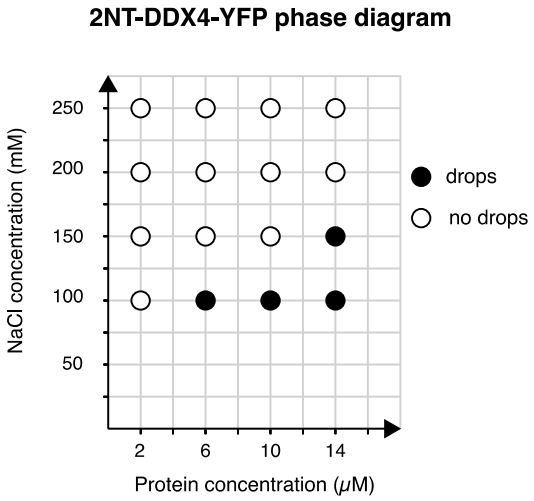

Figure 2. Two-dimensional phase diagram of 2NT-DDX4-YFP construct determined *in vitro* depicting dependence of droplet formation as a function of salt and protein concentrations.

Construction of a phase diagram and determination of the saturation concentration can also be derived from ***in vivo*** experiments. One approach can be to transiently transfect adherent, cultured cells with a plasmid encoding a fluorescently tagged protein of interest. The highly stochastic nature of transfection generates a population of cells with a wide range of concentrations of the expressed recombinant protein. Fluorescence intensity of each cell is then measured by microscopy. Using a calibration curve generated by imaging dilution series of purified protein of interest we can transform the fluorescence intensities to concentrations. Correlating protein concentration to the presence of dense, bright condensates inside the imaged cells allows generation of an *in vivo* phase diagram and determination of saturation concentration.

In this practical course we will apply a simple method to determine *in vivo* saturation concentration of a recombinant fluorescent derivative of human DEAD-box helicase 4 (DDX4) helicase using quantitative fluorescence microscopy in *HeLa* cells. DDX4 is a human helicase expressed specifically in testes (Meikar et al. 2011). Its homologs in other animals include VASA proteins in *Drosophila* and GLH proteins in *C. elegans*. In all cases the expression is restricted to the germline with the protein localizing to germline specific membranelles organelles. DDX4 proteins play an essential role in the formation of these germ granules referred to as nuage in mammals, P-granules in worms and polar granules in flies (Liang et al 1994). Apart from the well-structured DEAD-box containing helicase domain, the protein contains two disordered regions, one at each terminus. The over-expression of recombinant DDX4, where the helicase domain was replaced with YFP, resulted in formation of heterologous granules with liquid-like properties in the nuclei of HeLa cells (Nott et al. 2015). Duplicating the N-terminal domain increases the propensity of the protein to phase separate, suggesting that the N-terminal region is involved in the formation of liquid-like condensates and plays an important role in setting the saturation concentration (Figure 3). A precise measurement of the saturation concentration can be determined using a phase diagram.

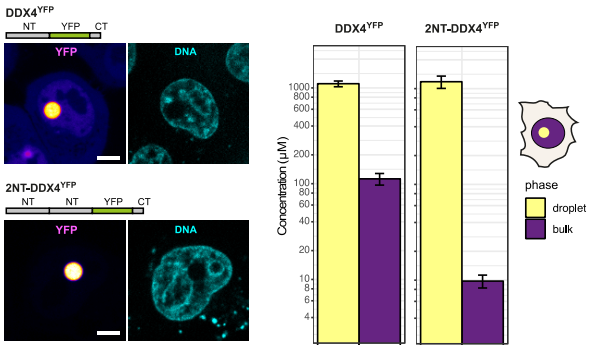

Figure 3. Duplicating the N-terminal disordered domain in DDX4^{YFP} increases propensity to phase-separate in cells by reducing saturation concentration. Quantification of the droplet and the bulk, nucleoplasmic phase in HeLa cells containing large droplets formed by DDX4^{YFP} (n=15 cells) or 2NT-DDX4^{YFP} (n= 25 cells). Scale bar = 10 μm.

Reagents and Materials

Cell line: HeLa Kyoto cell line expressing H2B:mCherry from a plasmid kept at stable level by selection with Blasticidin (Life Technologies, 2 µg/ml).

Cell culture reagents: high glucose DMEM (Gibco) with 0.5 mg/ml of Pencillin-Streptomycin (Gibco) and 5% fetal bovine serum (Gibco), PBS, Trypsin, disposable culture flasks, 8-well µ-slide (Ibidi) or 35/10 mm glass bottom culture dish with 4 compartments (Greiner Bio-One).

Transfection reagent: Lipofectamine 2000 Transfection Reagent (Invitrogen), Opti-MEM (Gibco).

Plasmid: engineered pcDNA 3.1+ (Invitrogen) plasmid encoding 2NT-DDX4-YFP under pCMV promoter was purified with PureLink HiPure Plasmid Filter Midiprep Kit (Invitrogen). Other kits that provide high concentration and purity can be used. We do not recommend using Miniprep kits.

Equipment

Cell culture equipment: Incubator at 37 °C and with 5% CO2, cell culture hood.

Microscopy equipment: Scanning Confocal or Spinning Disk Confocal microscope equipped with incubation chamber (37 °C and with 5% CO2) for long term cell imaging.

Experimental Procedure

Transfection

1. Seed cells into 8-well µ-slide at density of 10 000 cells/well and total medium volume of 250 µl/well. Proceed with transfection the following day.
2. For every well to be transfected, prepare appropriate amount of solutions A and B from reagents at room temperature in sterile conditions.
3. Solution A: 100 ng plasmid (in max 2 μl) + 30 μl Opti-MEM – mix well and incubate 5' at RT
   Solution B: 1 μl of Lipofectamine 2000 + 30 μl Opti-MEM – mix well and incubate 5' at RT
4. Mix solutions A and B and incubate 15' at RT
5. Add 200 μl DMEM + 5 % FBS and mix well to get the final transfection solution
6. Remove all medium from the well and immediately and very gently pipet in 250 μl of transfection solution
7. Medium may be changed after 4-6 hours.
8. Incubate for 20 h at 37 °C and with 5% CO2

Imaging

1. Next day, proceed with imaging of the transfected cells using a scanning confocal or spinning disk confocal microscope equipped with temperature controller set to 37 °C and with 5% CO2
2. Acquire 40 fields of view, recording the fluorescence intensity of two channels (mCherry and YFP) with a 40x objective. Image the hole adherent cells with Z-stacks, covering about 15 µm and 0.5 µm z-spacing.
3. The imaging conditions (laser power, exposure, camera gain) must be optimized for each sample to achieve maximum sensitivity without saturating the signal.

Droplet dissolution

1. 2xNT-DDX4-YFP droplets can be dissolved by adding 1,6-hexanediol to final concentration of 10% (v/v).
2. Add 60 μl of 50% 1,6-hexanediol solution to the well and image at the same, recorded positions immediately afterwards (1 – 5 min after addition, cells will begin to die in the presence of 10 % hexanediol)

Calibration curve

1. In order to convert the fluorescent intensities to protein concentration, a calibration curve needs to be prepared using purified fluorescent protein of interest.
2. Serial dilutions of the stock solution are made in buffer conditions that inhibit phase separation (high salt) and imaged at identical settings as the cells.

Data analysis

1. Image analysis can be performed using FIJI and a custom-built macro script.
2. The script uses the mCherry signal to identify and segment the nuclei. The YFP channel is used to identify the droplets inside the nuclei using fluorescence intensity thresholding.
3. The values extracted by the macro are the YFP mean fluorescence intensity in the nucleus including the droplets, as well as excluding them. These two values provide an estimation of the total and dilute phase protein concentrations.
4. Using the calibration curve, mean fluorescence intensities are converted to protein concentrations.
5. Presence and absence of droplets is plotted against the concentration of the protein inside the nuclei providing us with a one-dimensional phase diagram. Saturation concentration can be extracted directly from this data using a sigmoidal fit.
6. The average dilute phase concentration in droplet-containg cells provides an alternative estimate of saturation concentration.

Anticipated results

A large majority of the transfected cells express the fluorescent protein. A substantial fraction of those

cells contain spherical droplets of varying size and numbers within the nucleus. Some lethality is observed due to toxicity caused by transfection reagent, handing of the cells and the protein overexpression. The droplets appear inside cells in a concentration dependent manner – only the ones expressing the protein to a level exceeding the critical threshold for phase separation exhibit droplets of 2NT-DDX4-YFP. 2NT-DDX4-YFP enriches in the nucleus The reason for this enrichment is unknown. However, 2NT-DDX4-YFP droplets form exclusively in the nucleus. The anticipated saturation concentration is in the range of 5 – 10 μM.

Discussion and Conclusions

Saturation concentration of a phase separating protein is typically determined in vitro using purified protein and through varying buffer composition. The method presented here provides estimation of saturation concentration *in vivo*. This allows to tune the *in vitro* buffer composition, to better reflect the behavior of the protein in cell. This can be done by modulating salt concentration or by adding crowding agent such as PEG or dextran until the *in vitro* saturation concentration matches the one determined of in cells.

The dilute phase concentration is largely consistent among cells even with very different total protein content. Cells with high total protein concetraiton, and hence large and numerous droplets have similar concentration of the protein in the dilute phase as the cells with only few small condensates. This demonstrates the remarkable ability to buffer protein concentration by a liquid-like organelle. The small difference in the saturation concentration (dilute phase concertation) observed among cells might reflect different state of the individual cells in terms of their cell cycle state, pH, or concentration of micro- and macro-molecules.

The liquid compartments formed by the engineered DDX4 construct can be studied as model phase-separated organelles and used for more further studies beyond determination of the saturation concentration. However, not all proteins capable of phase separation are amenable to study using this technique. Possible complications include toxicity of the overexpressed protein, lack of an essential nucleator or modifier as well as interference of the fluorescent tag with the phase separation process. This might be circumvented to some extent by working with an isolated domain or concomitant overexpression of the missing nucleator/modifier (if known or suspected).

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