Quantification of molecular dynamics in phase separated protein solutions using fluorescence correlation spectroscopy

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Abstract

The goal of this practical course is to quantify protein dynamics (diffusion and protein-protein interactions) in the condensed and the dilute phase of a liquid-liquid phase separated protein solution. We will use fluorescence correlation spectroscopy (FCS) to measure the translational diffusion constant and the concentrations of purified FUS in its dispersed and condensed phase in an *in vitro* setup. We will use FCS with non-interacting tracer molecules to determine the apparent viscosity inside and outside the droplet. Together, the measurements will allow us to estimate the hydrodynamic radius of the phase separating protein, the apparent viscosity of the condensed phase and the timescale of the transient protein-protein interactions within the condensed phase.

Introduction

Liquid-liquid phase separation of proteins is typically characterized by observing fusion events of small droplets into larger ones and/or by measuring recovery after photo-bleaching (FRAP) inside the condensed phase. While this may indicate liquid like material properties, FCS can provide additional information, which may help to characterize the underlying protein-protein interactions that drive phase separation.

FCS evaluates the stochastic fluctuations caused by fluorescently labelled molecules diffusing through a focused laser spot [1]. With an appropriate setup and calibration FCS provides the concentration, the lateral diffusion constant and the brightness of the labelled molecules [2]. One of the advantages of FCS compared to single molecule tracking techniques is that the measurements are easy and fast to do both *in vitro* and in complex environments such as living cells. Nowadays, many extensions of FCS exist, which make the technique more robust or allow determination of additional parameters, but this typically requires specialized setups [3]–[6]. Here we will deploy simple single point FCS measurements, which can be performed on any confocal microscope equipped with single photon counting detectors.

To enable FCS measurements, our model phase separation protein FUS was labeled with SNAP-SIR, a photo-stable and bright far-red emitting fluorescent tag [7], [8]. Using FCS, we will determine the concentration, the lateral diffusion constant and the brightness of FUS inside and outside the condensed phase. The concentration difference can be used to determine the partitioning coefficient. The lateral diffusion constants outside the condensed phase provides information about the hydrodynamic radius of the protein, which is related to the conformation of intrinsically disordered proteins (extended versus collapsed). Additionally, the hydrodynamic radius together with the molecular brightness can be used to estimate the oligomeric state of the protein. Interpretation of the lateral diffusion constant inside the condensed phase is more difficult, because at least two processes contribute to the effective diffusion constant. First, the viscosity is typically higher inside the liquid protein phase compared to aqueous buffer, hence diffusion will be slowed down. Second, if the labelled protein is the scaffolding protein or a client, it will transiently interact with other proteins and may form higher order polymers. These binding reactions will result in an additional slowing down of the molecular movements. Therefore, in such complex environment the proteins may show deviations from simple free Brownian diffusion and undergo anomalous diffusion, i.e. the effective diffusion constant depends on the time and length scale [9]. To untangle the contributions of lateral diffusion and transient protein-protein interactions, we will determine the diffusion constant of non-interacting protein (SNAP-SIR), which has a comparable size as the scaffolding protein (FUS-SNAP), outside and inside the condensed phase. Together, the characterization of the system by FCS will help to estimate the apparent viscosity and the timescale of the transient protein-protein interactions within the condensed phase.

Reagents and Materials

1. Stock solutions of labelled and unlabeled protein (FUS-SNAP, FUS-SNAP-SIR) and a labelled control protein (SNAP-SIR).
2. Storage buffer: (500mM KCl, 1mM DTT, 5% Glycerol, 50mM TRIS, pH 7.4)
3. H_2O
4. PBS
5. 96 well glass bottom measurement chamber
6. PEG passivated glass coverslips #1.5

Equipment

FCS measurements are performed on confocal laser scanning microscope from Abberior Instruments (Göttingen, Germany), equipped with pulsed laser diode (635nm, 40mHz), Olympus 60x, NA 1.2 water objective. Fluorescence (650 nm - 700 nm) is detected with APD and recorded and auto-correlated using PicoHarp (PicoQuant, Berlin, Germany).

Software to fit FCS correlation data. We use a custom written software in MATLAB. Other, free to use software can be found here (refs).

Procedure

General FCS considerations:

Meaningful FCS measurements require thorough calibration of the laser focus parameters. Special attention should be placed on the following points:

1. The focus parameters depend on the depth at which you measure in the sample. Therefore, try to stay within 5-20 µm above the cover glass for all measurements including the calibration.
2. The average particle number N should be between 0.2-20. If that is not the case adjust the concentration of the labelled species accordingly. Sometimes, that requires addition of unlabeled protein of the same species.
3. In certain situations, diffusion can become very slow and photo-bleaching will occur before the molecules can pass the focal volume. In that case laser power needs to be reduced. In general, very slow processes (confocal diffusion times > 1s) cannot be studied reliably with conventional FCS.

Calibration of the confocal volume (ref):

10. Dilute Alexa647 to 10 nM in 100 µl PBS
11. Measure 5 FCS curves each 20s of diluted Alexa647 in 96 well plate 5 - 10µm above the cover glass using 20µW laser power
12. Determine diffusion time τ_D, focal aspect ratio k, triplet time τ_T and fraction $T$by fitting the curve with equation (1).
13. Calculate the focal beam diameter w_{xy} using equation (2) and assume a lateral diffusion constant of D_{lat} = 330 µm²/s.
14. Calculate the effective confocal volume using equation (3).

$$G(t) = \frac{1}{N}\frac{1}{\left(1+\frac{t}{\tau_D}\right)}\frac{1}{\sqrt{\left(1+\frac{t}{k^2\tau_D}\right)}}\left(1 + \frac{T}{1-T}e^{-\frac{t}{\tau_T}}\right) \quad (1)$$

$$w_{xy}{}^2 = 4D\tau_D \quad (2)$$

$$V_{eff} = \pi^{2/3} w_{xy}{}^3 k \quad (3)$$

Determine lateral diffusion constant of SNAP-SIR in the dilute and condensed phase:

1. Prepare a mixture of 5µM unlabeled FUS-SNAP with 10 nM SNAP-SIR in 10µl phase separation buffer (80mM KCl, 5mM TRIS, pH 7.4).
2. Measure 5 FCS curves each 20s inside and outside the droplets.
3. Fit curves and check that the particle number N = 1 – 20 in both phases.
4. Determine lateral diffusion constant D inside and outside phase separated droplets, equation (2).
5. Calculate the apparent viscosity η inside and outside the condensed phase using equation 4, assume a hydrodynamic radius of $r = 2.8\ nm$.

$$D = \frac{k_B \cdot T}{6\pi \cdot \eta \cdot r} \quad (4)$$

Determine lateral diffusion constant and concentration of FUS-SNAP in the dilute phase:

1. Prepare a mixture of 5µM unlabeled FUS-SNAP with 10 nM SNAP-SIR in 10µl phase separation buffer (80mM KCl, 5mM TRIS, pH 7.4).
2. Measure 5 FCS curves each 20s outside droplets at a distance of 5 - 10 µm above the cover glass, use 20 µW laser power.
3. Fit curves and make sure that the average particle number N is between 1 and 10. If this is not the case adjust the protein concentration repeat the measurements.

4. Calculate molar concentration c in the dilute phase using equation (5), remember to correct for the unlabeled protein ratio.
5. Calculate the lateral diffusion constant D according to equation (2).

$$V_{eff} = \frac{1}{N \cdot N_A \cdot c} \quad (5)$$

Determine effective lateral diffusion constant and concentration of FUS-SNAP-SIR in the condensed phase:

1. Prepare a mixture of 5 µM unlabeled FUS-SNAP with 5 fM SNAP-SIR in 10 µl phase separation buffer (80 mM KCl, 5 mM TRIS, pH 7.4).
2. Measure 5 FCS curves each 200s inside the phase separated droplets, use 20 µW laser power. Make sure to place the focus inside the middle of a droplet of size > 3 µm that is stably attached to the cover glass.
3. Fit curves and make sure that the average particle number N is between 1 and 30. If this is not the case adjust the labeled to unlabeled protein ratio and repeat the measurements.
4. Check if there are signs of photo-bleaching that occurred during the FCS measurements due to slow diffusion in the condensed phase. The offset of the FCS should be comparable to the previous measurements (very close to 1).
5. If the offset is > 1 photo-bleaching is an issue and measurements have to be repeated with decreased laser power (or correct bleaching in the photon-trace before autocorrelation or to use scanning FCS [6]).
6. Calculate molar concentration c in the condensed phase using equation (5), remember to correct for the protein ratio.
7. Calculate the effective lateral diffusion constant using equation (2).

Estimate protein interactions dynamics:

1. Calculate partition coefficient from the measured concentrations inside and outside the condensed phase using equation (6)
2. Compare the diffusion constant of FUS-SNAP-SIR inside and outside the condensed phase to the control protein SNAP-SIR. The effective diffusion constant of FUS-SNAP-SIR is expected to be lower compared SNAP-SIR, because FUS undergoes multivalent, transient interactions with other FUS molecules.
3. The effective diffusion constant determined by FCS inside the condensed phase is the result of the (1) lateral diffusion of the unbound FUS fraction, (2) the density of binding sites and (3) the binding equilibrium constant $K = k_{on}/k_{off}$ of FUS to the scaffold matrix. To estimate the reaction terms from the FCS data we use our measurement of the control protein to estimate the diffusion inside the phase. The additional slowing down is due to binding and unbinding to the scaffold matrix. Depending on the timescales of the binding process the diffusion term may be separable from the reaction term and the equilibrium binding constant with $K = k_{on}/k_{off}$ can be determined [10].

$$P = \frac{c_{in}}{c_{out}} \quad (6)$$

Anticipated results

1. Typical values for a confocal volume:

	Alexa647
D_{lat} [µm²/s]	330 [4]
Laser Power [µW]	20
τ_D [ms]	90
T [%]	20
τ_T [µs]	10
k	6
w_{xy} [nm]	330
V_{eff} [fl]	1.2
CPP [kHz]	20

2. Expected values for the measurements of SNAP-SIR and FUS-SNAP-SIR inside and outside the condensed phase:

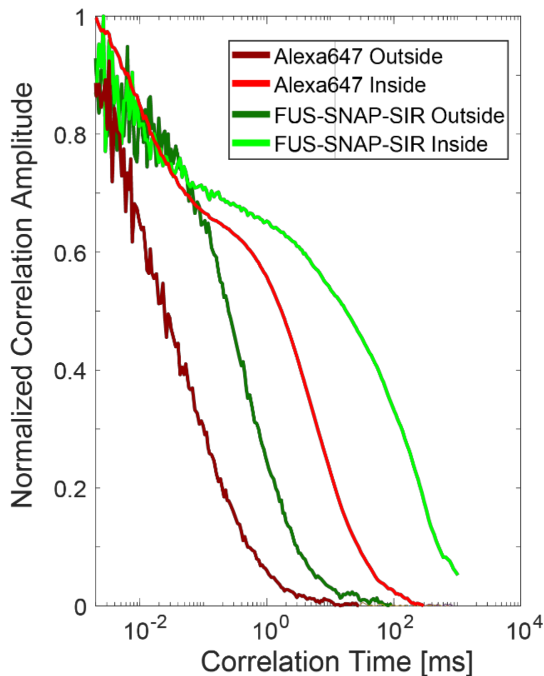

Figure 1: Preliminary FCS data for a small organic dye (Alexa647) and FUS-SNAP-SIR inside outside FUS droplets. Note the shift towards longer correlation times inside the droplet for both the dye as well as FUS-SNAP-SIR.

	SNAP-SIR *out*	SNAP-SIR *in*	FUS-SNAP-SIR *out*	FUS-SNAP-SIR *in*
Laser Power [μW]	20	20	20	20
$\tau 1_D$ *[ms]*	0.4	5	0.5	1
$\tau 2_D$ *[ms]*	-	-	-	200
$D1_{lat}$ *[μm²/s]*	60	2	60	2
$D1_{lat}$ *[μm²/s]*	-	-	-	0.1
Fr1 [%]	100	100	100	?
Fr2 [%]	-	-	-	?
CPP [kHz]	10	?	?	?
c [μM]	0.01	0.01	4	4000

Discussion and Conclusions

In this course we used FCS to quantify the molecular dynamics of a phase separating protein outside and inside the condensed phase. The results show that our model protein FUS, which we labelled with SNAP-SIR diffused with a hydrodynamic radius of r_H = *? nm* in the dilute phase. Its concentration in the dilute phase was c_{out} = *? μM*. Inside the condensed phase the effective diffusion constant of FUS was reduced ~100fold and its concentration was c_{in} = *? mM*. Therefore, the partitioning coefficient is *P = ?*.

To decompose the effective diffusion constant of FUS inside the condensed phase into the contribution from diffusion and binding reactions, we compared the dynamics of FUS to a non-phase separating protein (SNAP-SIR). We found that SNAP-SIR diffusion inside the condensed phase could be fitted with a single diffusive component, which indicates relatively homogenous and possibly free diffusion. However, its diffusion constant was reduced X-fold compared to outside. Therefore, the effective viscosity inside the condensed phase is X-fold higher then outside (aqueous solution). FUS diffusion inside droplets was slower than would be expected due to the reduced viscosity. In fact, the majority of FUS molecules moved ~X-fold slower than expected from pure diffusion in that viscous medium. Therefore, the effective movements of FUS inside droplets is dominated by its transient interactions with itself. If we for simplicity assume that FUS is moving much slower compared to its unbound state (D_{bound} << D_{free}) it is possible to estimate the reaction term $K = k_{on}/k_{off}$ from the FCS measurement [10].

We note that diffusion inside the droplet may be very anomalous, i.e. the diffusion constant depends on the length scale due to confinements and barriers. In that case our results and estimates are valid only for length scales of the confocal volume ~300 nm.

Taken together, FCS is a powerful tool to quantify the underlying molecular interactions of proteins in phase separated systems. In this course, we characterized a simple *in vitro* model system. However, this technique can be in principle also be used to study phase separated compartments in living cells.

References

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