Fluorescence Recovery after Photobleaching (FRAP) in protein condensates

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Abstract

Fluorescence recovery after photobleaching (FRAP) is a simple tool to estimate the viscosity of protein condensates both in vivo and in vitro. In this protocol we introduce FRAP, provide some examples of how it is used, outline typical pitfalls and describe the data analysis.

Introduction

The basic concept behind FRAP is to photobleach a small fluorescent region causing it to have decreased fluorescence intensity. The intensity of the sample recovers over time as the photobleached molecules are replaced by molecules which have not been photobleached. Analyzing the intensity of the sample over time can be a powerful way to assess the dynamics of proteins on a molecular level; however, to do this analysis properly requires understanding (or correctly assuming) the underlying molecular mechanisms through which the intensity recovers. For instance, if your sample is a simple liquid then one can assume that diffusion of molecules is the primary mechanism. Protein condensates, however, may also have elastic (non-liquid-like features) or have prominent binding/unbinding kinetics. Unfortunately, there is no theory that can account for all such molecular behaviors. In fact, even for the assumption that protein condensates are simple (Newtonian) liquids that recover from diffusion, no rigorous framework exists to account for the geometry and concentration profiles typically seen for protein condensates.

Noting these caveats, we nonetheless learn a lot about the properties of a protein condensate using FRAP. In this protocol, we describe how we estimate the viscosity of a protein condensate using this technique: We find an approximate time-scale of recovery from our FRAP measurements and assume the sample is dominated by diffusion. We know from comparing viscosities estimated from FRAP curves in protein condensates composed of PGL-3 shortly after formation to very accurate measures of droplet viscosity using oscillatory optical trap rheology that this seems to, at least, approximately yield the correct viscosity.

Moreover, we also outline some typical pitfalls of this approach and FRAPping in general.

Reagents and Materials

- Solution of fluorescently labelled protein droplets
- Glass-bottomed sample chamber of your choice.
    - We will sandwich our sample between PEG-passivated, coverslip and a PEG-passivated microscope slide using double-stick tape as a spacer.

Equipment

A confocal microscope equipped with a FRAPping unit and high powered objective (in our case, we will use a 60X or 100X objective).

Procedure

Prepare your sample

5. Prepare a solution of protein droplets.
6. Cut a small rectangle out of a piece of double stick tape adhered to parafilm.
7. Put the double stick tape on a passivated glass microscope slide.
8. Add 1-2 ul of droplet solution to the microscope slide.
9. Seal the sample by adding a passivated glass coverslip as the top of a sample chamber.
10. Flip the sample over so all the droplets settle onto the passivated coverslip side of the chamber.

FRAP and image acquisition

15. Find a droplet. Adjust the microscope stage to image approximately the mid-plane of the droplet.

   a. Set-up a region of interest that should be frapped. Typical geometries include:
   b. a small circle in the center.
   c. A so-called half frap in which half the droplet is bleached
   d. A so-called full frap in which the entire droplet is bleached.

16. Acquire a time-series of droplet frapping and recovery. For instance
   a. Acquire images at a rate of 0.1 seconds for 10 seconds
   b. FRAP the region
   c. Acquire images at a rate of 0.1 seconds for 3 minutes.

Image Processing

1. Find typical regions (reference droplet, background, frapped region) as illustrated in Fig. 1 and their intensities over time. This can be done using MATLAB or FIJI.
   a. Frapped region : I_F
   b. Not bleached protein condensate (reference): I_R
   c. Background: I_B
2. Measure the approximate radius of the frapped region. We will define this quantity to be “r”.

Figures

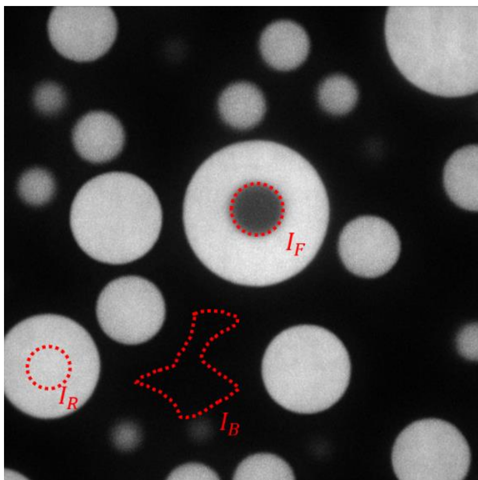

Figure 1: PGL-3 droplets shortly after photobleaching. Regions used for the analysis are denoted.

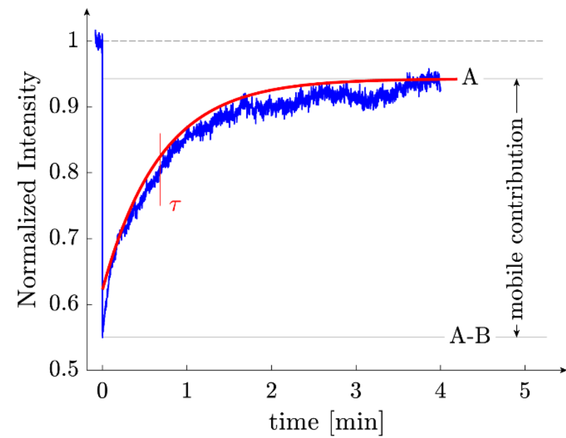

Figure 2: An example of a normalized FRAP curve fit with a one time exponential.

Data analysis

1. Normalize your FRAP curve. Your normalized intensity curve is given by the following formula:

$$I_{NORM}(t) = \frac{I_F(t) - I_B(t)}{I_F(t < t_0) - I_B(t < t_0)}$$

or

To account for any bleaching we use a reference droplet’s intensity:

$$I_{NORM}(t) = \frac{I_F(t) - I_B(t)}{I_F(t < t_0) - I_B(t < t_0)} \cdot \frac{I_R(t < t_0) - I_B(t < t_0)}{I_R(t) - I_B(t)}$$

Where $t_0$is the time at which the frap is applied and $<..>$ indicates that you take the mean value of all those times.

2. Fit a single time scale exponential to your curve with the form:

$$I_{Norm} = A - Be^{-\frac{t}{\tau}}$$

Alternatively you can fit a two-time exponential curve of the form:

$$I_{Norm} = A - Be^{-\frac{t}{\tau_1}} - Ce^{-\frac{t}{\tau_2}}$$

3. The recovery half-time $\tau_{1/2}$ of the curve is the point where the normalized sample reaches the half of its recovered value, see Fig. 1. You can also simply read this time off the graph. This is related to the characteristic recovery time through $\tau_{1/2} = \tau ln2$ (This is also a good consistency check – the τ

value determine in this way should be approximately equal to the value obtained from a fit to an exponential).

4. Determine τ. Either directly from the fit in step 2 or, if you used two time scales to fit the curve, use the time scale that is closest to the τ expected from inspecting the curve (as in step 3).
5. Determine the immobile fraction (if the curve does not recover to 1). From the one time-scale exponential fit this is:

$$Immobile\ Fraction = \frac{1 - A}{1 - (A - B)}$$

Approximate a viscosity from a characteristic time

To approximate the order of magnitude of the viscosity of a protein condensate from the characteristic time we assume that the sample recovering from diffusion. If this is the case then we expect that the timescale τ and the size of the frapped spot r (determined in the image processing section) should be related through:

$$r^2 = 2dD\tau$$

Where d is the dimension of the sample (typically 2 for FRAP experiments) and D is the diffusion constant. For a Newtonian liquid, we can find the viscosity of the sample using the Stoke's Einstein relation,

$$\eta = \frac{k_B T}{6\,\pi a\,D}$$

Where k_B is boltzmann's constant, T is the temperature of the sample, η is the sample viscosity and a is the radius of the protein. We have also assumed in this equation that an individual protein molecule acts approximately like a sphere of radius a diffusing through a liquid of viscosity η. We approximate the radius of the protein as a polymer of a given length l in a relatively good solvent ($\nu = 3/5$).

$$a = l^{\nu}$$

The length of the protein can be determined from its sequence. A good approximation is that a typical amino acid is approximately 0.8 nm; l is thus the product of the number of amino acids in the sequence and 0.8 nm.

The basic procedure is to use r and τ to solve for D. Then we subsequently solve for η to determine the viscosity.

Notes

1. **Reproducibility/Comparability**. To make your measurements as reproducible or comparable as possible. Try to maintain the following:
    a. Droplet size
    b. Frap region size and shape
2. **Accuracy of recovery time scale.** Keep in mind that it is very difficult (and probably inaccurate) to measure a recovery timescale that is much longer than your experiment time. For example, if you measure a typical time scale of 60 minutes and you have only watched the recovery for 30 seconds, you should not consider the typical recovery time accurate.
3. **Sample or droplet movement.** If your sample or FRAPped droplet moves significantly over time it may be that your intensities over time are not accurate. Carefully check this!
4. **Don't bleach your sample to 0!** You want that your sample is bleached but not to the point of damage. So do not bleach your sample to the point that the intensity reaches 0 in the normalized curve.

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