What do FRAP curves tell us?

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*To proceed, press Page Down or click the mouse button. To go back, Press page Up. Clicking hyperlink (blue fonts) leads you to a website in the Internet.
In this presentation, I will give a short overview on what we can know about molecular dynamics by studying the Fluorescence Recovery After Photobleaching (FRAP). The presentation is intended for beginners of FRAP.

The slides consists of the following topics.

- What is FRAP?
- Quantitative Analysis of FRAP
- Modeling and FRAP
1. What is FRAP?

Fluorescence Recovery After Photobleaching (FRAP)

We focus a strong laser beam to a spot (red dotted circle). Let's think of fluorescence molecules dispersed in a field. White circles represent the molecules. Then the strong irradiation BLEACHES the fluorescence at that spot.
1. What is FRAP?

Fluorescence Recovery After Photobleaching (FRAP)

Since molecules are moving driven by diffusion or active transport, bleached molecules exchange their place with un-bleached molecules. Then the average intensity at the bleached spot recovers.
1. What is FRAP?

Fluorescence Recovery After Photobleaching (FRAP)

In practice, the FRAP process looks like the following. Above is a microscope field filled with fluorophores.
1. What is FRAP?

Examples

**Drosophila Centrosome**
JORDAN RAFF, Cambridge

**GFP-sialyl transferase in CEF cells**
Banting LAB, MRC
a type 1 integral membrane protein which is localised to the trans face of the Golgi stack in control cells

**FRAP demo**
Federica Brandizzi, University of Saskatchewan

**Filament FRAP**
Leube group, Mainz
A section of a PK18-5 cell producing HK18-YFP; filamentous fluorescence recovery occurs preferentially in the cell periphery. dt=2 min.

**Virus (SV40) FRAP**
Pelkmans et al.
NATURE CELL BIOLOGY, vol 3 pp473-

Many FRAP movies can be found on the web. Above is a list of examples. You could also find many others by searching keywords “FRAP” and “movie”.
Fluorescence Recovery After Photobleaching (FRAP)

To gain information on molecular dynamics, time-course of the fluorescence recovery must be measured.

Measure the temporal changes of the fluorescence intensity!
2. Quantitative Analysis of FRAP

This is an example of FRAP curve, a result of measuring the fluorescence intensity at the bleached spot. X-axis is time, Y-axis is the fluorescence intensity.

Reits & Neefjes (2001)

Figure 1 Fluorescence recovery after photobleaching (FRAP). When a region in the fluorescent area (here the endoplasmic reticulum) is bleached at time $t_1$, the fluorescence decreases from the initial fluorescence $F_0$ to $F_1$. The fluorescence recovers over time by diffusion until it has fully recovered ($F_0$). The characteristic diffusion time $t_d$ indicates the time at which half of the fluorescence has recovered. The mobile fraction can be calculated by comparing the fluorescence in the bleached region after full recovery ($F_0$) with that before bleaching ($F_d$) and just after bleaching ($F_1$).
2. Quantitative Analysis of FRAP

First is the “half-life”, the time it takes for intensity to reach half the maximum of the plateau level. Plateau being lower than the pre-bleach intensity (in this graph, intensity during t<0).

What could be the reason of this incomplete recovery? The actual measurement of fluorescence intensity tends to result in a graph like this. We can extract several information out of this curve by drawing lines.
The incomplete recovery is due to some fraction of the molecules that are immobilized at the BLEACHED spot. We call these molecules the “immobile fraction”.

Rest of the molecules are contributing to the fluorescence recovery. We call them the “mobile fraction”.

Mobile Fraction and Immobile Fraction

Immobile molecule!
2. Quantitative Analysis of FRAP

In the FRAP curve, the immobile & mobile fraction can be measured by determining the plateau level.

The time constant and mobile / immobile fractions

Half Life ($\tau_{1/2}$)
2. Quantitative Analysis of FRAP

A more automatic way of obtaining Half life and mobile/immobile fractions is by "Curve Fitting". In curve fitting, parameters in an equation are optimized by computer. Here, we use a simple exponential equation for fitting.

This is the fitted curve… Optimized parameters can be directly related to the FRAP kinetics.

Half Life is the time when the recovery is the half of A, by definition.

Substitution of $f(t)$ by $A/2$ gives the formula below. Half Life can be calculated from $\tau$.

$$t_{1/2} = \frac{\ln 0.5}{-\tau}$$

Half Life $\tau_{1/2}$

$$f(t) = A \left( 1 - e^{-\tau t} \right)$$
2. Quantitative Analysis of FRAP

The FRAP curve equation we will use:

\[ f(t) = A\left(1 - e^{-\tau_1 t}\right)\left(\gamma_0 + B e^{-\tau_2 t}\right) \]

In this course, we will use the same type of exponential equation as it was shown in the previous slide. This equation however is corrected for the bleaching due to the image acquisition bleaching (underlined term). The kinetics of acquisition bleaching is actually unknown. It is also possible to directly correct the Frap curve by the acquisition bleaching at each time point.

In this graph, acquisition bleaching curve is plotted in blue color. Since we illuminate the sample for taking images, the fluorescence intensity decays slightly. This decay is considered in the above fitting equation.
What does the fitting and obtained parameters actually mean, in terms of molecular dynamics?

This question leads us to the world of "Modeling". We will consider following three molecular dynamics in next slides.

1. Specific Interactions
2. Diffusion
3. Advanced…
   a. Analytical Approach
   b. Numerical Approach

Specific interaction is also called chemical interaction. Specific interaction and diffusion are two basic models. In actual research, there are more various and advanced models.
Let’s consider a cluster of molecules, and the same molecular species unbound to the cluster. There are continuous exchange between the bound and the un-bound molecules. The system is in a steady state. The cluster size is not changing.
...then we bleach only the cluster. Immediately after the bleaching, all molecules consisting the cluster loses fluorescence.
3. Modeling & FRAP

A. Specific Interactions

Since there is a continuous exchange of the molecules, the cluster regains fluorescence by time.

We define the exchange rate $k_1$ and $k_2$ to see this event in more detail.
We consider a simplified model. The exchange of molecules between the cluster and the surrounding can be thought as molecule exchange between two chambers as above. The exchange is limited by a wall in between. The transfer rates in two directions are $k_1$ and $k_2$ respectively.

We define the number of molecules in the cluster as $[F]_1$. The number of molecules in surrounding environment as $[F]_2$. 
Solving the equation (1) and substituting the rate constants as shown in (1a), we get the equation (2). This is the same equation as we have used for the fitting in the previous slides. We now know the meaning of the parameters we obtained by fitting the FRAP curve.
3. Modeling & FRAP

B. Diffusion

FRAP could also occur without the specific interactions. Simply the diffusion drives the exchange of molecules between bleached and non-bleached area.
FRAP kinetics based on diffusion was examined theoretically many years ago by Axelrod et al. (1976). The formula they proposed are still in use by many researchers with small modifications.

The equation shown in the left needs a parameter before fitting. Bleaching constant $K$ is obtained by measuring the laser intensity distribution $C_u(r)$.

$$ F(t) = C_0 \sum_{n=0}^{\infty} \left[ \frac{(-K)^n}{n!} \right] \left[ 1 + n \left( 1 + \frac{2t}{\tau_D} \right) \right]^{-1} $$

- $C_0$: plateau fluorescence
- $\tau_D$: the characteristic diffusion time.
- $\tau_D = \frac{w^2}{4D}$

$C_u(r) = e^{-K \frac{I(r)}{I(0)}}$

- $Cu(r)$: unbleached fluorophore concentration
- $r$: radial distance
- $K$: bleach constant
- $I(r)$ is the Gaussian intensity profile of the laser
- $w$: half width at $e^{-2}$ intensity.

Phair & Misteli (2000)
B. Diffusion

Empirical approach

\[
\begin{align*}
    f(t) &= I_{final} \left(1 - \frac{w^2}{w^2 + 4\pi D t}\right)^{1/2}
\end{align*}
\]

Ellenberg et al. 1997

There are also empirical formulas for fitting the FRAP curves. Above equation is an example. \( w \) is the width of bleached spot. \( I_{final} \) is the plateau level after recovery. The diffusion coefficient \( D \) can be directly obtained by fitting this equation.
Advanced techniques for the modeling deal with complex molecular interactions such as the one shown above. This is a diagram of molecular interactions involved in vesicle trafficking. For all these interactions, it is possible to design differential equations for each molecular species involved in the system. These equations can be solved and fitted to the FRAP curves from the experiment to study their dynamics.

\[
\begin{align*}
\frac{df_{ARF_g}}{dt} &= k_{gof} f_{ARF_cyt} - k_{ac} f_{ARF} \cdot effector - k_{ac} f_{ARF_g} \cdot COP_{cyto} - k_{bleachARF} \cdot f_{ARF_g} \\
\frac{df_{ARF}}{dt} &= k_{ac} f_{ARF} \cdot effector - k_{gpase1} f_{ARF}_{effector} - k_{bleachARF} f_{ARF}_{effector} \\
\frac{df_{ARF_cop}}{dt} &= k_{ac} f_{ARF} \cdot COP_{cyto} - k_{gpase2} f_{ARF} \cdot cop - k_{bleachARF} f_{ARF_cop} \\
\frac{df_{ARF_cyt}}{dt} &= k_{gpase1} f_{ARF}_{effector} + k_{gpase2} f_{ARF} \cdot cop - k_{gof} f_{ARF_cyt} \\
\frac{df_{ARF_\text{exch}}}{dt} &= k_{f_\text{araf}} f_{ARF_g} + k_{e_\text{araf}} f_{ARF_\text{exch}} - k_{bleachARF} f_{ARF_\text{exch}} \\
\frac{df_{COP_Xg}}{dt} &= k_{gpase2} ARF \cdot f_{COP} - k_{uncoat} f_{COP} - k_{bleachCOP} f_{COP} - k_{f_\text{exch}} f_{COP} + k_{e_\text{exch}} f_{COP_\text{exch}} \\
\frac{df_{ARF_\text{fCOP}}}{dt} &= k_{ac} ARF \cdot f_{COP} - k_{gpase2} ARF \cdot f_{COP} - k_{bleachCOP} ARF \cdot f_{COP} \\
\frac{df_{COP_\text{exch}}}{dt} &= k_{f_\text{exch}} f_{COP_Xg} - k_{e_\text{exch}} f_{COP_\text{exch}} - k_{bleachCOP} f_{COP_\text{exch}} 
\end{align*}
\]
C. More Advanced…

Numerical Approach

The example shown in the previous slide was a modeling by analytical approach – which is to compose differential equations and solve them for the fitting.

There is another approach for an advanced modeling of biological system. That is to derive the estimated FRAP curve by simulation and compare it with the FRAP curve from the experiments. Simulation has potential in including many more parameters than the analytical approach. This approach is powerful but not yet widely used and still in progress.

Simulations in this sense can be viewed on the web, such as Adriaan Houtsmuller Lab’s web site.
What is the significance of FRAP technology? Let's compare it with traditional biochemistry. Biochemistry studies enzymes in the solution. Within beakers such as the one shown in above, enzyme-substrate kinetics is measured and characterized.

Now, we can measure the molecular interaction directly inside cells through microscope. This enables us to consider the spatial information that was disregarded in biochemistry. Maybe it would be more suited to call this technology as "topo-biochemistry." Since the site of interaction does matter within cell, these technology are now inevitable in understanding the cell as a system.
The European Advanced Light Microscopy Network (EAMNET) is a EU funded network of eight European laboratories and two industrial partners working in the field of light microscopy. The aim of EAMNET is to assist scientists in exploiting the power of imaging by organizing practical teaching courses, creating online teaching modules and offering software packages for microscopy. All EAMNET partners are also members of the European Light Microscopy Initiative (ELMI).

For Suggestions, Comments and Questions, please e-mail Kota Miura (miura@embl.de)
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"From fixed to FRAP: measuring protein mobility and activity in living cells."