FRAP analysis of photosynthetic membranes

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Abstract

Fluorescence Recovery after Photobleaching (FRAP) is a technique widely used in cell biology to observe the dynamics of biological systems, including the diffusion of membrane components. More information is needed on the dynamics of photosynthetic membranes in order to help to understand processes such as photosynthetic electron transport, regulation of light-harvesting, and biogenesis and turnover of the photosynthetic apparatus. FRAP has the potential to provide this information, although applying the technique to photosynthetic membranes is not always straightforward. This review explains the potential and the problems, and gives a brief guide to performing FRAP measurements and analysing the data.

Key words: Chlamydomonas reinhardtii, confocal microscopy, cyanobacteria, diffusion, Fluorescence Recovery after Photobleaching (FRAP), green algae, Synechococcus sp., photosynthesis, thylakoid membrane.

Photosynthetic membranes as dynamic systems

Thylakoid membranes are potentially a wonderful model biological membrane system. Not only is there outstanding structural information on many of the thylakoid membrane protein complexes, but also a battery of functional assays of light-harvesting and electron transport that make it very easy to probe membrane function in detail. However, there is one area where there is a distinct gap in current knowledge of thylakoid membrane function. There is very little direct information about the dynamics of thylakoid membranes. Diffusion of membrane components has been invoked in a number of contexts, for example, to explain electron transport (Kirchhoff et al., 2002), adaptation of the light-harvesting apparatus (Allen and Forsberg, 2001), and the biogenesis, turnover, and repair of the photosystems (Baena-Gonzalez et al., 1999). Diffusion coefficients for certain thylakoid membrane components have been estimated by indirect means (Drepper et al., 1993; Blackwell et al., 1994), but current knowledge of thylakoid membrane dynamics remains very fragmentary. For example, it is not clear whether the major membrane protein complexes are locked into a semi-crystalline array, or whether they are freely mobile and interact transiently by collision, as is more normal in biological systems (Storrie and Kreis, 1996). In the absence of this basic information, detailed models cannot be built for many aspects of thylakoid membrane function, from electron transport to the turnover and repair of Photosystem II.

Fluorescence Recovery after Photobleaching

FRAP and related optical techniques are the method of choice for observing diffusion in biological systems. FRAP measurements are now normally performed with a laser-scanning confocal microscope (Kubitscheck et al., 1994). The component whose diffusion is to be observed must be tagged with a fluorophore so that it can be imaged in the confocal microscope. To observe the diffusion of the fluorophore, the laser power is increased and the confocal spot is scanned briefly over a small area of the sample, so as to bleach the fluorophore. There are several possible mechanisms of photochemical bleaching, of which the commonest is probably photo-oxidation (Xie and Trautmann, 1998). After bleaching, the laser power is decreased again and the whole sample is imaged. The bleached area of the sample will be seen as a dark, non-fluorescent patch on the image. The sample is repeatedly imaged, and if the fluorophore is mobile the bleaching will change in a characteristic way. As the fluorophore diffuses, the fluorescence in the centre of the bleach will recover, and the bleach will become broader and shallower. In favourable cases, it is then possible to analyse the images...
to obtain an accurate value for the diffusion coefficient for the fluorophore. The requirements for quantitative FRAP are that the membrane geometry is known, and the membrane environment is uniform over an area considerably larger than the area of the bleach. As FRAP is an optical technique, it has limited resolution (the theoretical limit is about half the wavelength of light being used). When performing FRAP measurements in vivo the resolution may be further reduced by sample scattering, and diffusion of the fluorophore in the short time-interval between bleaching and recording the first post-bleach image. In practice, it was found that the minimum bleach width in this study was about 0.5–1 μm.

Ideal membrane systems for FRAP include the extensive, flat, plasma membranes of cultured fibroblasts (Kubitscheck et al., 1994). Chromophores used for FRAP measurements include native photosynthetic pigments (Mullineaux et al., 1997), GFP (Reits and Neefjes, 2001), and a range of dyes which may be conjugated to proteins or to lipid analogues. The use of GFP-fusions to tag specific proteins for FRAP is becoming routine (Reits and Neefjes, 2001). Photo-activatable forms of GFP have now been developed. In these variants of GFP, exposure to specific wavelengths of light induces a transition to a stable conformational with greatly increased fluorescence yield. This offers the promise of being able to carry out FRAP measurements at lower laser intensities, using the laser to photo-activate GFP and increase fluorescence in a limited region of the sample (Patterson and Lippincott-Schwartz, 2002). It is conceivable that the GFP tag could alter the mobility of the membrane protein, although this is perhaps unlikely, given that diffusion in membranes is generally very much slower than in the cytoplasm (compare Zhang et al., 1993, with Elowitz et al., 1999). Thus, an additional cytoplasmic domain is unlikely to be a significant drag on an integral membrane protein unless it specifically binds to other components in the cytoplasm. Where possible, a functional assay should be carried out on the GFP-tagged transmembrane to see if the function of the tagged protein is perturbed.

### Photosynthetic membranes as model systems for FRAP

One great advantage of photosynthetic membranes is that some of the protein complexes are naturally fluorescent (Table 1), so their diffusion can be observed without any necessity for specific fluorophore binding or GFP gene fusions. In other respects, the majority of thylakoid membranes are far from ideal for FRAP measurements. The membrane systems are enclosed in bacteria or chloroplasts whose overall dimensions are usually rather small. Furthermore, most green plant thylakoid membranes have an intricate, convoluted structure, and extensive lateral heterogeneity on small scales (Mustardy and Garab, 2003). Thus they do not meet the basic requirements for quantitative FRAP, that the membrane geometry is predictable and the membrane environment is uniform over the area of the measurement. While it will not be possible to use FRAP to obtain accurate diffusion coefficients for components of typical green plant thylakoids, it will be possible to see if particular components are mobile or not, and to get a rough idea of time-scales.

By far the most regular thylakoid membranes are found in certain species of cyanobacteria, which have elongated cells, and thylakoid membrane whose conformation approximates to a set of concentric cylinders aligned along the long axis of the cell (Mullineaux and Sarcina, 2002). In contrast to typical green plants, there is no thylakoid membrane stacking and no extensive lateral heterogeneity on small scales (Mustardy and Garab, 2003). Thus they do not meet the basic requirements for quantitative FRAP, that the membrane geometry is predictable and the membrane environment is uniform over the area of the measurement. While it will not be possible to use FRAP to obtain accurate diffusion coefficients for components of typical green plant thylakoids, it will be possible to see if particular components are mobile or not, and to get a rough idea of time-scales.
cell division inhibitors: under these conditions, cells 10–20 μm long can be found in the culture (Sarcina and Mullineaux, 2000). Growth for about 24 h in the presence of 0.5% DMSO is an efficient way to produce elongated cells (Aspinwall et al., 2004). With cylindrical membranes like Synechococcus thylakoids, the best way to do a FRAP measurement is to bleach a narrow line across the cell, by scanning the confocal spot for about 1–3 s in the X-direction (Mullineaux et al., 1997; Fig. 1). Diffusion can then be followed in one dimension, along the long-axis of

Fig. 1. Extracting data from a FRAP experiment on a cell of Synechococcus 7942. This experiment shows fluorescence from the phycobilisomes. An image (a) is recorded prior to bleaching, by scanning the confocal spot in the XY mode. The scan is then switched to the X-mode, the laser power is increased by a factor of 8, and the confocal spot is scanned across the cell for about 2 s to bleach a line. The laser power is then reduced again, the scan is switched back to the XY mode, and a second image (b) is recorded. A series of further images is recorded, at time intervals appropriate to the rate of diffusion. To extract data, image analysis software is used to obtain a fluorescence profile along the long axis of the cell (in the Y-direction), summing all the pixel values in the X-direction for each Y co-ordinate (c). Pre-bleach and post-bleach profiles are aligned (d). The post-bleach profile (shown in black) is corrected by subtracting the pre-bleach profile (shown in grey). A Gaussian curve is then fitted to the corrected fluorescence profile (e). The fitted Gaussian curve is used to obtain the depth (C) and the half-width (1/e²) (R) of the bleach (e). Finally, a suitable function of C is plotted versus time and regression analysis is used to obtain the diffusion coefficient (f). R₀ and C₀ are the values of R and C at time t=0. For one-dimensional diffusion, the plot shown in (f) should be linear with gradient equal to the diffusion coefficient (Mullineaux et al., 1997).
the cell (Mullineaux et al., 1997; Mullineaux and Sarcina, 2002). These measurements are fully quantitative. This system has been used to show that the phycobilisome light-harvesting antennae are highly mobile, and to investigate a number of factors that affect their diffusion coefficient (Sarcina et al., 2001; Aspinwall et al., 2004). It has also been shown that Photosystem II core complexes are astonishingly immobile: no diffusion can be detected even on very long time-scales (Sarcina et al., 2001). This raised the possibility that all the integral membrane complexes might be locked into a rigid array. However, it has recently been found that this is not the case: the IsiA protein, an additional chlorophyll-protein induced under iron-stress, is quite mobile (M Sarcina, CW Mullineaux, unpublished data). In addition, BODIPY FL C12, a lipid-soluble green-fluorescent dye, has been used to probe the mobility of thylakoid membrane lipids (Sarcina et al., 2003). It was shown that the diffusion coefficient is strongly increased in a transformant with more desaturated thylakoid membrane lipids, but that lipid lateral diffusion is rather slow in thylakoid membranes, compared with, for example, eukaryotic plasma membranes. This may reflect differences in membrane lipid composition, but also the exceptionally high protein content of thylakoid membranes (Sarcina et al., 2003).

The author does not know of any green plant whose thylakoid membrane conformation is as amenable to FRAP as that of *Synechococcus*. However, some green algae have the advantages of large chloroplast size and low levels of membrane stacking and lateral heterogeneity. Mullineaux and Sarcina (2002) have started a study of *Chlamydomonas reinhardtii*. It was found that a proportion of the fluorescent chlorophyll in the membrane is mobile. Studies in a range of mutant backgrounds suggest that Photosystem II is immobile, as in cyanobacteria, but a part of the LHCII pool is mobile (M Sarcina, CW Mullineaux, unpublished results). Chloroplast division mutants (Osteryoung and Pyke, 1998) may, potentially, be useful systems for thylakoid FRAP in higher plants.

**Doing a FRAP measurement**

FRAP measurements can be carried out with most modern laser-scanning confocal microscopes. Currently a Nikon PCM2000, which has been equipped with a range of lasers and extra filters, has been used to give the necessary wavelengths required. Table 1 shows the wavelengths that were used with a range of photosynthetic pigments, GFP, and dyes. During a measurement, a series of images of the cell is recorded, and it is important to keep the laser power low enough not to cause significant further bleaching of the cell during repeated imaging. This should be tested at the start, and the laser power reduced if necessary. The basic procedure for the measurement is to record an image of the sample cell. The laser power is then increased. This is usually done by removing a neutral density filter to increase the power by a factor of 8. The confocal spot is scanned over a restricted area of the sample to bleach out most of the fluorescence in that area. The best way to scan for the bleach depends on the geometry of the sample (see above). The laser power is then reduced again, and a series of post-bleach images is recorded. The best time-scale for the measurement depends on the rate of diffusion and has to be found by trial and error. For a rapidly-diffusing chromophore the bleach must obviously be performed very quickly and post-bleach images recorded rapidly. In thylakoid membranes, the diffusion of both lipids and proteins is generally rather slow compared with, for example, that often observed in eukaryotic plasma membranes. It was found that a 1s bleach, followed by a series of images recorded at 3 s intervals, was adequate to capture even the relatively rapid diffusion of a fluorescent lipid analogue (Sarcina et al., 2003).

It is important to check that the bleaching of the fluorophore is irreversible, as some pigments (including GFP) can exhibit complex photochemical behaviour including the formation of states which are temporarily bleached, but whose fluorescence eventually recovers. This is an obvious complication in FRAP measurements (Cowan et al., 2003). For this possibility is routinely controlled by bleaching an entire, small cell and checking that the fluorescence does not recover on the time-scale of the measurement.

**Analysing FRAP data**

Figure 1 summarizes the steps involved in analysing FRAP data. In this case the experiment is a one-dimensional diffusion measurement in a cell of the cyanobacterium *Synechococcus* 7942. Optimas software (Optimas Corporation) was used to extract the fluorescence profiles from the images, and SigmaPlot (Jandel Scientific) for curve fitting. Fitting Gaussian curves to the bleaching profile (Fig. 1e) yields numerical values for *C*, the bleach depth, and *R*, the half-width of the bleach, at a series of time-points. To obtain the diffusion coefficient (*D*), it is necessary to have an equation which predicts the change in *C* and *R* with time (t) and diffusion distance (x), according to the value of *D*. The equation is a solution of the basic diffusion equation

\[ \frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \]

according to the particular geometry of the membrane and the bleach. An appropriate equation for the one-dimensional diffusion experiment shown in Fig. 1 is given by Mullineaux et al. (1997). Figure 1f shows a plot used to obtain the diffusion coefficient from a series of values for *C* according to this equation. Other equations are appropriate
for other experimental geometries (Cowan et al., 2003; Kubitscheck et al., 1994), but in all cases it is necessary to have an explicit model of the membrane topology in order to obtain the diffusion coefficient. In some cases, fluorescence recovery is only partial. This indicates that only a part of the pool of the fluorescent chromophore is mobile, and in these cases the equation should take that into account. It is then possible to estimate the proportion of the fluorophore that is mobile.

The future of thylakoid FRAP

To gain a more complete picture of thylakoid membrane dynamics, it will be necessary to add fluorescent tags to those membrane proteins that are not naturally fluorescent. Unfortunately, GFP gene-fusions appear not to work in Synechococcus 7942 (Susan Golden, Texas A&M University, personal communication). However, GFP can be successfully expressed in other cyanobacteria, and mutant forms are available whose fluorescence excitation and emission does not overlap severely with that of the photosynthetic pigments (Spence et al., 2003).

There is scope for considerable further work on the dynamics of thylakoid membranes in green plants and green algae. Although it will not be possible to obtain quantitative diffusion coefficients for membrane components in stacked, laterally heterogeneous thylakoid membrane systems, it will be possible to see if there is an exchange of fluorescently tagged proteins between the grana and stroma lamellae, for example. This should give some fascinating insights into membrane biogenesis, electron transport, and the Photosystem II repair cycle.

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