Published in final edited form as:

Curr Protoc Cytom. 2012 October ; CHAPTER: Unit2.19. doi:10.1002/0471142956.cy0219s62.

Analysis of protein and lipid dynamics using confocal fluorescence recovery after photobleaching (FRAP)

Charles A. Day^{1,*}, Lewis J. Kraft^{2,*}, Minchul Kang^{1,*}, and Anne K. Kenworthy^{1,2,3}

¹Department of Molecular Physiology & Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

²Chemical and Physical Biology Program, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

³Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Abstract

Fluorescence recovery after photobleaching (FRAP) is a powerful, versatile and widely accessible tool to monitor molecular dynamics in living cells that can be performed using modern confocal microscopes. Although the basic principles of FRAP are simple, quantitative FRAP analysis requires careful experimental design, data collection and analysis. In this review we discuss the theoretical basis for confocal FRAP, followed by step-by-step protocols for FRAP data acquisition using a laser scanning confocal microscope for (1) measuring the diffusion of a membrane protein, (2) measuring the diffusion of a soluble protein, and (3) analysis of intracellular trafficking. Finally, data analysis procedures are discussed and an equation for determining the diffusion coefficient of a molecular species undergoing pure diffusion is presented.

Keywords

FRAP; diffusion; Confocal laser scanning microscopes; protein trafficking; fluorescence microscopy; GFP

Introduction

Fluorescence recovery after photobleaching (FRAP) is a fluorescence-based biophysical tool often utilized to investigate the dynamics of molecules within live cells. It has proven to be a highly versatile technique, allowing for the characterization of a wide variety of phenomena. For example, FRAP is commonly utilized, either quantitatively or qualitatively, to examine the diffusion of membrane bound or soluble particles. Additionally, FRAP is an important tool for studying the movement of particles between organelles or to observe import and export through nuclear pores. In addition to being flexible in its applications, most commercially available confocal microscopes on the market today come equipped to perform FRAP. Due to its wide accessibility and high versatility, FRAP has become an important tool for the cell biologist.

In a FRAP experiment, a group of fluorescently tagged molecules in a defined region of interest (ROI) is rapidly photobleached using a high intensity laser. The exchange of the bleached molecules with unbleached molecules from the surrounding region is then

*These authors contributed equally to this work.

followed over time using low intensity excitation light to minimize further photobleaching. The time course of change in fluorescence intensity in the ROI after the photobleaching is called the FRAP curve (Figure 1). FRAP curves yield information about both the recovery kinetics and the fraction of molecules free to diffuse. Since a wide variety of factors can impact these values, well planned FRAP experiments and careful analysis of the data can yield a wealth of information, including but not limited to the underlying mode of diffusion or transport, the organization inside the cell or organelle, binding reaction rates, and the size of the diffusing species (Reits and Neefjes, 2001; Kang and Kenworthy, 2008; McNally, 2008; Mueller et al., 2010).

Originally, FRAP measurements were performed using a focused, static laser beam to bleach molecules (conventional FRAP) (Axelrod et al., 1976; Thompson et al., 1981; Elson, 1985). A theoretical basis for the analysis of FRAP measurements of lateral diffusion by spot photobleaching approaches for a static laser was established shortly after the development of conventional FRAP (Axelrod et al., 1976). The earliest applications of FRAP were predominantly confined to measurements of cell surface proteins or lipids, which could be fluorescently labeled by exogenous probes (Axelrod et al., 1976; Thompson et al., 1981; Elson, 1985). The discovery of GFP and molecular techniques to tag proteins with GFP, or similar fluorescent proteins, has vastly expanded the number of proteins that can be studied by FRAP. Furthermore, technical advances in confocal laser scanning microscopes in the late 80s to early 90s have made confocal microscopy available to many researchers. These two factors have allowed for a growing interest in techniques sensitive to protein dynamics such as FRAP. Even today the technique continues to evolve thanks to advances in mathematical analysis of FRAP data (Braga et al., 2004; Sprague et al., 2004; Kang et al., 2009; Kang et al., 2010).

While FRAP is a common tool for measuring dynamics *in vivo*, other methods for quantifying diffusion exist, such as fluorescence photoactivation, single particle tracking (SPT), fluorescence correlation spectroscopy (FCS), and image correlation spectroscopy (ICS) (Lippincott-Schwartz et al., 2003; Haustein and Schwille, 2007; Kolin and Wiseman, 2007; Toprak and Selvin, 2007; Alcor et al., 2009; Bancaud et al., 2010; Petrusek et al., 2010). While these are powerful techniques with their own strengths and weakness, FRAP has many advantages over these alternative methods. For example, the ratio of mobile to immobile particles within the total population is accessible using FRAP, a variable not easily acquired with other techniques. Furthermore, FRAP can be performed on most commercially available confocal setups without any special modifications.

In the current protocol, we describe the confocal FRAP methods developed and utilized by our group to study the dynamics and trafficking of proteins and lipids in living cells (Kenworthy et al., 2004; Goodwin et al., 2005a; Goodwin et al., 2005b; Hinow et al., 2006; Kenworthy, 2006; Kenworthy, 2007; Kang and Kenworthy, 2008; Wolf et al., 2008; Kang et al., 2009; Drake et al., 2010; Kang et al., 2010; Day and Kenworthy, 2012; Kraft and Kenworthy, 2012). This includes presenting step by step instructions for carrying out FRAP experiments to characterize diffusion of membrane associated proteins, diffusion of soluble proteins, and trafficking/transport between organelles. We have included in this protocol advice on presenting and interpreting the data. Additionally, we go into detail regarding the extraction of diffusion coefficients and mobile fractions from FRAP experiments where pure diffusion is being examined. This protocol should serve as useful tool for cell biology labs that seek to begin using confocal fluorescence recovery after photobleaching.

Basic protocol

Materials

Mammalian cultured cell lines (e.g. COS-7, ATCC #CRL-1651)

Fluorescent protein vector (e.g. EGFP)

cDNA of protein of interest (e.g. H-Ras)

Amine reactive dye pack (e.g. Alexa Fluor 488 (A488) protein labeling kit, available through Invitrogen)

Purified protein of interest (e.g. cholera toxin B subunit (CTxB), available through Sigma)

Cell culture media (DMEM + 10% FBS + phenol red)

Imaging media (e.g. phenol red-free DMEM + 10% FBS, 25 mM HEPES)

35-mm glass-bottom dish (e.g., MatTek Corp., glass bottom microwell dish)

Pipette and tips

Transfection agent (e.g. Lipofectamine, available through Invitrogen)

Line scanning confocal microscope (e.g. Zeiss LSM 510 or comparable system), with the appropriate laser and filter configuration for the fluorophore to be used:

- Argon ion laser (488 nm laser line for bleaching and for imaging EGFP and Alexa-488)
- HeNe lasers (only necessary if imaging red and far red fluorophore (i.e. Cy3, mCherry, Alexa-555, Cy5, Alexa-647, etc)
- Band-pass emissions filters (i.e. 505–550 for EGFP and Alexa-488)
- Acquisition software (e.g. Zeiss LSM)
- Objectives (e.g. 40×/1.3 Oil Plan-Neofluar lens)

Stage and objective heater or imaging chamber

Software for image analysis (ex. Zeiss LSM software, ImageJ)

Software for data analysis (ex. Excel, MATLAB)

Prepare plasmids and/or exogenous fluorescent probes and cells

- 1) Produce the fluorescently tagged protein of interest. For example, for exogenous proteins that bind cell surface receptors such as cholera toxin B subunit, use an amine reactive dye pack to attach fluorophore to purified proteins (using dye manufacturer's instructions). For expressed proteins, prepare plasmid encoding fluorescent fusion protein DNA using molecular biology techniques.
- 2) Grow cells in culture media in glass bottom dishes.

Unless restricted for biological reasons, it will make FRAP data collection much easier if a cell line with a morphology well suited for FRAP is selected. For example, when doing FRAP on the plasma membrane we prefer COS-7 cells as they produce large expanses of flat plasma membrane. Similarly, if you wish to monitor a cytoplasmic or nuclear protein, select a cell line with large cytoplasmic or nuclear space. COS-7 cells are also well suited for these types of experiments.

- 3) For genetically encoded proteins, transfect cells with the plasmid expression vector produced in step (1) using Lipofectamine approximately 24 hours after plating.
- 4) Incubate transfected cells for 24 hours at 37°C in 5% CO₂ to allow fluorescent protein expression to occur.
- 5) The cells will be ready for FRAP 48 hours after plating. For the study of expressed fusion proteins, rinse the cells with imaging media and move the cells (in imaging media) to the microscope. For exogenous protein labeling, rinse cells with imaging media and perform labeling. Then rinse again and move the cells (in imaging media) to the microscope.
- 6) Perform control experiments to ensure correct production of expressed fluorescent conjugated protein, efficient labeling of exogenous protein, and correct protein localization.

Quantitative FRAP analysis of lateral diffusion is most reliable when there is an optically uniform (or nearly uniform) distribution of the fluorescent particles. If this not the case, other methods may be better suited for characterizing diffusion. See “Comparison of FRAP to other techniques sensitive to protein and lipid dynamics” section for more information.

Configuring the confocal LSM for time-lapse imaging

- 7) Turn on the confocal microscope according to the manufacturer’s operating instructions.
- 8) Find a representative cell through the eyepiece and place the cell in the center of the image window.
- 9) Optimize the imaging configuration such that the fluorescence intensity per pixel is high (but not saturating), by adjusting the laser transmission, pinhole, and detector gain. To minimize photofading (i.e., the loss of fluorescence due to repetitive imaging), we recommend setting the detector gain high and lowering the laser transmission as much as possible. The goal here is not to take a publication quality image but to fill the pixels at the detector so that a good fluorescence signal is obtained. Collect time lapse images of a single cell and further adjust the microscope settings so that a bright image is maintained, as best as can be, over sufficient time span required for FRAP (see the examples below to select general time courses for your application). There is no need to be too meticulous at this point, as these settings will be fine tuned later.

Configuring the confocal for FRAP

This section will walk through the general microscope configurations that are necessary for the FRAP data acquisition. This set up will need to be repeated for every fluorophore and/or marker you wish to study with FRAP. However, once the proper photobleaching parameters have been worked out for each marker they can be saved and reused, with little or no adjustment, on subsequent days. These methods are relatively unchanged between FRAP applications, and any special variations between different FRAP applications will be discussed in detail in the examples. This can be done on live cells, although using fixed cells at this stage has the advantage that it allows for the optimization of bleaching conditions independent of the temporal resolution of the experiment.

- 10) Set the image window with the appropriate zoom to the area of interest. Generally when working with COS-7 cells, a 512 × 512 pixel image at 4X

electronic zoom from an image collected using a 40X lens is a good starting point and the electronic zoom can be adjusted from there depending on the FRAP application.

- 11) Using the software ROI selection tool, draw a circular bleach region in the center of the image window. Once a bleach region has been created, save the ROI into the computer for use on subsequent samples.

Choosing the right size bleach ROI can take some time as it needs to be determined on a case-by-case basis. If the bleach region is too small, fluorescence recovery may occur so quickly that it will become difficult to analyze the FRAP curve or the FRAP curve may be very noisy. On the other hand, the ROI should not be so large that a significant fraction of the total fluorophores is lost due to the bleaching event, as this will artificially lower the mobile fraction. It may take several rounds of performing pilot FRAP experiments in order to achieve this balance. For plasma membrane proteins, cytoplasmic proteins, and nuclear proteins in COS-7 cells, we suggest 4.1, 2.3 and 1.7 μm diameter ROI's, respectively. Note that geometry and size of bleach ROI can be varied depending on the goal of particular experiments. For FRAP measurements of diffusion, circular ROIs are recommended in order to take advantage of analytical FRAP equations developed specifically for this geometry, as discussed in more detail below.

- 12) Configure the software to perform the bleach event after multiple prebleach images are scanned. These prebleach images provide steady state fluorescence intensity before photobleaching, which will be required to normalize the FRAP data later.

The number of prebleach images needed varies depending on the type of experiment being carried out and the rate at which the images are acquired. A general rule of thumb is to collect a minimum of 3 prebleach images when imaging at a rate of ~1 frame per second to establish a stable fluorescence baseline prior to FRAP.

- 13) As a starting point for determining the bleaching conditions, in the software, set the number of bleach iterations between 5 and 20 with no delay between them. This is the number of times that the laser will pass over each pixel in the bleach region during the bleach event.

The number of bleach iterations required will depend on the power of the bleach laser, the scan speed, the photostability of the fluorophore, and the rate of diffusion of the particle being studied. Between five and twenty passes of the bleaching laser is a reasonable range of bleach scans to start with, but this iteration number will be fine tuned in subsequent steps.

- 14) The proper bleach laser wavelength for photobleaching will now need to be determined. Select a bleach laser wavelength and set it to the maximum power allowed on your setup. Ideally, there should be a large difference between the laser transmission used to repetitively image the sample during the recovery phase and the transmission used for the bleach event itself.

For GFP or similar fluorophores, run a test FRAP using the 488 Argon laser line at 50% output and 100% transmission.

- 15) Select the preset "start FRAP" button in the software to begin FRAP.

- 16) If needed, repeat steps 14 and 15, using the different laser lines available for photobleaching until the best laser line for bleaching your specific fluorophore is determined.

Photobleaching for a specific fluorophore is most efficient when performed with a wavelength of light matched to the maximal excitation energy of that fluorophore. However, Argon lasers are typically significantly more powerful than HeNe lasers. For this reason, when bleaching with a fluorophore which does not excite optimally at 488 nm, such as Cy3 for example, bleaching with a 488 nm Argon laser line will often produce better bleach depth than could be achieved by bleaching with a less-powerful HeNe laser tuned to the maximum excitation energy of Cy3.

- 17) Once the optimum wavelength of light to bleach with has been determined, the ideal number of iterations for bleaching will need to be established. Try changing the iterations from between 1 to 50; saving the data after each attempt. Then examine the FRAP curves and postbleach images side by side to find a setting that maximizes the bleach depth while minimizing the time required to bleach. Once an ideal number of iterations has been selected, this iteration number and wavelength should be reused in subsequent studies.

The goal here is to find the number of iterations that will produce as short a bleaching event as possible, while also giving good bleach depth. Ideally, this means a bleach depth of 80%, however anything greater than a 50% drop in fluorescence immediately following the bleach is typically usable. However, if the number of iterations is too high, a large percentage of fluorophores could potentially diffuse out of the bleach region during the bleach. This effect, if uncorrected, can give rise to artificially low values of D and also can create an artificially low mobile fraction. This is especially problematic for soluble proteins, as will be discussed in Example 2 below. If a setup cannot be achieved which yields deep bleaching, try adjusting the ROI, changing frame rate (by altering delay, scan speed, image window size or line averaging) or consider changing fluorophores.

- 18) FRAP data should be collected for a long enough period of time that the total time series is significantly longer than the time it takes for recovery to level off after the bleach. Additionally, multiple timepoints need to be obtained during the early stages of recovery in order to extract a diffusion coefficient from the data. Adjust the number of images and the delay between each image to satisfy both requirements.
- 19) Once the appropriate number of images has been determined, levels of photofading should be assessed. To do so, carry out an experiment in which the photobleaching step is omitted. Ideally, the levels of fluorescence should remain constant for the entire timeseries. Photofading will result in a systematic loss of fluorescence over time. Keep in mind a small amount of photofading is acceptable as it can be corrected for during data processing without impacting the experimental results, as discussed below. However, if significant photofading is seen in the recovery readjust the pinhole, laser transmission, and detector gain to minimize photofading. If photofading is still a significant problem, consider changing fluorophores.

Performing confocal FRAP

The previous section discussed the general steps involved in setting up a FRAP experiment. However, the actually data collection process will vary depending on the particle or

biological process being studied. In this section, we will go through the actual steps of FRAP data acquisition for three common FRAP applications: (1) measurements of diffusion of a membrane protein at the cell surface, (2) measurements of diffusion of a soluble cytoplasmic or nuclear protein, and (3) analysis of intracellular trafficking kinetics.

Example 1. Lateral diffusion measurement for slowly diffusing membrane proteins in cells with large flat plasma membranes.

This subsection presents a FRAP protocol which is ideal for slowly diffusing membrane markers. This includes transmembrane proteins and most lipid anchored proteins, such as GPI-anchored proteins or bacterial toxins that bind lipid receptors at the cell surface (as illustrated in Figures 1 and 2). However, FRAP can also be done on fluorescent lipid analogs, such as the DiIC class of membrane markers, which have much faster rates of diffusion. For these fast-moving molecules, the method outlined here may not produce the temporal resolution necessary to resolve the recoveries. In that case, the following example will need to be modified to allow for faster data acquisition, as outlined for soluble markers in Example 2.

1. Locate a representative cell in the eyepiece.
2. Begin imaging, and use the fine z-adjustment to align the confocal slice to obtain a large, flat expanse of plasma membrane.
3. At this point the bleaching parameters and bleach ROI should be saved in the computer, as they were determined in the “Configuring the Confocal for FRAP Section”. Load these settings. The flat membrane region selected should be significantly larger than the bleach ROI (Figure 1A). If not, find another cell, or create a smaller bleach ROI. Note that for ease of data analysis it is recommended that the position of the bleach region within the image window be held constant from cell to cell, i.e. moving the cells as needed rather than moving the bleach region, as this enables batch processing of data sets.
4. Using a 512×512 pixel window with the appropriate electronic zoom and the ROI centered in the image window, move the stage in the X and Y planes such that the bleach ROI is centered in the flat membrane region. It will become important during data analysis that a background region outside the cell is also visible in the imaging window (Figure 1B). If not, collect at least 3 examples of background under the identical conditions.
5. Perform FRAP by selecting the preset “start FRAP” tool on your microscope’s software.
6. Examine the FRAP curve on the computer. As with the FRAP done in the “Configuring the Confocal FRAP” section, be sure that there are at least 3 prebleach images, good bleach depth, multiple data points during the recovery period and minimal photofading due to imaging. For a membrane protein, the recovery will generally level off 1–2 minutes after the bleach, although the exact recovery time will vary depending on the size of the bleach ROI and diffusional mobility of a given protein or lipid (Figures 1 and 2). Note that for slower moving particles, line averaging can be used. This will decrease the noise in the data but may increase photofading.
7. Make any necessary adjustments to the time series set up.
8. Once the experimental setup has been perfected, repeat FRAP on 12–14 individual cells per sample group. It is acceptable to readjust the detector gain used for collecting pre- and postbleach images between cells, since the degree of labeling/

protein expression will likely change between cells. However, all other settings must be kept the same from cell to cell (including zoom, frame rate and count, scan speed, line averaging, bleach iterations, and bleaching laser wavelength and intensity). This allows comparisons to be made between sample groups.

9. Repeat the experiment on multiple days to ensure reproducibility.

Example 2. Lateral diffusion measurements for a rapidly diffusing cytoplasmic protein

In addition to measuring slow diffusion, as is the case for most membrane proteins, FRAP can also be employed to study faster diffusing molecules, such as soluble proteins in the cytoplasm or nucleus (Figures 3 and 4). However, the protocol must be changed from the method presented for membrane proteins (Example 1) in order to achieve the necessary temporal resolution required when studying soluble markers. To monitor fast diffusion, this example illustrates how to crop the image window around the ROI allowing for faster FRAP data acquisition.

1. Locate a representative cell using the eyepieces.
2. While continuously imaging, use the fine Z-adjustment to align the confocal slice with a large expanse of cytoplasm.
3. At this point the bleaching parameters and ROI should be saved in the computer, as they were determined in the “Configuring the Confocal for FRAP Section”. Load these settings.
4. Using an imaging window at the appropriate electronic zoom with the ROI centered in the image frame, move the stage in the X and Y so that the cytoplasm fills the entire image window.
5. Next, the number of pixels imaged needs to be reduced. Using the LSM software, this can be done by specifying a digital zoom or by reducing the pixel number in the image window. For this application of FRAP, crop the image around the ROI to reduce the number of pixels imaged. For the best results make the imaging window a rectangle that is as tall as the bleaching ROI, and at least 3 fold wider than the bleaching ROI. For example, we often use a bleaching ROI 2.3 μm in diameter, centered in a rectangular imaging window of $13.7 \times 3.4 \mu\text{m}$ when photobleaching in the cytoplasm (Figure 3). To further increase the rate of data acquisition, bidirectional scanning can be used.

Unlike the FRAP performed on a full 512×512 pixel window (as described for membrane proteins), reducing the image window for soluble proteins makes measuring whole cell fluorescence decay and background fluorescence at the same time as the FRAP data difficult. Therefore, do not worry about aligning the image window to show a region outside of the cell (as was done for the membrane protein FRAP). In a subsequent step, we will discuss collecting separate whole cell decay and background data that can be used to correct the final FRAP curves.

6. Collect a single image to verify that the fluorescence intensity is high for most pixels, but with only a few pixels saturated. If not, adjust the pinhole, laser intensity, or detector gain.
7. Perform FRAP by selecting the preset “start FRAP” tool on your microscope’s software.

In the case of very fast moving soluble proteins, the bleached molecules will disperse so quickly that a defined ROI may not be recognizable in the postbleach image. Instead, the entire image may darken immediately after the bleach. This is

well illustrated by comparing the recoveries of EGFP and p53-EGFP for FRAP experiments performed under the same conditions (Figure 4). In cases where the bleach region is unrecognizable in the images, a fixed sample can always be used as a control to confirm that bleaching is in fact occurring and that the absence of a discrete bleach spot is due to rapid recoveries.

8. Examine the FRAP curve on the computer. As with the FRAP done in the “Configuring the Confocal FRAP” section be sure to collect a minimum of 3 prebleach images, obtain a good bleach depth, collect multiple data points during the recovery phase, and minimize photofading. Additionally, the FRAP curves will need to be carried out well past the initial recovery period, which for a soluble protein typically takes 2–20 seconds depending on the size of the bleach ROI and the protein being studied (Figures 3 and 4).
9. Make any necessary adjustments to the time series set up. Once the experimental setup has been perfected, repeat FRAP on 12–14 individual cells per sample group. The degree of labeling/vector expression will likely change between cells. Therefore, it is acceptable to readjust the detector gain between cells. However, all other settings (including zoom, frame rate and count, scan speed, line averaging, bleach iterations, and bleaching laser wavelength and intensity) must be kept the same from cell to cell or else the data cannot be easily grouped for batch analysis later.
10. Next, collect some control data which will be used later to correct the FRAP curves for photofading caused by imaging. To do this set up the ROI’s just as with the FRAP experiment (Step 1–9). However, run the experiment without the bleach. Repeat on 3–5 cells.
11. Finally, collect background fluorescence by running a time series (as in Step 10) on the background fluorescence in an area where no cells are present or for unlabeled cells.
12. Repeat the experiment on multiple days to ensure reproducibility.

Example 3. FRAP analysis of intracellular trafficking kinetics

In addition to measuring the diffusion of a particle in a single compartment (i.e. nucleoplasm, cytoplasm, or membrane), FRAP can also be used to measure trafficking between compartments. In this example, we will explain how FRAP can be used to measure nucleocytoplasmic transport (Figure 5). Similarly, by moving the ROI to another organelle one could measure molecular trafficking between compartments, as has been done to study Ras trafficking to and from the Golgi complex (Goodwin et al., 2005b).

1. Locate a representative cell in the eyepiece.
2. Use the fine Z-adjustment to align the confocal slice with the center of the nucleus on the computer screen. Then move the stage in the X and Y planes such that the nucleus is centered in the imaging window.
3. At this point the bleaching parameters for the fluorophore being used should be saved in the computer as they were determined in the “Configuring the Confocal for FRAP Section”. Load these settings.
4. In a 512×512 image window, choose an electronic zoom setting that allows imaging of the whole cell. Draw a new ROI around the nucleus. Try to exclude any cytoplasm from the ROI.

Unlike when measuring diffusion in a single compartment, this ROI does not have to be perfectly circular.

5. Take a single image to verify that most pixels have a high fluorescence intensity, but with only a few pixels saturated. If this is not the case adjust the pinhole, laser intensity, or detector gain.
6. Perform FRAP by selecting the preset “start FRAP” tool on your microscope’s software.
7. Examine the FRAP curve on the computer. As with the FRAP done in the “Configuring the Confocal FRAP” section be sure that there are least 3 prebleach images, good bleach depth (of 50% or greater drop in fluorescence), multiple data points during the recovery, and minimal photofading.

Keep in mind that for this application of FRAP the recovery times will likely be much longer than when FRAP is done to measure free diffusion, as transport can be much slower than diffusion. For example, EGFP takes approximately 5 minutes following the bleach to equilibrate between the cytoplasm and nucleus (Figure 5). However, when examining diffusion of EGFP within the cytoplasm using a small bleach ROI, the post bleach fluorescence will reach equilibrium in a matter of seconds (Figure 4).

8. Make any necessary adjustments to the time series setup. Once the experimental setup has been perfected, repeat FRAP on 12–14 individual cells per sample group. A new ROI will need to be drawn in each cell as the size and shape of the nucleus may vary between cells. The degree of labeling/vector expression will also likely change between cells. Therefore, it is acceptable to readjust the detector gain between cells. However, all other settings (including zoom, frame rate and count, scan speed, line averaging, iterations, and bleaching laser wavelength and intensity) must be kept the same from cell to cell, or else the data cannot be easily grouped for batch analysis.
9. Repeat the experiment on multiple days to ensure reproducibility.

Initial analysis of FRAP data at the microscope

Before leaving the microscope, it is a good idea to undertake a quick review of the data so that if any major flaw is detected in the experiment, new data can be quickly generated. Also, data tables will need generated and exported for subsequent analysis. Here we describe both steps.

1. On the microscope computer, play back the timelapse images collected during the FRAP experiment. There are three common artifacts which can render data unusable and that can be easily identified at this step. First, the focal plane may have drifted during the FRAP experiment. This will compromise the data as the focal plane will not stay aligned with the bleach region. This usually produces a FRAP curve which first increases as normal, then decreases as the specimen moves out of focus. Another error that can be observed in the image series is photofading due to imaging. A small degree of decay is acceptable as it can be corrected later (Figure 1). A third common artifact is when a large structure (such as an endosome) passes through the ROI during the FRAP experiment. This will cause a significant spike or dip in the FRAP curve.
2. Examine the individual raw FRAP curves for any irregularities. If the curves look acceptable, export the mean fluorescence intensity in the bleach ROI for each time

point. The times should be contained within the timestamps for each image and will be automatically exported as part of the ROI analysis in the Zeiss software.

3. In a case where the FRAP was done using the whole image window, as opposed to the cropped window method discussed for cytoplasmic/nuclear proteins, draw an ROI around the whole cell and another around a region in the background (Figure 1B). Export this data as well.

Normalizing the FRAP data and calculating mobile fraction and $t_{1/2}$ values

The following steps will guide you through normalizing the data and plotting the data for documentation and publication. Additionally, the calculations for acquiring the mobile fraction (M_f) and half time of recovery ($t_{1/2}$) values are also presented. The mobile fraction is the portion of molecules that can undergo diffusion. The $t_{1/2}$ is the point in the recovery curve at which half of the fluorescence recovery has occurred. Since recovery is the result of molecular movement, $t_{1/2}$ is related to the rate of diffusion (or kinetics of transport). It is important to bear in mind that the $t_{1/2}$ values are influenced by a number of factors in the experiment, including the amount of time taken to photobleach the ROI and the ROI spot size. The math involved in determining M_f and $t_{1/2}$ is relatively simple and this step in the data processing can be easily automated in Excel. In the subsequent “Further analysis of FRAP data to obtain diffusion coefficients” section, we presents the more complex math involved in extracting diffusion coefficients which are independent of bleach time or spot size.

1. It is customary to plot the FRAP curve with the first point after the bleach being set as $t = 0$. To do so, subtract the time of the first postbleach image from each timepoint in the series.
2. For further data analysis and to produce publication quality graphs the FRAP curves will need to be corrected for background fluorescence (F_{bkgd}), photofading, and loss of fluorescent material due to the bleach, as well as normalized to correct for differences in protein expression levels between cells:

$$F(t)_{norm} = \frac{F(t)_{ROI} - F_{bkgd}}{F(t)_{cell} - F_{bkgd}} \times \frac{F(i)_{cell} - F_{bkgd}}{F(i)_{ROI} - F_{bkgd}} \quad (1)$$

The first part of this normalization accounts for irreversible loss of molecules owing to the bleaching event, as well as any photofading that may have occurred. To correct for this, the bleaching ROI intensity $F(t)_{ROI}$ is divided by the whole cell intensity $F(t)_{cell}$ for each time point $F(t)$. The second part of the normalization rescales the data in terms of percentage of initial fluorescence by multiplying by the initial whole cell intensity $F(i)_{cell}$ divided by the initial intensity in the ROI $F(i)_{ROI}$ (Figure 1D).

3. Plot $F(t)$ over time for each sample for a given sample group on the same graph so that any outliers in the data can be easily identified.
4. Calculate the mean and standard deviation of $F(t)$ for each group of samples. Then plot this mean and SD of $F(t)$ against time.
5. M_f can be calculated from the data obtained from the normalized recovery curves (eq. 2) as

$$M_f = \frac{(F_{\infty} - F_0)}{(F_i - F_0)} \times 100 \quad (2)$$

where F_{∞} , F_0 , and F_i are the normalized fluorescence intensities inside the bleach ROI after full recovery (at the asymptote), immediately following the bleach, and before the bleach, respectively (Figure 1D). Mobile fractions are customarily reported as a percentage, and therefore the product is multiplied by 100.

6. The M_f values for each FRAP curve can then be averaged across all the cells, thus providing statistics for the cell population.
7. As a first approach to data quantification, FRAP recovery curves can be described in terms of the half time of recovery ($t_{1/2}$). This can be approximated by fitting to the equation (Feder et al., 1996)

$$F(t) = [F_0 + F_{\infty}(t/t_{1/2})] / [1 + (t/t_{1/2})] \quad (3)$$

The $t_{1/2}$ values can then be averaged, thus providing statistics for the cell population.

Further analysis of FRAP data to obtain diffusion coefficients

In many cases, the fluorescent molecules of interest are uniformly distributed throughout the cytoplasm or nucleus or within the plasma membrane. As a starting point, under these conditions FRAP data can be analyzed quantitatively using a closed form analytical equation describing a purely diffusive process (Kang et al., 2009). Here we describe the protocol for analyzing FRAP data using a single component pure diffusion model to obtain diffusion coefficients. For quantification of more complex modes of diffusion or transport, including reaction-diffusion type behavior or anomalous diffusion, alternative mathematical models should be used to extract quantitative results from the FRAP data (Axelrod et al., 1976; Peters, 1983; Sprague et al., 2004; Hallen et al., 2008; Kang et al., 2010).

The initial treatise on extracting diffusion coefficients did not take into account scenarios where diffusion is occurring during the bleach (Axelrod et al., 1976). This is not a tremendous concern in some cases where either the bleach event is extremely short and/or diffusion is extremely slow. However, this becomes a major concern when using line scanning confocal microscopes, as molecules have time to recover during the bleaching event. Additionally, the extent of diffusion during the bleach becomes greater as the rate of diffusion of the molecule becomes greater (Figures 2 and 3). We and others have found a method to correct for diffusion during the bleach prior to extracting the diffusing coefficient (Braga et al., 2004; Kang et al., 2009). This is done by altering the radius of the user-defined bleach spot (known as the nominal radius or r_n) to an empirically determined radius that takes into account diffusion that may have occurred during the bleach (called the effective radius or r_e).

To determine the effective radius, the bleaching profile of the ROI must be established. To do this, first obtain the fluorescence intensities as a function of distance along any line bisecting the post-bleach image ($Profile_{post}$). Next, the post-bleach profile must be normalized. To accomplish this, measure the fluorescence intensities immediately prior to photobleaching ($Profile_{pre}$) along the same axis as before. Now the normalized post-bleach profile is obtained by,

$$\frac{Profile_{post}}{Profile_{pre}} \quad (4)$$

Assuming the center of the circular bleaching ROI is the origin, the post-bleach profile can be approximated by an exponential of a Gaussian laser profile:

$$\varphi(x, y) = F_i \exp \left(-K \exp \left(-\frac{2(x^2 + y^2)}{r_e^2} \right) \right) \quad (5)$$

where $F_i = 1$ for a normalized post-bleach profile, K is a bleach depth parameter, and r_e is the effective radius. Both K and r_e contain information about the initial conditions required to solve the diffusion equation, and approximate the diffusion that occurred before acquisition of the post-bleach image (Braga et al., 2004). By solving the diffusion equation in two dimensions with an initial condition given by Eq. 5, the FRAP equation can be derived as a closed-form analytical solution in series form (Kang et al., 2009),

$$F(t) = F_i \sum_{m=0}^{\infty} \frac{(-K)^m r_e^2}{m! (r_e^2 + m(8Dt + r_n^2))} M_f + (1 - M_f) F_0 \quad (6)$$

where $F_i = 1$ for a normalized FRAP curve and r_n is the nominal radius of the circular bleaching ROI. Due to a fast convergence rate of this Taylor series, summation over the first 30 terms approximates Eq. 6 excellently. Finally, by fitting normalized FRAP data (Eq. 1) with the FRAP equation for D (Eq. 6), the diffusion coefficient can be determined. MatLab codes to fit FRAP data are available from the authors upon request.

Sometimes there is a discrepancy between the initial fluorescence intensities of the FRAP fit and the FRAP data (Eq. 1, 6). This is largely due to the difference between the experimental and theoretical postbleach profiles (Eq. 4, 5). We recommended resolving this by numerically solving

$$F_0 = \sum_{m=0}^{\infty} \frac{(-K)^m r_e^2}{m! (r_e^2 + m r_n^2)} \quad (7)$$

where F_0 is the initial fluorescence intensity of FRAP data.

Commentary

Background information

FRAP was developed using a focused, static laser beam in the early 1970's. A few years later, conventional FRAP theory for quantitative FRAP analysis was established (Axelrod et al., 1976). At the time, performing FRAP required high levels of expertise. General procedural issues in early FRAP applications are discussed in detail elsewhere (Axelrod et al., 1976; Thompson et al., 1981; Elson, 1985). With the help of advances in computer and laser technology in the 70's and 80's, the first form of a laser scanning confocal microscope for imaging fluorescent biological specimens appeared in the late 1980's.

In addition to limitations from the microscopes, early FRAP experiments were also restricted to markers which could be exogenously introduced and the application was very specialized for plasma membrane studies. Although GFP was discovered in the 60's, it wasn't until the early 90's when the application of GFP fusion was elucidated. Today, GFP (or other fluorescent proteins or organic dyes) can be attached to almost any protein of interest, making FRAP possible with a wide variety of proteins.

As the tools for performing FRAP have developed, so have the ways in which scientists apply FRAP. Common variations on FRAP include fluorescence loss in photobleaching (FLIP) (Cole et al., 1996), fluorescence localization after photobleaching (FLAP) (Dunn et

al., 2002), inverse FRAP (iFRAP) (Dundr et al., 2002), two-color FRAP (Picard et al., 2006), FRAP-FRET (Vermeer et al., 2004) and photoconversion (Patterson and Lippincott-Schwartz, 2002). Additionally, while FRAP is conventionally used to measure diffusion/transport, recent mathematical advances have made extraction of *in vivo* binding kinetics values from FRAP data possible (Carrero et al., 2004; Sprague et al., 2004; Kang et al., 2010). While many exciting variations on and applications of FRAP have been developed, for the purposes of this chapter we have focused on application of FRAP to the study of diffusion and trafficking using a confocal laser scanning microscope.

Mathematical description of FRAP

Quantitative FRAP analysis requires a mathematical description of fluorescence recovery for a given underlying transport/reaction kinetics as well as the two different modes of laser excitation: photo-illumination and photobleaching (Kang and Kenworthy, 2009). For small bleaching spot size, r_n , it has been reported that the scanning profile of a confocal laser is well approximated by a Gaussian function (Braga et al., 2004):

$$L_{r_n}(x, y) = \frac{2L_0}{\pi r_n^2} \exp\left(-\frac{2(x^2 + y^2)}{r_n^2}\right) \quad (8)$$

where L_0 is the maximal laser intensity. Similarly, a scanning profile of photo-illumination mode can be described as $\epsilon L_{r_n}(x, y)$ for an attenuation factor $\epsilon \ll 1$. If we let $F(x, y, t)$ be the fluorescence intensity at a location (x, y) at time t , then $F(x, y, t)$ is proportional to the illumination mode laser intensity at (x, y) , and the number of fluorescent proteins at that location at time t . If the concentration of fluorescent proteins is described by $u(x, y, t)$, then

$$\text{the number of fluorescent proteins in } A = \left[x - \frac{\delta x}{2}, x + \frac{\delta x}{2}\right] \times \left[y - \frac{\delta y}{2}, y + \frac{\delta y}{2}\right] \text{ is}$$

$$u(x, y, t) \delta x \delta y, \quad (9)$$

and the local fluorescence intensity in A is computed as

$$F(x, y, t) = q \cdot \epsilon L_{r_n}(x, y) u(x, y, t) \delta x \delta y \quad (10)$$

where a proportionality constant q is referred to as a quantum yield or quantum efficiency.

Finally, the total fluorescence intensity from the ROI can be found by integrating this local fluorescence intensity over the ROI

$$F(t) = q \epsilon \iint_{\mathbb{R}^2} L_{r_n}(x, y) u(x, y, t) dx dy \quad (11)$$

which is called a FRAP equation for u .

Note that different underlying kinetics for u yields different FRAP equations. For free diffusion, the evolution of $u(x, y, t)$ can be described by the diffusion equation, subject to the initial conditions given by a postbleach profile right after photobleaching

$$\begin{cases} u_t = D \Delta u \\ u(x, y, 0) = \varphi(x, y) \end{cases} \quad (12)$$

where $D(\mu\text{m}^2/\text{s})$ is the diffusion coefficient, the Laplacian $\Delta = \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2}$, and ϕ is given by Eq. 5, which describes the postbleach fluorescence intensity profile. The solution of the diffusion equation can be found as

$$u(x, y, t) = \Phi_D * \phi = \iint \Phi_D(x - x', y - y', t) \phi(x', y') dx' dy' \quad (13)$$

where the fundamental solution of the diffusion equation $\Phi_D(x, y, t)$ is defined as

$$\Phi_D(x, y, t) = \frac{1}{4\pi Dt} \exp\left(-\frac{x^2 + y^2}{2Dt}\right). \quad (14)$$

The postbleach profile, $\phi(x, y)$ is a function that describes an experimental postbleach profile. In theory, $\phi(x, y)$ can be obtained by solving the photobleaching equation. Assuming a first order photobleaching process with a photobleaching rate α , a governing equation for a photobleaching process of freely diffusing fluorescent proteins can be described as a reaction diffusion equation:

$$\begin{cases} u_t = D\Delta u - \alpha L_{r_n}(x, y)u \\ u(x, y, 0) = u_0 \end{cases} \quad (15)$$

where u_0 is the prebleach steady state fluorescence intensity, which is regarded as a constant.

Unlike the diffusion equation, this photobleaching equation cannot be solved explicitly, so an approximated form of the solution is used for $u(x, y, T)$ where T is the duration of photobleaching. From the empirical observation, $\phi(x, y)$ is chosen as an exponential function of Gaussian as in Eq. 5.

With this consideration (Eqs. 5–8), the FRAP equation for free diffusion becomes (Kang and Kenworthy, 2008; Kang et al., 2010)

$$F(t) = q\epsilon \iint_{\mathbb{R}^2} I_{r_n}(x, y) \left(\iint_{\mathbb{R}^2} \Phi_D(x - x', y - y', t) \phi(x', y') dx' dy' \right) dx dy = \sum_{n=0}^{\infty} \frac{(-K)^n}{n! (1 + n(\gamma^2 + 2t/\tau_{De}))} \quad (16)$$

where $\gamma = r_H/r_e$ and $\tau_{De} = r_e^2/(4D)$. Using this equation, the τ_{De} values that provide the best fit to the actual data can be determined and from there, $\tau_{De} = r_e^2/(4D)$ can be easily solved for D .

For FRAP equations addressing dynamics other than free diffusion, the governing equation that describes the evolution of fluorescent protein concentration (u) for a given a postbleach profile as an initial condition has to be found first. Then, the integral in Eq. 16 should be evaluated. For example, in the case of binding diffusion kinetics, some postbleach profiles can be described as $\phi = \beta\phi_1(x, y; r_e) + (1 - \beta)\phi_2(x, y; \bar{r}_e)$ for $0 < \beta < 1$ where $\phi_1(x, y; r_e)$ and $\phi_2(x, y; \bar{r}_e)$ are as defined in Eq. 5 (Kang et al., 2010).

Comparison of FRAP to other techniques sensitive to protein and lipid dynamics

Photoactivation and photoconversion are techniques in which fluorescent molecules in a certain area of a cell are selectively converted to an active fluorescent state or different color and then the fluorescent signal is tracked over time to get information on the mobility of proteins as well as the viscosity of the cellular milieu (Politz, 1999; Lippincott-Schwartz et

al., 2003; Lippincott-Schwartz and Patterson, 2008; Patterson, 2008). To do so, photoactivation and photocoverseion experiments require specific fluorescent protein variants such as photoactivatable GFP (PA-GFP), Kaede80, kindling fluorescent protein 1 (KFP1), and others (Lukyanov et al., 2005; Kremers et al., 2009; Kremers et al., 2011). Since photoactivation shares the same theoretical framework as FRAP, it can be understood as a complementary counterpart to FRAP where the same analytical equations will apply.

Fluorescence Loss in Photobleaching (FLIP) is another fluorescence based technique that utilizes the photobleaching property of fluorescent proteins (Lippincott-Schwartz et al., 2001; Lippincott-Schwartz et al., 2003). In a FLIP experiment, a photobleaching ROI is selected in a cell and then the ROI is repeatedly photobleached while the whole cell is continuously imaged. From the rate of loss in fluorescence at observation ROIs outside of the photobleaching ROI, FLIP can assess not only the connectivity between the ROIs but also whether a protein moves uniformly or undergoes interactions that impede its transport between the ROIs. Since the total number of fluorophores (photobleached fluorophores + fluorescent fluorophores) remain constant before and after photobleaching, FRAP and FLIP are complementary and FLIP data can also be analyzed in a quantitatively similar way as FRAP to obtain kinetic rate constants.

Another group of fluorescence based methods such as fluorescence correlation spectroscopy (FCS) and image correlation spectroscopy (ICS) utilize fluorescence fluctuations in time and/or space rather than photobleaching (Bacia and Schwille, 2003; Elson, 2004; Hausteint and Schwille, 2007; Kolin and Wiseman, 2007; Petrasek et al., 2010). Therefore, these methods may serve as independent cross validation tools for quantitative FRAP analysis. In FCS, fluorescence fluctuations due to movement of fluorescent molecules in and out of a small volume (confocal volume) is analyzed via a time dependent correlation function. One strength of FCS is that it can measure the mean fluorescent protein concentration in the picoliter confocal volume. FCS is best suited for investigating fast kinetics, as slow moving molecules may become photobleached as they pass through the confocal volume creating a serious problem for studying slow diffusion with FCS. FCS generally requires very low concentrations of fluorescent particles in order to correlate the data, making FCS a good alternative to FRAP when studying low concentrations of markers. However, one strength of FRAP over FCS is that FCS cannot yield information on the immobile fraction.

Whereas FCS utilizes a time correlation function of fluorescence fluctuation from a confocal volume, image correlation spectroscopy (ICS) deals with spatial autocorrelation functions calculated from spatial fluorescence fluctuations in the fluorescence microscopy images (Kolin and Wiseman, 2007). Therefore, ICS can be understood as an imaging analog of FCS. ICS can determine velocity and aggregation state of fluorescently labeled proteins in addition to the fluorescent protein concentration and mobility. However, since this technique requires images to be collected in scanning mode, which takes time, it may not be as well suited as FCS for examining fast diffusing species. For this reason, ICS analysis is mostly applied to membrane-associated receptors, receptor clusters, or even immobile objects (Kolin and Wiseman, 2007).

The fluorescence microscopy techniques discussed to this point all report on diffusion of multiple particles at once. On the other hand, single particle tracking (SPT) exclusively focuses on the motion of individual molecules (Saxton and Jacobson, 1997; Chen et al., 2006; Levi and Gratton, 2007; Alcor et al., 2009; Kusumi et al., 2010). By analyzing the trajectories of the tracer particles in time, not only the mobility of the tracers but also heterogeneities in their cellular environment can be uncovered. Hence, mobility analysis by FRAP can be validated by SPT analysis. However, this method requires special camera systems and tracking software making it less accessible than FRAP. Furthermore, as its

name implies, SPT is limited to observing only a very small number of particles at one time, although recently these approaches have been extended to allow for visualization of multiple molecules simultaneously (Jaqaman et al., 2008; Manley et al., 2008).

Fluorescence based techniques have become among the most powerful tools in modern biology. As we have seen, a wide variety of fluorescence based techniques for diffusion measurements are currently available making selection of a proper technical approach challenging. Therefore, it is important to keep in mind the strengths and weaknesses of each technique when choosing how to best answer a given biological question.

Considerations for data analysis

With appropriate mathematical models that describe changes in fluorescence protein concentration in time, FRAP has been used successfully to analyze lateral diffusion, free diffusion, reaction-diffusion, anomalous diffusion, as well as active transport (Axelrod et al., 1976; Feder et al., 1996; Sprague et al., 2004; Kang et al., 2009; Roth et al., 2009; Kang et al., 2010). Because diffusion coefficients determine the transport and reaction rates of proteins in living cells, they are extremely important to understand how biological processes are orchestrated in time and space in a living cell.

Since the recovery kinetics are different in different spatial dimensions, 1D, 2D and 3D FRAP equations have to be considered separately. For example, the mean square displacement of proteins undergoing free diffusion with a diffusion coefficient D is approximated as

$$\langle x^2 \rangle = 2NDt \quad (17)$$

where N is the spatial dimensions ($\mathbb{R}^N$). Therefore, the number of dimensions in which a particle is free to diffuse will significantly impact both the x and D values.

In theory, the kinetic rate constants obtained by FRAP should be independent of experimental conditions such as bleaching spot sizes and photobleaching iterations. However, in some cases, a dependence on experimental conditions is observed as the result of diffusion and reaction during photobleaching. This is particularly true for large spot sizes, long bleaching events or for rapidly diffusing molecules, as illustrated in Figures 3 and 4. This dependence can be removed by using initial conditions obtained from the experimental postbleach profiles. i.e. by measuring the effective radius r_e from the experimental postbleach profiles (Braga et al., 2004; Kang et al., 2009). Other cases where diffusion coefficients may vary as a function of bleach spot size is when anomalous diffusion occurs. This effect can be used to obtain information about underlying structures that hinder diffusion, such as the cytoskeletal meshwork (Lenne et al., 2006).

FRAP analysis also depends on the size and geometry of the cell. Cells cannot necessarily be treated as an infinite space because the boundary effects become significant. In addition, due to the finite size of a cell, the recovery curve may not reach the level of prebleach steady state even when there is no immobile pool of proteins in the ROIs due to loss of a significant fraction of fluorescent molecules during the photobleach. Although one can develop a realistic recovery model taking the cell size and boundary effects into consideration, these types of problems often do not have an explicit solution in a closed form and have to be evaluated by time consuming numerical computations, which are not practical for analyzing large amounts of data. Therefore, it is important to find an optimal photobleaching condition where the bleach ROI is small while at the same time obtaining a reasonable bleaching depth.

Other possible artifacts in FRAP are linked to the properties of the fluorophores themselves, including photofading and reversible photobleaching/photoswitching. Photofading is well characterized by a slow single exponential decay and is easily observed from time-lapse images obtained under the same imaging conditions used to acquire FRAP curves. Photofading may interfere with analysis of FRAP, especially when long recoveries are being followed. In cases where only a small degree of photofading occurs, the FRAP curves can be corrected using Eq. 1 (Figure 1).

The photobleaching process in FRAP is often assumed to be an irreversible process. However, this is not always true as some fluorophores, such as GFP, can reversibly recover from photobleaching as the result of photoswitching (Dickson et al., 1997; Dayel et al., 1999; Sinnecker et al., 2005; Mueller et al., 2012). The percent of photobleached molecules that undergo this process is dependent on the fluorophore in question as well as bleaching conditions and may represent a measureable percentage of the total bleached fluorophores. It is important to use a photo-stable fluorophore and to standardize bleaching protocols as well as intensity of the excitation light to minimize photoswitching (Dickson et al., 1997; Sinnecker et al., 2005; Mueller et al., 2012). Recently, a procedure for detecting, minimizing, and correcting for the effects of photoswitching of fluorescent proteins was reported (Mueller et al., 2012).

Artifacts in FRAP analysis may also arise from the microscope detection system (Mueller et al., 2008). For example, when the laser intensity is switched from photobleaching model to photo-illumination model, a transient reduction in fluorescence intensity can occur due to detector blinding. Although the effect of detector blinding on the FRAP curve is similar to that of irreversible photobleaching, they are distinguishable in that the detector blinding is also observed for the photo-illumination mode of laser. In addition, detector blinding happens only when the photobleaching area is relatively large, whereas no detector blinding is observed for a small spot bleaching FRAP data. Therefore, a correction for detector blinding may be required for FRAP experiments utilizing large bleaching areas (Mueller et al., 2008).

Other useful equations for benchmarking data analysis

The absolute value of D obtained experimentally contains important information about both the environment and structure of the diffusing molecule. The expected value of D can be approximated by theoretical predictions based on the molecular size, viscosity of the medium and the absolute temperature. The diffusion coefficient of a spherical object in 3D space can be approximated by the Stokes-Einstein relation:

$$D = \frac{\kappa_B T}{6\pi\eta r} \quad (18)$$

where κ_B , T , η and r are the Boltzmann's constant, the absolute temperature, viscosity of the medium and the radius of the spherical particle, respectively. This equation also provides a way to compare the diffusion coefficients of two different proteins with molecular weights, M_1 and M_2 as

$$\frac{D_1}{D_2} = \sqrt[3]{\frac{M_2}{M_1}} \quad (19)$$

assuming the proteins can be approximated as spheres (i.e. $M_i = \frac{4}{3}\pi r^3$). This relation also has a practical importance to predict whether a protein exists as a freely diffusing monomer, is part of a high molecular weight complex, or reversibly binds to cellular components. For

example, Drake et al. demonstrated that the autophagy-associated protein LC3 may exist as part of a large complex based on a comparison of the diffusion coefficients and molecular weights of EGFP and EGFP-LC3 (Drake et al., 2010).

For lateral diffusion in membranes, the Saffman and Delbruck equation (Saffman and Delbruck, 1975) can be applied to approximate the diffusion coefficient of membrane proteins with radius r as

$$D = \frac{\kappa_b T}{4\pi\mu h} \left(\log \frac{\mu h}{\mu r} - 0.5772 \right) \quad (20)$$

where η and μ are viscosities of the aqueous environment and membrane, and h is the thickness of the membrane. In practice however, membrane proteins do not diffuse as rapidly as predicted by this equation in cell membranes due to the complex environment of the cell (Jacobson et al., 1995).

Critical Parameters

Choice of fluorophores

The choice of a fluorophore is a critical consideration for FRAP studies, and no single fluorophore excels in all situations. Some important considerations are: 1) Can the molecule of interest be labeled with an organic dye, and exogenously added to the cell? 2) Is the molecule of interest easily fused with a fluorescent protein using genetic engineering?

If the molecule of interest is a protein, and can be easily genetically engineered with either an N- or C-terminal fluorescent protein tag we recommend using monomeric EGFP (~ 27 kDa), Venus, or Emerald. EGFP has a high brightness (defined as the product of quantum yield and molar extinction coefficient), good photostability during imaging, and is easily irreversibly photobleached. Venus is a yellow GFP variant with very high brightness, and reasonably good photostability. Emerald is spectrally similar to EGFP, and is also an extremely bright and photostable option if photostability is an issue. Another important consideration is that tagging a molecule of interest with a fluorescent protein may alter the native function or distribution of the molecule of interest. Therefore, when possible, we recommend confirming that the fluorescent fusion protein retains the native behavior of the molecule of interest. For further discussion of the properties of specific fluorescent proteins, see (Day and Davidson, 2009; Kremers et al., 2011).

For molecules of interest that can be easily isolated and added exogenously to a cell population, using a fluorescent small molecule to tag the protein is advantageous. The organic Alexa Fluor dyes are a very good choice of fluorophore if FRAP is to be performed on the tagged molecule. These dyes are extremely bright, photostable, and easily photolysed under higher intensity radiation. This class of fluorescent small molecules is commercially available in a wide range of colors, as well as different variants for the desired means of attaching them to the molecule of interest. For example, Alexa Fluor 488 (which is spectrally similar to GFP) can be ordered in a variety of reactive forms, including amine reactive, carboxylic acid reactive, aldehyde reactive, and thiol reactive allowing direct labeling of the molecule of interest. As with fluorescent fusion proteins, labeling a protein with a fluorescent dye may alter the native function or distribution of the molecule of interest and we recommend confirming that the fluorescent fusion protein retains the native behavior of the molecule of interest.

Choice of bleach ROIs and geometries

The quantitative method for obtaining the diffusion coefficient we described here requires a circular bleach region. The bleaching ROI should be defined as small as possible in order to approximate the post-bleach profile as an exponential of a Gaussian laser profile. In practice, the signal to noise ratio of the measurements dictates how small the bleaching spot can be defined. In our experience, a bleaching ROI with a 1 μm radius using a 1.4 NA 40X objective is a good starting point. It is important not to change any microscope settings between samples in order to qualitatively interpret the data. For qualitative experiments, any size or shape bleaching geometry may be used.

Bleaching times and sampling rate

The bleaching time should be minimized, while still resulting in an adequate amount of bleaching. The sampling rate should be maximized to obtain as many early time points in the recovery as possible, while being careful not to increase the amount of unintentional photobleaching (photofading) during imaging.

Troubleshooting

Molecule does not appear to bleach

In the case of very fast moving molecules, such as soluble GFP, recovery may be so rapid that a defined ROI bleach region is not visible to the eye in the postbleach image (Figure 4). The lack of visible bleach region is a characteristic of rapid diffusion during the bleach. If no bleach region is visible, first see if there is a general darkening of the image at the moment after the bleach event. This effect is the result of the bleached molecules diffusing out of the bleach ROI before the first post bleach image is acquired. If this effect is too great, it can become difficult to accurately measure the post bleach profile, potentially leading to underestimates of the diffusion coefficient. Next, check the FRAP curve to validate the bleaching event occurred. If you are still not confident the FRAP experiment is set up properly, the experiment can be repeated on a fixed sample, where the bleached ROI should be clearly visible to the eye.

If the molecules did not bleach in the fixed sample, double check to make sure the software is properly configured to bleach the ROI. It may be necessary to increase the number of bleaching iterations, the bleaching laser intensity, and/or the bleaching wavelength. If the molecules still do not bleach we recommend switching to a fluorophore that is more easily bleached. If instead the apparent lack of bleaching is due to fast recoveries, try decreasing the size of the region being imaged, decreasing the time delay between images, or performing bidirectional scans.

Unintentional bleaching occurs during the recovery phase

We recommend reducing the intensity of the excitation light used for imaging as much as possible to avoid photodecay during imaging. To do this, try increasing the detector gain and decreasing the laser intensity and line averaging. Additionally, the confocal pinhole can be opened to allow more fluorescent light to the detector. Finally, the frame number and rate can be adjusted to allow for less photobleaching, as long as a sufficient amount of data is collected to produce the FRAP curve. All of these adjustments may help minimize photodecay. For slowly moving molecules such as membrane proteins the intensity from the entire cell ROI can be used for normalization (Figure 1). Alternatively, a small amount of decay can be corrected by fitting a single exponential decay to data collected under identical conditions but without a photobleaching event. If none of these methods are adequate to remove photodecay, another fluorescent marker will need to be selected.

Drift in focal plane

At times cells drift out of focus or particles move into or out of the ROI causing aberrations in the recovery curve. In this case, the data is not suitable for further analysis and should be dropped. To eliminate shifts in focal plane, make sure the sample has reached thermal equilibrium. The use of autofocus, available on most current confocal systems, will help to alleviate drift from occurring.

Anticipated Results—When performing FRAP for the first time it may be helpful to evaluate your results with previously determined diffusion coefficients and mobile fractions. Our lab has used the FRAP methods presented above to characterize the diffusion of a wide range of molecules (Table 1), which may be used as benchmarks. While these values should be fairly representative it is important to keep in mind that a variety of factors, including cell line and temperature, can impact these values. Alternatively, FRAP analysis can be performed for purified EGFP in aqueous glycerol solutions of known viscosity (Kang et al., 2009). Additionally, the way in which the data is fit can play a major factor in the final diffusion coefficient. It is therefore important to take great care in finding the best model to fit the FRAP curves.

Time considerations

It may take several sessions on the microscope to optimize a particular FRAP experiment depending on the degree of complexity. Once all the necessary conditions have been worked out, a FRAP experiment can be completed in three to four days, with only a small amount of time given to the experiment on most days. On the first day, cells will need to be plated. On the second day, any transient transfections will need to be performed. On the third day, FRAP can be carried out. A minimum of another day will be required for data analysis.

The first time FRAP is performed in the lab, Excel spreadsheets and/or MatLab code may need to be created *de novo* to analyze the data. This could take a considerable amount of time to accomplish. However, once these tools are assembled, analysis of future FRAP data can be reduced to between a few minutes to a few hours. MatLab codes for analyzing FRAP data are available from the authors upon request.

Acknowledgments

We thank Kimberly Drake for expert technical assistance. Support for development of the methods described here was provided by the Molecular Biophysics Training grant (NIH T32 GM08320), Vanderbilt Biomath Initiative, American Cancer Society-Institutional Research grant (No. IRG-58-009-48), the Sartain-Lanier Family Foundation, R01 GM073846, 3R01 GM73846-4S1, and NSF/DMS 0970008. The funding sources had no role in the study design, collection, analysis or interpretation of data, writing the report, or the decision to submit the paper for publication.

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Figure 1. Confocal FRAP assay

COS-7 cells were transiently transfected with the GPI-anchored protein YFP-GL-GPI 24 hours prior to FRAP. FRAP was performed at 37°C. **(A)** Representative time series images of YFP-GL-GPI on the plasma membrane of a COS-7 cell immediately following the photobleach ($t = 0$). Bar, 10 μm . **(B)** YFP-GL-GPI expressing COS-7 cell at $t = 0$ sec in the FRAP time series, showing ROI's denoting the bleaching ROI, whole cell, and background regions. **(C)** FRAP recovery in the bleach ROI, compared to whole cell fluorescence and background fluorescence. **(D)** Recovery curve of YFP-GL-GPI corrected for fluorescence decay and background fluorescence. Notation denotes the initial prebleach fluorescence (F_i), the initial postbleach fluorescence at $t = 0$ (F_0), and the fluorescence after recovery (F_∞). Additionally, the half maximal fluorescence recovery ($F_{1/2}$) denotes the $\tau_{1/2}$ position in the recovery curve.

Figure 2. Confocal FRAP assay of a membrane protein

COS-7 cells were labeled with 1 μM of Alexa546 tagged cholera toxin B subunit (A546-CTxB), which binds ganglioside GM_1 on the plasma membrane. FRAP was then performed using a 4.1 μm diameter bleach spot at 37°C. **(A)** Representative images of A546-CTxB during a FRAP experiment. Bar, 10 μm . **(B)** Raw fluorescence intensity profile of cross section through the bleach ROI and fitted curve with mathematically derived effective bleach ROI. **(C)** Representative recovery curve of Alexa546-CTxB corrected for fluorescence decay, along with mathematically fitted FRAP curve. **(D)** Diffusion coefficients of Alexa546-CTxB derived using the nominal bleach radius r_n and the effective bleach radius r_e for the same FRAP curves. Data show the mean $\pm$ standard deviation for 35 cells.

Figure 3. Confocal FRAP assay of a soluble protein

COS-7 cells were transiently transfected with mVenus. FRAP of mVenus in the nucleus was performed on the following day, using a 1 μm radius bleach spot. **(A)** Representative whole cell images of mVenus. Bar, 10 μm . **(B)** Representative images from a FRAP experiment of mVenus in the nucleus. Bar, 10 μm . **(C)** Normalized fluorescence intensity profile of cross section through the bleach ROI, and fitted curve with mathematically derived effective bleach ROI. **(D)** Representative recovery curve of mVenus corrected for fluorescence decay, along with mathematically fitted FRAP curve. **(E)** Diffusion coefficients of mVenus in the

nucleus derived using the nominal radius r_n or the effective radius r_e for the same FRAP curves. Data show the mean $\pm$ standard deviation for 12 cells.

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Figure 4. Comparison of FRAP data for different soluble proteins

COS-7 cells were transfected with the indicated soluble proteins. The following day, confocal FRAP experiments were performed at 37°C. (A) Representative images collected during a FRAP experiment in COS-7 cells. Data are shown for measurements in the nucleus. Note that the imaging ROI was cropped to enable more rapid data collection. Scale bar = 2 μm . (B) Recovery curves for EGFP (black circles), EGFP-LC3 (blue squares), tfLC3 (red triangles), and p53-GFP (green crosses) in the nucleus of COS-7 cells. Data show the mean recoveries for 10 cells for each protein from a representative experiment. For clarity, error bars are not shown. (C) Effective diffusion coefficients for EGFP, EGFP-LC3, tfLC3, and p53-GFP in the cytosol (black bars) and nucleus (gray bars) of COS-7 cells. Data show the mean $\pm$ SD for 30 cells from a total of 3 independent experiments. Figure adapted from Drake et al. (Drake et al., 2010).

Figure 5. Confocal FRAP assay of nucleo-cytoplasmic transport

COS-7 cells were transiently transfected with EGFP-LC3 or EGFP. The following day, FRAP experiments were performed to assess the rates of nuclear import and nuclear export. Representative images obtained before the photobleach (prebleach), immediately after the photobleach ($t=0$) and during the recovery phase ($t=150$ s or $t=300$ s) are shown. **(A)** To monitor nuclear import, the pool of EGFP-LC3 or EGFP in the nucleus was photobleached. **(B)** To monitor nuclear export, the cytoplasmic pool of EGFP-LC3 or EGFP was photobleached and fluorescence recovery recorded over time. Notice that both the nuclear

import and nuclear export of EGFP occur significantly more quickly than for EGFP-LC3. Bar, μm . Figure adapted from Drake et al. (Drake et al., 2010).

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Table 1

Diffusion coefficients of various molecules determined by FRAP

Particle	Class	Cell Line/Medium	r_n (μm)	D ($\mu\text{m}^2/\text{s}$)	Mf (%)	Citation
cholera toxin B subunit	plasma membrane	COS-7, MEF	2.05	0.2	80	Day and Kenworthy
YFP-GL-GPI	plasma membrane	COS-7, MEF	2.05	1.1	85	Day and Kenworthy
YFP-GT46	plasma membrane	COS-7, MEF	2.05	0.5	80	Day and Kenworthy
DiIC ₁₆	plasma membrane	COS-7, MEF	2.05	2.7	85	Day and Kenworthy
EGFP-HRas C184S	ER membrane/cytoplasm	COS-7	0.6–1.1	7.0	84	Kang et al. 2010
EGFP	Cytoplasm	COS-7	0.55–1.375	36–48	95	Kang et al. 2009, Drake et al. 2010
Venus	Cytoplasm	COS-7	0.99	44	99	Kraft and Kenworthy, 2012
EGFP-LC3	Cytoplasm	COS-7	1.375	10	93	Drake et al. 2010
Venus-LC3	Cytoplasm	COS-7	0.99	17	98	Kraft and Kenworthy, 2012
EGFP	Nucleus	COS-7	0.55–1.375	20–34	80	Kang et al. 2009, Drake et al. 2010
Venus	Nucleus	COS-7	0.99	49	99	Kraft and Kenworthy, 2012
EGFP-LC3	Nucleus	COS-7	1.375	10	78	Drake et al. 2010
Venus-LC3	Nucleus	COS-7	0.99	12	99	Kraft and Kenworthy, 2012
p53-GFP	Nucleus	COS-7	1.375	2	70	Drake et al. 2010
EGFP		40% glycerol	1.15	38	90	Kang et al. 2009
EGFP		50% glycerol	1.15	26	87	Kang et al. 2009
EGFP		70% glycerol	1.15	11	82	Kang et al. 2009

All FRAP was performed on a Zeiss LSM510 LSCM at 37°C under steady state conditions.

Abbreviations: DiIC₁₆, 1,1'-dihexadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; EGFP, enhanced green fluorescent protein; YFP, yellow fluorescent protein; MEF, mouse embryonic fibroblast.