Measuring protein mobility by photobleaching GFP-chimeras in living cells

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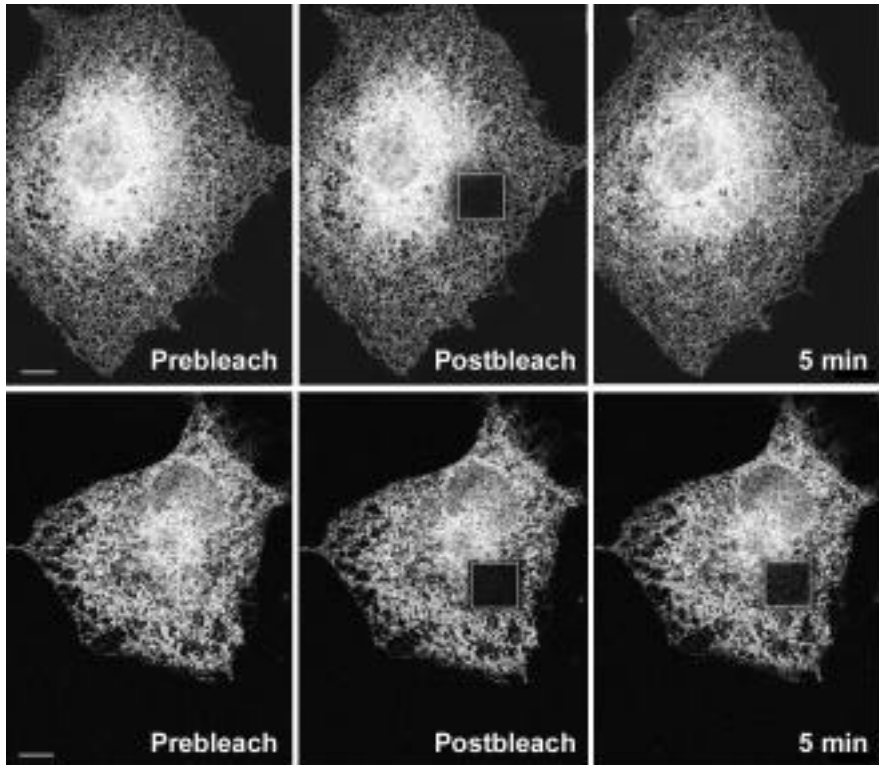
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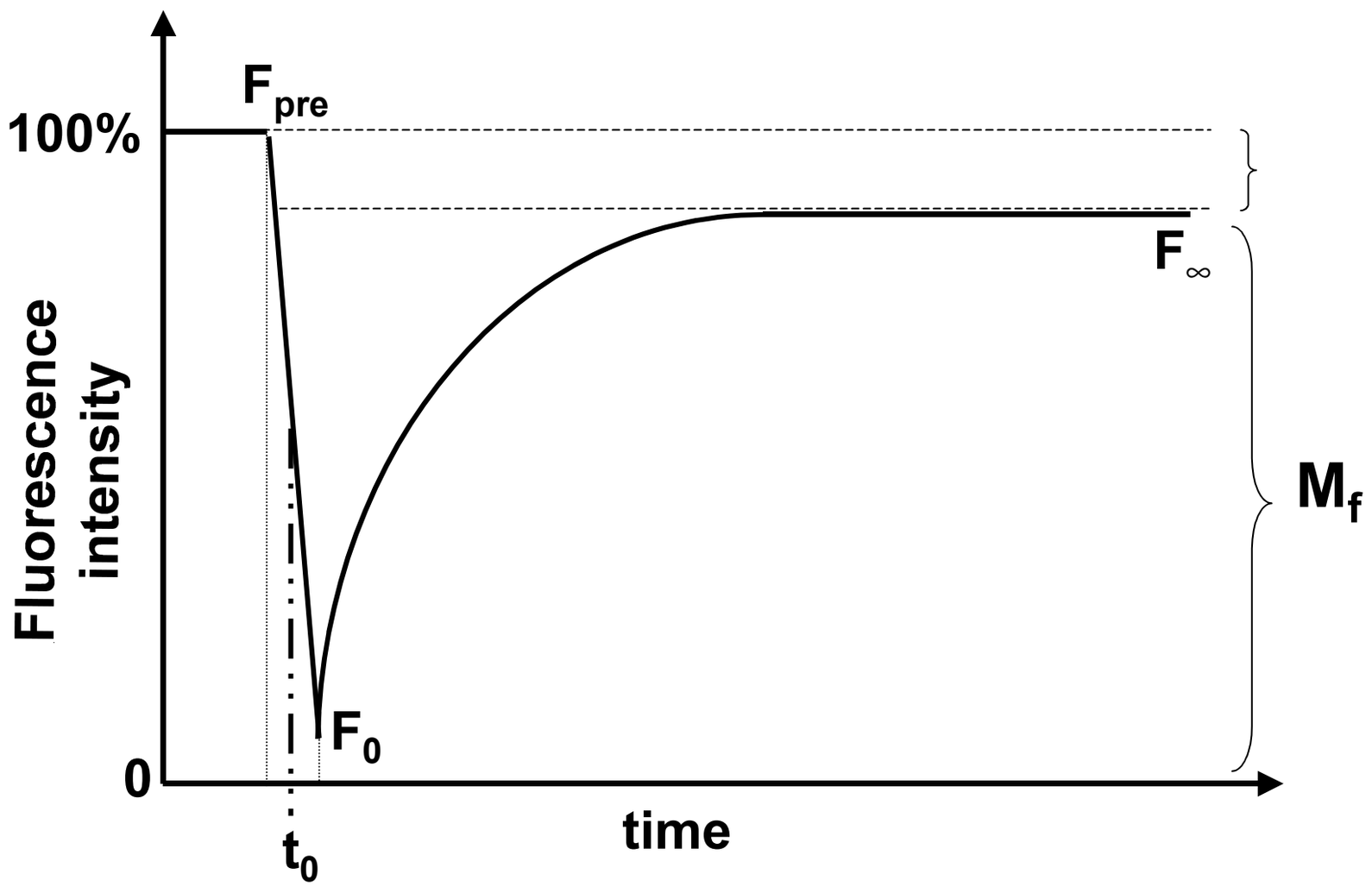
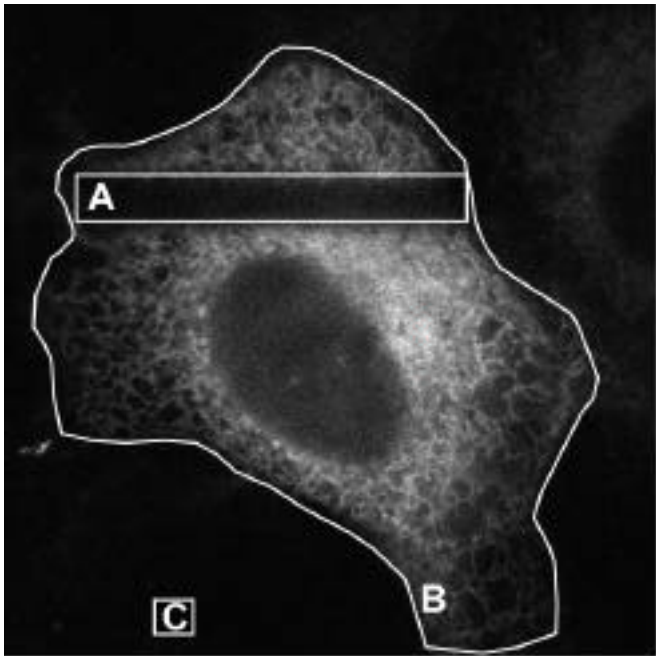


Fig. 1 B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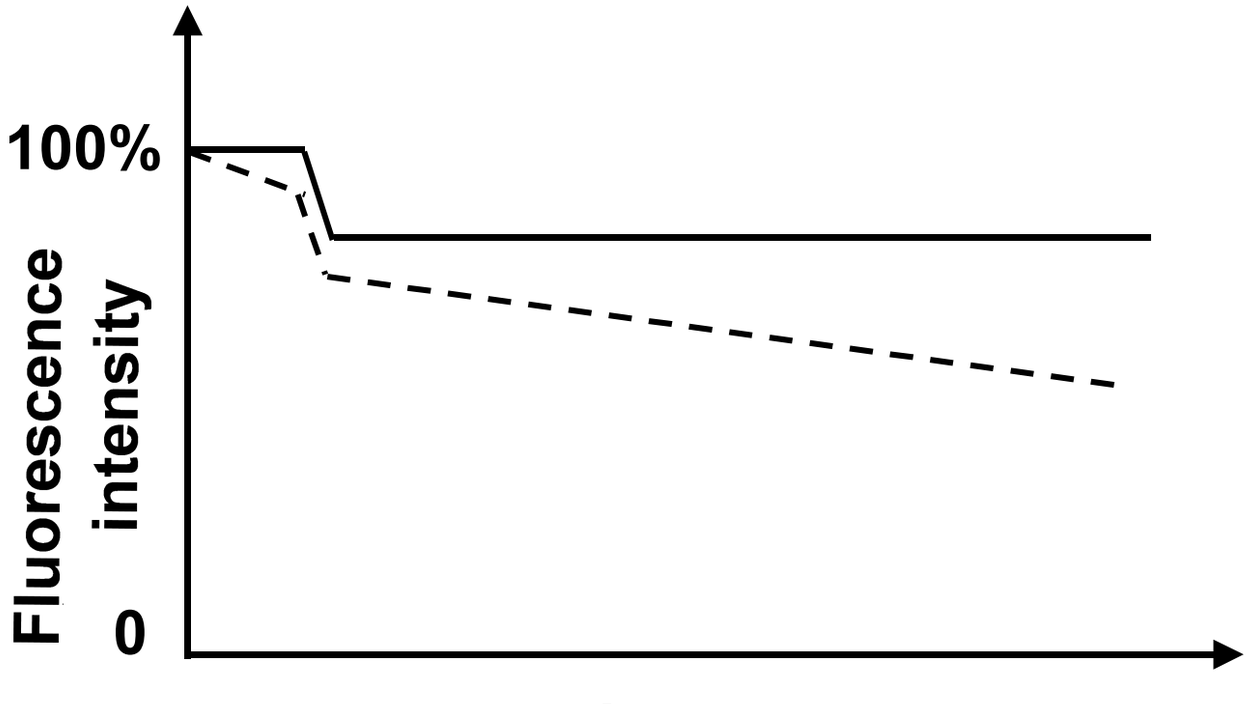
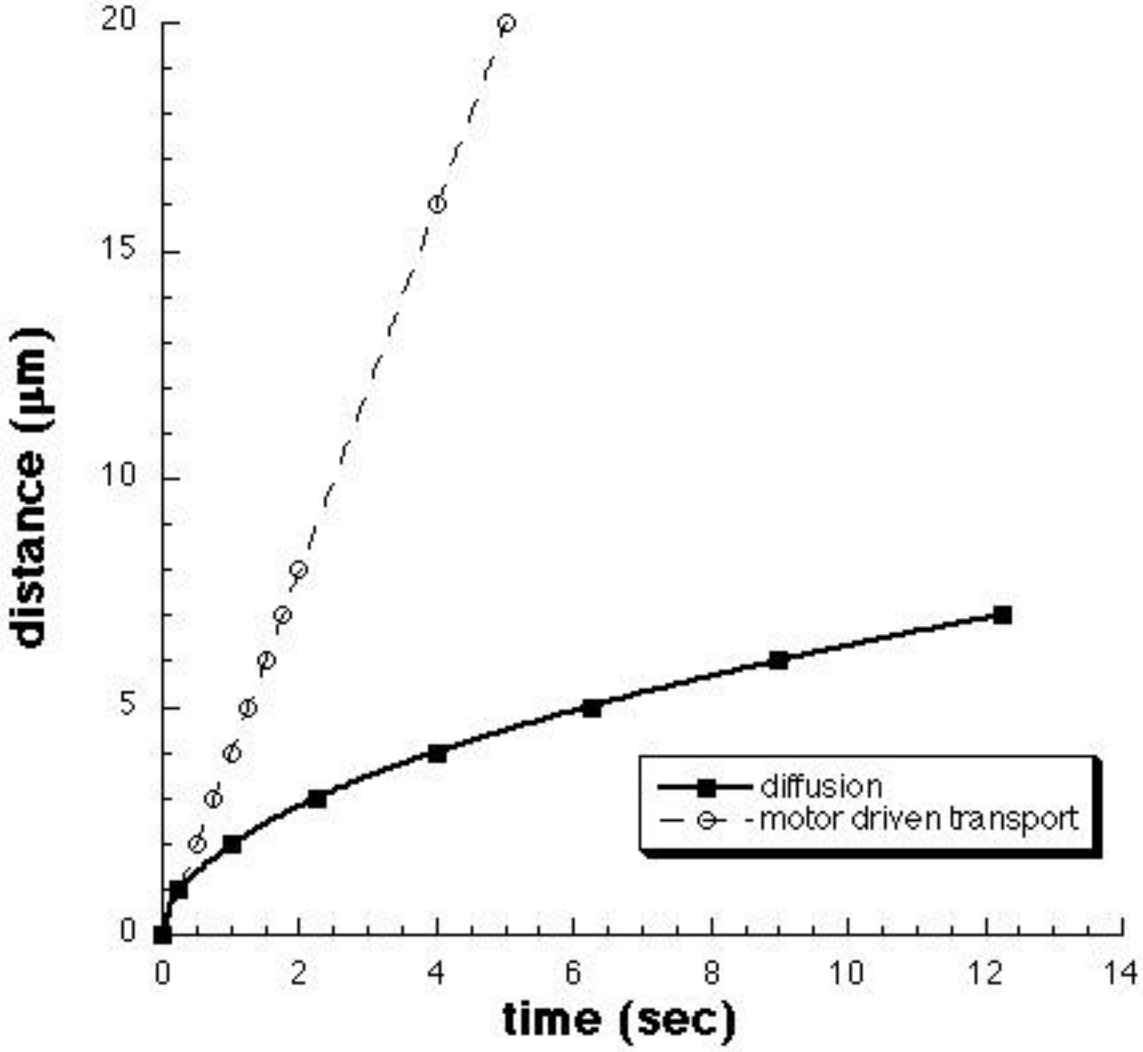


Fig. 2B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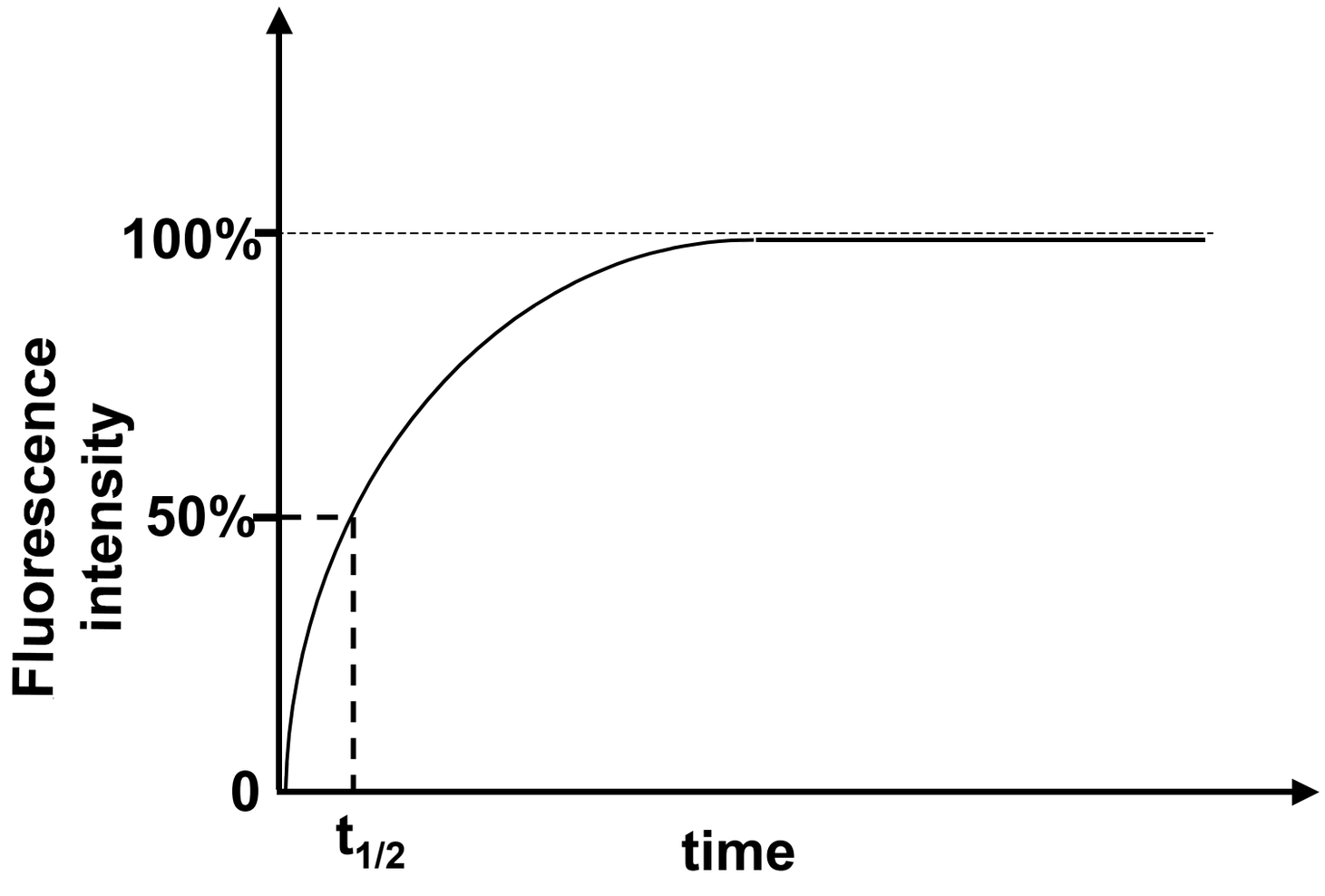
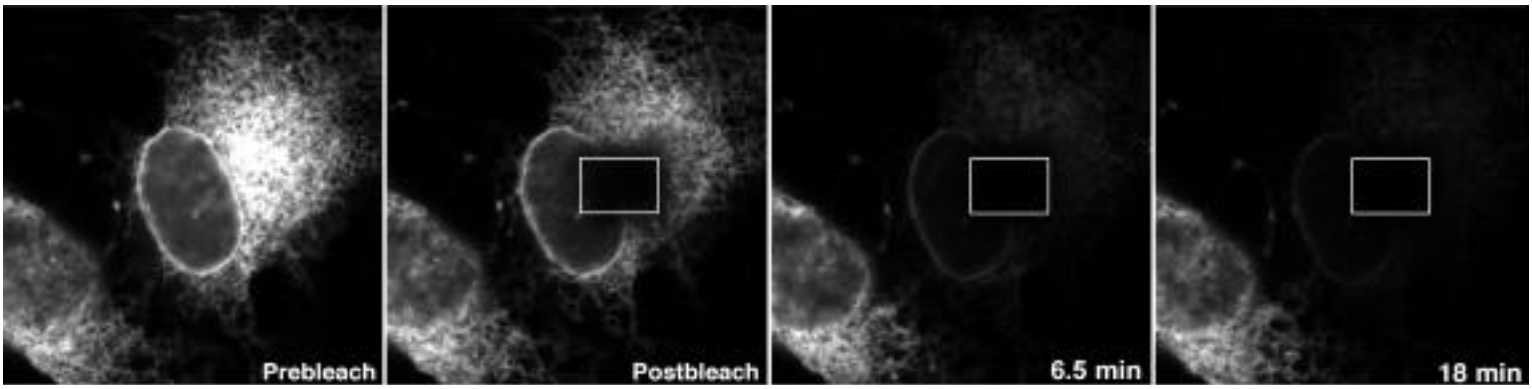


Fig. 4


Prebleach
Postbleach
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18 min

This unit describes protocols for Fluorescence Recovery after Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP) using commercially available confocal laser scanning microscopes (CLSM). Photobleaching is the photo-induced alteration of a fluorophore that abolishes the fluorophore's fluorescence. The diffusive characteristics of green fluorescent protein (GFP)-chimeras in a cell can be studied by FRAP, in which a selected region of a cell is photobleached with intense light and then the movement of nonbleached fluorescent molecules into the photobleached area is quantified by imaging using an attenuated light source. The movement of proteins between cellular compartments can be determined by FLIP, in which the same region of a cell expressing a GFP-chimera is repetitively photobleached and the loss of fluorescence from regions outside of the photobleach region is monitored. Together, these techniques are providing fundamentally new insights into the kinetic properties of proteins in living cells.

This unit describes photobleaching methods used in combination with green fluorescent protein (GFP) chimeras to analyze protein and organelle dynamics in living cells. Photobleaching is the photo-induced alteration of a fluorophore that abolishes the fluorophore's fluorescence. The diffusive characteristics of fluorescently tagged proteins or organelles in the cell can be studied by photobleaching a selected region of a fluorescently labeled cell with intense light and then quantitating the movement of nonbleached fluorescent molecules into the photobleached area using an attenuated light source (Lippincott-Schwartz et al., 1999; Lippincott-Schwartz et al., 2001; Meyvis et al., 1999). Other processes that lead to recovery into the bleached area (including vesicle transport or flow based movement) also may be quantitated and studied by this method. Insights into a variety of aspects of protein and organelle dynamics thus can be addressed using photobleaching. These include: (1) estimation of the diffusion coefficient, ***D***, of a fluorescent protein, (2) determination of the fraction of fluorescent molecules able to move under different cellular conditions, (3) assessment of continuity or discontinuity of an organelle, and (4) characterization of protein rates to or from an organelle (Dayel et al., 1999; Ellenberg et al., 1997; Hirschberg et al., 1998; Marguet et al., 1999; Nehls et al., 2000; Partikian et al., 1998). Photobleaching can also be used to reduce fluorescence from background noise to reveal faint populations of fluorescent proteins.

The usefulness of photobleaching methodologies depends on the availability of fluorescently labeled molecules. Until recently, these were limited to fluorescent dyes and their labeled antibodies, which only permitted labeling of the plasma membrane, unless microinjected into cells. With the advent of GFP, a naturally fluorescent protein from the jellyfish ***A. victoria*** (Tsien, 1998), this has changed. Virtually any protein of interest can be tagged with GFP. When expressed within cells, these proteins usually retain their parent protein targeting behavior. An important property of GFP is that it can be bleached without detectable damage to the surrounding environment. This is presumably because the GFP's cage-like structure, which surrounds a small cyclic peptide fluorophore (Prendergast, 1999), shields the external environment from any damaging effects caused by reactive photobleaching intermediates. These characteristics of GFP-chimeras make them ideal reagents in photobleaching experiments.

In this unit, we describe two photobleaching techniques for confocal laser scanning microscopes (CLSM), FRAP (Fluorescence Recovery after Photobleaching) and FLIP (Fluorescence Loss in Photobleaching). In FRAP, a distinct region-of-interest in a cell expressing a GFP-chimera is briefly photobleached with a high intensity laser and the movement of unbleached fluorescent molecules into the bleached region is followed with low intensity laser light. FRAP is useful for determining a protein's diffusion coefficient (***D***), which measures the random movement or Brownian motion of a molecule. Knowing a protein's ***D*** can be used in turn to obtain information about the viscosity of a protein's environment and whether the protein is part a of much larger complex. FRAP also can be used to determine the fraction of molecules capable of

recovering into a photobleached area, referred to as the mobile fraction (M_f). FLIP differs from FRAP in that photobleaching is repeated several times, alternating each photobleach with a low laser intensity image of the whole cell. FLIP reveals the connectedness of cellular compartments and whether immobile pools of proteins are spatially segregated. The protocols are described for mammalian cells and the concepts can apply to other systems, including plant cells, yeast cells, and bacteria. SUPPORT PROTOCOL 1 discusses some practical issues of expressing and imaging GFP-chimeras in living cells. Preparation of transfected mammalian cells for imaging on a CLSM and setup of the CLSM is described. Next, the basic and alternate protocols describe photobleaching techniques that can be used to visualize and quantitate protein mobility and organelle dynamics. In addition, a selective photobleaching protocol is discussed, which can be applied to the study of protein trafficking and protein flux through organelles.

PHOTOBLEACHING APPLICATIONS

FRAP

Fluorescence Recovery After Photobleaching (FRAP) can be performed by irreversibly photobleaching a fluorescent marker in a region of a cell with a high intensity laser beam and then following diffusion of unbleached fluorescent proteins into the bleached region by imaging with nonbleaching attenuated laser light (Fig. 1A). Several forms of FRAP or FPR (Fluorescence Photobleaching Recovery) have been described. Popular methods include spot bleaching, pattern photobleaching, polarized photobleaching, and strip bleaching. The protocols in this unit will focus on the strip photobleach method for confocal laser scanning microscopes. Strip photobleaching permits imaging of the whole cell, while monitoring the fluorescence recovery into the photobleached region-of-interest (ROI). It allows information concerning the spatial distribution of fluorescence to be readily determined. It also can reveal whether the cell, stage, or focus have moved during imaging, which makes FRAP analysis difficult.

There are two types of strip FRAP protocols, quantitative and qualitative. Using the former FRAP protocol, it is possible to obtain an effective diffusion coefficient, ***D***, and to calculate the mobile fraction, M_f, of a fluorescent protein. Images are acquired rapidly and the data can be used to plot a recovery curve from which ***D*** can be determined (Fig. 1B). The qualitative protocol is used to obtain high quality images for visualizing the diffusion process in a single cell. Because the images are obtained by line averaging and slow scan speeds, they take longer to acquire and therefore are usually unsuitable for calculation of ***D***, which requires time points immediately after bleaching.

BASIC PROTOCOL 1

QUANTITATIVE FRAP BY STRIP PHOTOBLEACHING USING A LASER SCANNING CONFOCAL MICROSCOPE CAPABLE OF SELECTIVE PHOTOBLEACHING

This FRAP protocol has been designed for the Zeiss LSM 510 microscope (using the physiology software package) and for other laser scanning confocal microscopes capable of selective photobleaching (from Leica and Biorad). Selective photobleaching means that the investigator can bleach any shape or size ROI. Specific settings will vary for each microscope. The user must determine conditions for photobleaching and postbleach recovery imaging that do not result in significant photobleaching of the cell (steps 1-6). The actual FRAP experiment is described in steps 7-9, and data analysis is described in the FRAP data processing section.

ESTABLISHING FRAP CONDITIONS

1. Pre-warm the imaging stage to 37^{o}C or desired temperature and warm up the microscope lasers for at least 5 minutes to avoid power fluctuations during imaging. Image living cells in imaging medium in a chamber as described in Strategic Planning.

2. Identify the cell of interest on the confocal microscope. Bring it to the desired focus. Scan an image of the whole cell at the desired excitation light intensity, line averaging, zoom, etc. Modify pinhole and detector gain for maximal fluorescence signal with no pixel saturation (pixel intensities that exceed the detector scale, i.e. > 255 for an 8-bit image). Saturated pixels only register as the maximum detector value, 255, so the true intensity of the pixel cannot be calculated under these conditions. Detector gain and offset will vary depending on the concentration of the fluorophore, the laser power, and the width and thickness of the fluorescently labeled organelle or region. The imaging parameters used for this image should be used for the fluorescence recovery time series. It may be useful to record the detector gain settings to compare recoveries in cells expressing high versus low amounts of fluorescent protein.

3. Define a region-of-interest (ROI) for the photobleach (see Fig 2A), usually a 2-4 µm strip across the width of the cell. For soluble proteins that diffuse rapidly, the photobleach ROI may need to be relatively large (i.e. one third to one half of the cell). When performing FRAP on a discrete organelle, such as the nucleus or the Golgi complex, the photobleach ROI does not need to extend across the entire cell. It is sufficient for the edges of the strip to extend beyond the edges of the structure being analyzed. Note that larger photobleach ROIs will require longer recovery times.

4. Empirically determine photobleaching conditions (i.e. scan speed, zoom, laser power, microscope objective, and the minimal number of laser iterations required for photobleaching) so that after photobleaching, the fluorescent signal of the photobleach ROI decreases to within background intensity levels. Use imaging software to quantitate fluorescence intensity in the photobleach ROI and the whole cell prior to bleaching (Fig 2A). This is necessary for determining the

extent to which the photobleach ROI and the whole cell undergo bleaching during the course of imaging (Fig. 2B).

> ***It is useful to establish photobleaching conditions on fixed samples, as rapidly diffusing species may be impossible to bleach to background levels. Fix the plated (either on a coverslip or in a Lab-Tek chamber) GFP expressing cells for 15 minutes in 4% formaldehyde/PBS at room temperature. Wash twice with PBS and then either place the coverslip on an imaging chamber with imaging medium or fill the Lab-Tek Chamber with imaging medium. DO NOT use an anti-fade reagent (such as fluoromount-G or phenyl-enediamine) because this will significantly alter the photobleaching properties of the fluorophore. Image the fixed cells in imaging media.***

Typical bleaching conditions require a 100-1000-fold increase in laser power (decrease in attenuation) for 1-5 bleach iterations (roughly 0.01-0.5 seconds) for many organelles. If an ROI requires more than 20 seconds to photobleach, a more powerful laser is necessary (see below).

> ***To ensure that bleaching laser power does not damage the cell, it is useful to repeat FRAP on the same ROI for the same cell. The diffusion coefficient (see Data Processing) now should not change, but the mobile fraction (see Data Processing) should be close to 100%. This is because any immobile fluorophores in the first FRAP will have been bleached and therefore will not contribute to the percent recovery observed will in the second photobleach.***

5. For acquisition of fluorescence recovery time points, empirically determine imaging conditions that do not significantly photobleach the cell (outside of the bleach ROI)(Fig. 2B)

6. Recommended conditions for photobleaching with a 40 mW 488/514 nm Argon or 25 mW Argon laser are 45-60% power with 100% transmission. For acquisition of recovery time points, use the same power with 0.1-1.0% transmission. For quantitative FRAP, the whole cell is usually scanned at scan speed 8 to 10 (0.798-3 sec per 512x512 frame) with either two line averaging or no line averaging. In qualitative FRAP experiments, where the goal is to obtain high quality images, the intervals at which images are collected during recovery need not be rapid (3-10 second intervals, for membrane proteins). Soluble and lumenal proteins tend to diffuse rapidly and both quantitative and qualitative FRAP may require the bleaching of a large ROI and very rapid imaging conditions.

FRAP Data Collection

To perform data analysis of a FRAP experiment, the user must collect a prebleach image of the cell, a series of postbleach images of the whole cell that

extends from an immediate postbleach image to several images after the bleach ROI fluorescence intensity reaches a plateau, and a table of fluorescence intensity values including the photobleach ROI, the whole cell ROI, and a background ROI. The user also needs to know the corresponding time for each image and the pixel size in microns. For the 510 microscope, the physiology software contains several windows which permit the user to determine the number of images to collect, laser intensities for bleaching and imaging, number of bleach iterations, how many images to collect before bleaching, and drawing of the photobleach ROI. Other microscopes may contain their own software package or may require writing macros for the photobleach time series.

7. Collect multiple prebleach images to establish the prebleach fluorescence intensity and to confirm that the prebleach fluorescence intensities of the cell and the bleach ROI do not fluctuate significantly.

8. Photobleach the ROI with intense laser illumination. Continue to image the whole cell at low laser illumination (the same conditions as the prebleach images), until the recovery process has reached a steady state. This must be determined quantitatively, as the human eye is incapable of distinguishing small differences in intensities. Typically, a 4 µm wide bleach ROI strip will recover within 90-120 seconds for most freely mobile soluble lumenal proteins and within 350 seconds for membrane proteins in the plasma membrane, endoplasmic reticulum, or Golgi. Cytoplasmic proteins may recover as rapidly as 5-20 seconds. In this way, the investigator can establish conditions to obtain data sufficient for estimation of $t_{1/2}$ and M_f (see below). The investigator should not attempt to refocus a cell during a FRAP experiment, because the fluorescence intensities will shift and the recovery curves will no longer be smooth. Time series that contain a shift in focal plane should be discarded.

> ***If a protein has a high protein diffusion coefficient (i.e. $\geq$ 5 μm^2/sec), the investigator may not observe a defined bleach region following the photobleach. Rapidly diffusing fluorescent proteins may appear homogeneously distributed immediately following a photobleach. The investigator must either 1) increase the image collection rate, by changing the scan speed or reducing the size of the total frame to be collected, 2) increase the bleach ROI size, 3) increase the laser power, or employ a combination of these suggestions.***

9. Collect at least 10-20 data sets for each fluorescently labeled protein and treatment for statistical analysis. A fraction of the data sets are usually discarded because of problems that potentially bias imaging results (i.e. recovery wasn't complete, the focal plane shifted, the recovery curve fitting method fails, etc.).

> ***In Lippincott-Schwartz et al. (Lippincott-Schwartz et al., 1999), a method for imaging the bleach ROI alone for rapid collection of a large number of data points is described. The caveat of the method is that the cell may***

shift in focus or position during the course of imaging. Imaging the whole cell offers the advantage of visualizing any movement of the cell or focal plane, thus allowing the researcher to reject unusable data upon visual inspection.

ALTERNATE PROTOCOL 1
FRAP WITH CONFOCAL LASER SCANNING MICROSCOPES WITHOUT THE CAPACITY FOR SELECTIVE PHOTOBLEACHING

FRAP can also be performed with older confocal laser scanning microscopes. Photobleaching is accomplished by zooming into a small region of the cell and scanning with full laser power. The procedure for photobleaching and some useful macros for the Zeiss 410 are described below. The actual FRAP protocol is otherwise identical to Basic Protocol 1. For quantitative FRAP experiments for determining ***D***, images should be acquired rapidly after the bleach and this is best done if fluorescence is collected from the ROI only. If the investigator acquires a prebleach image of the whole cell and a postrecovery image of the whole cell, it will be possible to calculate the M_f and ***D***.

Photobleaching by zooming
Define a region-of-interest (ROI) for the photobleach. Define the ROI to be bleached at the highest zoom possible (usually zoom 8). At high zooms the laser will dwell longer on an ROI per line scan and thus will deliver more bleaching radiation. To photobleach the ROI, set laser power at maximum and remove all neutral density filters from the path of the laser beam. Scan (photobleach) the ROI.

Macros
Write a macro that will bleach a desired ROI with high intensity light and then quickly switch to scanning the ROI with low levels of light and collecting emission will allow for rapid and reliable switching from bleach mode to emission collection mode. For a more extensive discussion on writing this macro and photobleaching applications for CLSMs in general see the review by Lippincott-Schwartz et al. (Lippincott-Schwartz et al., 1999). Sample macros for the Zeiss 410 are available from the Internet at: http://dir.nichd.nih.gov/cbmb/pb2labob.htm

FRAP DATA PROCESSING
After collecting FRAP data using either of the preceding protocols, the data needs to be processed for quantitation. The three parameters that can be determined are the mobile fraction (M_f) of the fluorescent fusion protein, its diffusion coefficient (***D***), and the $t_{1/2}$ of the fluorescence recovery. When first performing FRAP experiments, it is an excellent idea to perform calibration experiments with a fluorescent protein whose ***D*** and M_f have been previously characterized (see Table 1.)

Mobile Fraction (M_f)

M_f refers to the percentage of fluorescent proteins capable of diffusing into a bleached ROI during the time course of the experiment (see Fig 1B). M_f and ***D*** are distinct parameters and must be understood as such. ***D*** is a characteristic of the mobile pool of fluorescent proteins. The proteins in the immobile fraction do not diffuse. A slow ***D*** does not bear any relation to the fraction of immobile proteins.

An approximation of M_f can be calculated using the following equation, based on Feder et al. (Feder et al., 1996) with the inclusion of a photobleaching correction (Lippincott-Schwartz et al., 1999):

$$M_f = 100 * \frac{(F_{precell} - F_{background})}{(F_{\infty cell} - F_{background})} * \frac{((F_{\infty} - F_{background}) - (F_0 - F_{background}))}{((F_{pre} - F_{background}) - (F_0 - F_{background}))}$$

Where:
$F_{precell}$ is the whole cell prebleach intensity
F_{pre} is the bleach ROI prebleach intensity
$F_{\infty cell}$ is the asymptote of fluorescence recovery of the whole cell
$F_{background}$ is the mean background intensity
F_{∞} is the bleach ROI asymptote
F_0 is the bleach ROI immediate postbleach intensity

In the equation, the bleach ROI and whole cell ROI's are background subtracted. Next, the bleach ROI data are transformed such that the prebleach fluorescence intensity is defined as 100% fluorescence intensity. The equation includes a correction for the loss of total cellular fluorescence (due to the photobleach of the ROI and bleaching of the whole cell during imaging). The correction is calculated by determining the prebleach fluorescence intensity of the whole cell ROI ($F_{cell\ pre}$) and dividing it by the whole cell ROI intensity at time "t." Without this bleach correction, the bleach ROI intensity can never recover to 100% of the prebleach fluorescence, because the photobleach depletes 5-20% of the total cellular fluorescence. Finally, to convert the fraction to a percentage, the data is multiplied by 100.

> ***The F^o value is an approximation for the fluorescence intensity immediately following the photobleach. For conditions in which the ROI is bleached to near background levels, F^o will be relatively accurate. However, for rapidly diffusing proteins or for narrow bleach ROI's, the immediate postbleach ROI intensity may be closer to 30-40% of the prebleach fluorescence intensity. This can lead to the appearance of an artificial immobile fraction. The true F^o may be much lower and can potentially be derived by rapidly collecting images of the bleach ROI alone or by attempting to determine F^o by nonlinear regression analysis.***

Diffusion Analysis

The diffusion coefficient, ***D***, reflects the mean squared displacement (usually μm^2/sec or cm^2/sec, which can be multiplied by 10^8 to get μm^2/sec) that a protein explores through a random walk (i.e. Brownian motion) over time. It is important to recognize that ***D*** is NOT a simple linear rate (see Fig. 3). That is, the time required to cover increasing distances will not increase in a linear fashion. Time increases as the square of the distance covered divided by the ***D***. The primary constraints on protein diffusion are the viscosity of the environment, whether the protein is soluble or integrated into a membrane, and to a lesser extent, the molecular radius of the protein. Protein-protein interactions and collisions with other molecules also hinder free diffusion. By measuring ***D***, the investigator can obtain information related to a protein's environment and in some cases, whether the protein is interacting with large complexes.

Several groups have derived a number of equations and computer simulations to determine ***D***. The majority of available equations have been written for spot photobleaching. Related equations and simulations are available for strip photobleaching, including those described in Ellenberg et al (Ellenberg et al., 1997) and in Wey and Cone (Wey and Cone, 1981). These equations, however, make several assumptions about the experimental system, which may or may not be met. For example, the equation from Ellenberg et al. (Ellenberg et al., 1997) assumes that the bleach is complete, there is no immobile fraction, the cell is a uniform rectangle, and the width of the bleach ROI is much less than the distance to either end of the cell. ***D*** values calculated with this equation can differ by 50% or more from an assumed ***D***. Given these concerns, investigators are cautioned in the use this equation.

The best method we have found for obtaining ***D*** is through simulation of diffusive recovery into a strip bleach (Siggia et al., 2000). The Siggia simulation models inhomogeneous diffusion of nonbleached proteins in the cell into the photobleach ROI. The simulation then compares the simulated recovery to the actual data to determine ***D*** (Siggia et al., 2000). The program has been used for ER membrane proteins (Ellenberg et al., 1997; Zaal et al., 1999) and has been used to calculate ***D***'s comparable to values observed by other labs using different methods. Like other methods for determining ***D***, simulation encounters difficulties when a significant fraction of the fluorescent protein is immobilized (Siggia et al., 2000). In such cases, the simulation either fails to fit the simulation data to the experimental recovery data or the simulation cannot calculate ***D***.

> ***The Siggia simulation can be obtained by contacting Dr. Eric Siggia at siggia@eds1.rockefeller.edu. The simulation has been written for UNIX systems and has been compiled for Macintosh computers. After signing the licensing agreement with Dr. Siggia, the Macintosh compiled simulation can be obtained from the authors of this UNIT. Appendix 1 is a simple user's guide to use the simulation.***

After running any simulation or calculating a least squares fit to determine ***D***, it is essential to compare the simulation or fit data to the experimental data by plotting the data sets in a spreadsheet program. Even if a program or equation produces ***D***, a poor fit means that ***D*** is questionable at best. "Goodness of fit" describes how well the simulation or equation data overlaps the experimental data. The majority of the experimental data must overlap the simulation or equation plot. If the simulation misses the experimental data, the calculated ***D*** is questionable, at best. If there are any doubts about "goodness of fit," the user should consider another method of calculating the ***D***.

> ***A number of potential errors may occur in the course of data fitting. These errors are not unique to D calculations. They are part of the general problem of nonlinear regression analysis. Individuals that anticipate performing diffusion analysis are encouraged to become familiar with nonlinear regression analysis. An excellent introduction to the theory and problems of nonlinear regression analysis has been written by Dr. Harvey Motulsky and is available as a free download at http://www.graphpad.com/www/nonlin.pdf.***

$t_{1/2}$

The mobility of several proteins depends on complex behaviors, such as binding and release or populations with multiple ***D***'s. Such conditions complicate the analysis of ***D*** and may prevent fitting of data by traditional diffusion analysis. When a diffusion equation or simulation is unable to fit fluorescence recoveries, the $t_{1/2}$ measure can be used to compare relative recovery rates between samples. The $t_{1/2}$ is the time required for the fluorescence intensity in the bleach ROI to recover to 50% of the asymptote or plateau fluorescence intensity. This measure is independent of the prebleach ROI fluorescence intensity. In addition, it should be emphasized that while the $t_{1/2}$ can be a useful tool, it is only relevant for the user's system and conditions. It cannot be used to relate to $t_{1/2}$ values obtained from other experiments since the $t_{1/2}$ value is relative to a particular experimental setup. The $t_{1/2}$ value requires two data manipulations 1) conversion of time with the half time of the bleach as time zero (t_0) and 2) conversion of bleach ROI recovery data into fractional fluorescence. Note that fluorescence recovery into a photobleach ROI must plateau or $t_{1/2}$ analysis can not be performed. The $t_{1/2}$ can be determined visually or by solving the following equation:

$$F(t) = [F_0 + F_\infty (t/t_{1/2})]/[(1+ (t/t_{1/2})] \text{ (Feder et al., 1996)}$$

F_0 is the bleach ROI immediate postbleach intensity
F_∞ is the asymptote of the bleach ROI fluorescence recovery
t is the time for each ROI intensity value, usually in seconds or milliseconds
$t_{1/2}$ is the time required for the bleach ROI to recover to 50% of the asymptote.

Fractional Fluorescence

To directly visualize and determine the $t_{1/2}$, transform the fluorescence intensity (F(t)) data to a 0-100% scale (see Fig 4). The measurement is independent of the prebleach intensity and is not bleach corrected, as the relevant data occurs after the photobleach. It is critical that the bleach ROI fluorescence recovery reaches a true plateau for accurate measurements. It is also important to have a large signal difference between the first postbleach intensity and the plateau. If the signal difference is too small, the plotted recovery will tend to be very broad and difficult to interpret. The following equation will convert the bleach ROI fluorescence recovery into fractional fluorescence data:

$$F_2(t) = 100 \times [F(t)-F_0]/[F_\infty - F_0]$$

F(t) is the bleach ROI fluorescence intensity at time "t"
F_0 is the immediate postbleach bleach ROI fluorescence intensity
F_∞ is the asymptote of the bleach ROI fluorescence recovery.
Time "t" must be rescaled relative to t_0, as described above. The $F_2(t)$ data is then plotted versus time (in seconds) to determine $t_{1/2}$.

The measurement is sensitive to the initial time (or t_0) of the bleach. When varying the size of the bleach ROI, the time to bleach the ROI will change. Practically, this means that the time for collection of the first postbleach image will vary between samples with different sized bleach ROIs, which will alter the start time for data collection for each sample. Different groups define t_0 slightly differently. For this UNIT, t_0 is defined as the halftime of the bleach. To modify the time, subtract t_0 from each time value, where:

$$t_0 = (((t_{post} - t_{pre})-(\text{time per image frame}))/2)+ t_{pre}$$

and t_{post} = time of first image after photobleach, and t_{pre} = time of image immediately prior to photobleach

SELECTIVE PHOTOBLEACHING TO ENHANCE DIM STRUCTURES WHILE IMAGING OR TO VISUALIZE AND MEASURE NONDIFFUSIVE TRANSPORT INTO AN ORGANELLE

Photobleaching has additional uses. For example, a GFP-chimera may localize to two organelles in close proximity. One organelle may accumulate a substantial amount of the protein, while the adjacent organelle is dim. The bright organelle can be photobleached to permit imaging of the dim structure. Another application is to use photobleaching to visualize trafficking or flux through an organelle. For example, the Golgi complex (Nichols et al., 2001) or lysosomes can be photobleached and then fluorescence recovery of either fluorescence into the photobleached organelle or fluorescence trafficking out of the unbleached organelle into the photobleached area surrounding the organelle can be imaged for both qualitative and quantitative analysis. Selective photobleaching essentially follows the FRAP protocol. Instead of a strip, the investigator defines an ROI appropriate to the organelle or structure of interest. Imaging conditions

must be determined to photobleach either the structure of interest or around the structure of interest and to avoid bleaching of the overall cell during the course of imaging. When bleaching a bright structure to visualize dimmer structures, the conditions need to be set such that the dim structure(s) are sufficiently bright, but not saturating. The bright structure can be saturated, as it will be photobleached.

Basic Protocol 2
FLIP

FLIP (Fluorescence Loss in Photobleaching) is similar to FRAP in that an ROI is photobleached with a high power laser, but in FLIP, the ROI is repeatedly bleached over time to examine the behavior of the entire fluorescent pool. If the fluorescent molecules are completely mobile and have access to the photobleaching ROI, the entire fluorescent pool will be depleted (See Fig. 5). Results from FLIP experiments therefore provide information about the connectedness of structures containing GFP-chimeras.
For FLIP analysis, the user must obtain a prebleach image, an immediate postbleach image following the first photobleach, images following successive photobleaches (often 2-3 images following each photobleach) until the entire cell is depleted of fluorescence or several successive photobleaches do not further deplete cell fluorescence, the time for each image, pixel size, and fluorescence intensity values for the whole cell and the background intensity.

1. Pre-warm the imaging stage to 37^0C or desired temperature and warm up the microscope lasers for at least 5 minutes to avoid power fluctuations during imaging. Image living cells in imaging medium in a chamber as described in Basic Protocol 1.

2. Identify the cell of interest on the confocal microscope. The ideal situation is to have two adjacent cells of similar fluorescence intensity in the imaging field. The cell that is not bleached provides a control to insure that the imaging conditions do not cause nonspecific photobleaching of all fluorescence in the field of view. It is essential that imaging conditions do not significantly photobleach any adjacent cells during the experiment. Bring the desired cell into focus. Scan an image of the whole cell at the desired excitation light intensity, averaging, etc. Modify pinhole and detector gain for maximal fluorescence signal and minimal pixel saturation. Detector gain and offset will vary depending on the concentration of the fluorophore, the laser power, and the width and thickness of the fluorescently labeled organelle or region. The imaging parameters used for this image should be used for the fluorescence recovery time series.

3. Define a region-of-interest (ROI) for the photobleach. The bleach ROI should be between 5-20% of the structure of interest. The bleach ROI does not need to be a strip. A square is equally appropriate.

4. Determine imaging and photobleaching conditions (i.e. scan speed, zoom, laser power, number of laser iterations required for photobleaching, microscope objective) that photobleach 90% or more of the fluorescent signal in the ROI, without causing significant photobleaching of the cell outside the photobleach ROI. A 40 mW 488/514 nm Argon laser can be used at 45-60% power with 100% transmission for photobleaching and 1% transmission for imaging. Usually, cells are scanned at 0.8 to 3 seconds per image with two to eight line averaging.

5. Collect 3-5 prebleach images to establish the prebleach fluorescence intensity and to confirm that the prebleach fluorescence intensity does not significantly fluctuate.

6. Collect 2-5 prebleach images and then photobleach the bleach ROI. Collect 2-4 images of the whole cell and photobleach the ROI again. Repeat the process until the fluorescent structure intensity is similar to background. The pauses allow unbleached molecules to diffuse into the photobleached ROI. In addition, collecting images between bleaches to permits monitoring of progress and confirms that the cell remains in focus and hasn't moved. As this protocol requires longer time periods, the focal plane may shift. It is acceptable to refocus the cell during the experiment. However, if the cell or focus moves such that the region being photobleached also moves laterally, then the experiment should be aborted. Successful FLIP requires that the bleach ROI position within the cell is stable. If the cell migrates during the course of the experiment, then the data will be unusable.

7. Execution of a quantitative FLIP can be automated with a macro to program the bleaching and imaging timing. A FLIP macro (the advanced time series macro in combination with the physiology software package) is available for the Zeiss 510, for purchase from Zeiss. It is also possible to perform FLIP manually. Photobleach the bleach ROI in cells for several (i.e. 20-50) bleach iterations. Next, collect 3 images of the cell. Repeat the bleach and image collection many times, up to 15-20 minutes (or longer). If the proteins are mobile and the fluorescent structure is continuous, the total structure fluorescence will be depleted. If the protein diffuses relatively slowly, the investigator can modify the routine to include a time delay between images, to reduce overall photobleaching due to laser scanning of the cell.

8. Collect at least 3-5 data sets for each fluorescently labeled protein and treatment. A fraction of data sets may be unusable because the focal plane shifted, the cell moved, etc.

For CLSMs that are unable to perform selective photobleaching of an ROI, it is possible to perform FLIP, using essentially the same protocol. The primary differences are the need to photobleach the cell by zooming (see Alternate Protocol 1) and the requirement for a macro for automation of the FLIP

experiment. Our lab has provided a FLIP macro for the Zeiss 410 at http://dir.nichd.nih.gov/CBMB/pb2labob.htm. Essentially, the protocol must alternate between photobleaching the ROI and scanning images of the whole cell between photobleaching. The photobleaching and imaging scans must be performed at regular time intervals such that it will be possible to directly compare the rates of fluorescence depletion between two cells. For a more extensive discussion on writing this macro see the review by Lippincott-Schwartz et al. (Lippincott-Schwartz et al., 1999). Some FLIP experiments may not need to be quantitative and it is possible to manually perform FLIP.

REAGENTS AND SOLUTIONS

Imaging medium

Phenol red free medium (such as RPMI or DMEM)
10% fetal bovine serum
2 mM glutamine
25 mM HEPES pH 7.4

Formaldehyde/PBS

PBS (***APPENDIX 2A***) containing
4% formaldehyde

Block solution

PBS (***APPENDIX 2A***) containing
10% heat inactivated fetal calf serum

COMMENTARY

Background Information

Diffusion is defined as the rate of movement or flux of particles due to random or Brownian motion. An example of diffusion is the mixing of a drop of dye in a liquid solution. Random movements of proteins in a lipid bilayer (lateral diffusion), in the cytosol, or within an organelle are all examples of diffusion in a cell. Diffusion primarily depends on the viscosity of the environment and the size of the molecule. For soluble spherical molecules, the diffusion coefficient, ***D***, is proportional to the cube root of the molecular weight of the molecule (Stokes-Einstein equation). This means that a molecule must increase 8-10 fold in size to decrease ***D*** by one half. The size dependence of ***D*** for membrane proteins relates primarily to the radius of the transmembrane segments, rather than aqueous domains. This is because the lipid bilayer is more viscous than cytoplasm or organelle lumena. Other factors, such as protein-protein interactions or binding to a matrix can also profoundly affect the ***D*** of a protein.

After Frye and Edidin demonstrated that proteins on the plasma membrane of cells move by diffusion (Frye and Edidin, 1970), investigators have sought to directly measure diffusion coefficients of cellular proteins in their cellular context. Poo and Cone and Liebman performed the some of the first FRAP experiments by photobleaching retinal rod outer segments and

successfully measured ***D*** of the transmembrane protein rhodopsin (Liebman and Entine, 1974; Poo and Cone, 1974). The analytical equations and methodology that form the basis of FRAP were described originally by Axelrod et al. (Axelrod et al., 1976). Labeling proteins exposed to the extracellular face of the plasma membrane of living cells with fluorescent antibodies has been the primary labeling method for FRAP studies for 20 years. However, the method confined studies primarily to proteins on the cell surface, leaving all of the vesicles, organelles, and cytoskeletal components of cells unexplored. Some researchers have exploited microinjection techniques to place fluorescent antibodies or fluorescently labeled proteins in the cytoplasm. However, standard microinjection does not deliver proteins to the lumena of organelles, leaving these important cell environments unprobed. An additional barrier to investigating protein mobility was that traditional FRAP methodology required a dedicated custom microscope (Wolf, 1989).

Today, the combination of advances in fluorescence imaging methods, user-friendly commercially available laser scanning microscopes, and powerful cost-effective computing resources has made it possible for nonspecialists to exploit GFP-chimeras and to probe their mobility and interactions. For examples and more information, the reader is referred to several reviews of FRAP theory and techniques (Edidin, ; Ellenberg et al., 1997; Lippincott-Schwartz et al., 1999; Lippincott-Schwartz et al., 2001; Meyvis et al., 1999; Nichols et al., 2001; White and Stelzer, 1999).

Critical Parameters

The critical parameters for data collection are described in the protocols. There are several considerations for data analysis and interpretation that require additional emphasis. The major concerns are discussed below.

EGFP is better suited than other spectral variants for photobleaching experiments. Photoconversion and reversible photobleaching are phenomena that can seriously complicate photobleaching analysis by generating artificially high ***D*** values. Photoconversion is the process by which a fluorophore is excited and becomes transiently or permanently altered in its fluorescence excitation and emission spectra. Reversible photobleaching occurs when a fluorophore's excitation state is changed by intense illumination, which appears to an observer as destruction of the fluorophore. However, the fluorophore reverts to its native excitation and emission spectra and becomes fluorescent again. While many fluorophores exhibit varying degrees of both phenomena, photoconversion and reversible photobleaching are minimal for EGFP. In contrast, wild type GFP readily undergoes photoconversion. In addition, our lab has observed significant reversible photobleaching with YFP. Thus, FRAP experiments using YFP chimeras should be avoided.

Correct for photobleaching due to bleaching of the ROI and repeated scanning of the cell. One method to measure the amount of photobleaching is to directly measure the change in the fluorescence of the whole cell used in an experiment. A decrease in fluorescence can be used for data correction and can also help the investigator modify imaging conditions to achieve minimal sample

bleaching. In addition, this method offers an additional advantage that the investigator can monitor fluorescence changes in every frame and can be alerted to cell movement and focal drift, which can change the apparent fluorescence recovery. Alternatively, the investigator can fix the sample (4% formaldehyde in PBS for 15 min) and acquire the time-lapse images of the fixed sample using exactly the same imaging parameters (e.g., excitation light intensity, duration, zoom, number of images, etc.) as in living cells. Loss of fluorescence under these conditions will be due only to photobleaching and not to biological degradation processes. Once the photobleaching rate has been determined, the experimental values obtained from living cells can be corrected.

Photobleaching experiments must produce recoveries with a true plateau or asymptote. M_f and $t_{1/2}$ calculations cannot be performed reliably when the asymptote has not been reached. Often in the course of data processing, what appears to be a flat plateau with raw data may become an incomplete recovery following data processing. This result emphasizes the importance of determining the optimal imaging time to obtain a true plateau.

D* and M_f must be interpreted in the context of the cell.** Data analysis requires a commitment on the part of the investigator to not blindly accept numbers generated by the equations, without attempting to determine whether the results are biologically and physically reasonable. For example, a ***D of 2 μm^2/sec for an integral membrane protein exceeds the highest recorded diffusion coefficient (0.4-0.5 μm^2/sec) for a membrane protein in a cell by four fold. Such a number could indicate that a protein is no longer membrane associated or that the GFP-chimera is incorrectly processed or targeted. Another less likely possibility is that processes besides diffusion are occurring (i.e. flow)(Sciaky et al., 1997) which don't exhibit diffusive behavior.

Interpretation of *D* and M_f can be confounded by the connectedness of fluorescent structures. The problem concerning compartment connectedness is not always readily apparent. That is, an organelle or compartment may appear connected at the light level, but not actually be connected. This situation will result in a low apparent mobile fraction, since fluorescent molecules in nearby, but unconnected areas will be unable to diffuse into the recovery box. One way to clarify whether a structure is continuous or disconnected is to perform FLIP and check to see whether fluorescence of an otherwise mobile protein is depleted from all areas of the compartment (indicating connectivity) or only specific areas (indicating a lack of connectedness). If the molecule being studied resides within a discontinuous compartment, it will be difficult to estimate its ***D***, since the equations and simulation programs used for calculating ***D*** assume free diffusion throughout a compartment.

Anticipated results

The methods described in this UNIT should permit the investigator to obtain and interpret fluorescence intensity recovery data following photobleaching of GFP-chimeras with a CLSM. The investigator should be able to calculate the M_f and either the ***D*** or $t_{1/2}$ of fluorescence recovery of a protein in

a cell. The investigator should also be able to perform a FLIP experiment to determine the connectivity of GFP labeled cellular compartments and organelles. Finally, the investigator may take advantage of selective photobleaching to either reveal dimmer fluorescent structures obscured by bright structures or to visualize dynamic processes under steady state conditions, such as vesicular trafficking.

Time considerations

Creation of GFP-chimeras consists of standard cloning procedures and biochemical or genetic assays to confirm that the properties of the chimera are similar to the parent protein. Preparation for photobleaching experiments consists of transferring cells to coverslips or imaging chambers and transfecting the cells with a GFP construct 16-48 hours prior to imaging. The initial setup for photobleaching experiments requires a time investment of a few hours to determine conditions for each protein to be bleached. The actual FRAP experiments can be very rapid, ranging from 90 seconds for the recovery of a soluble lumenal ER protein into a 4 μm bleach ROI to six minutes for a membrane protein. FLIP experiments often take 10-20 minutes. FRAP experiments require at least 5 (and preferably 10) data sets for useful statistics to compare ***D***'s. Data processing depends on the method used and the power of the computer. The Siggia simulation will process a 250 image data series in 2-3 minutes on a Macintosh G4 450 MHz. Due to the large numbers of data sets to be processed, data analysis can be time consuming.

To ensure that FRAP did not damage the area that was being scanned, repeat FRAP on the same area and compare the diffusion coefficients obtained from both experiments. ***D*** should remain the same but the mobile fraction should now be close to 100%. This is because the immobile fraction was bleached in the previous experiment.

Table Trouble shooting guide

In the table below, a number of common problems and potential solutions are presented.

Problem	Possible cause and solution
Autofluorescence noise	Lysosomes are notorious for autofluorescence when excited with light between 400-488 nm. To decrease autofluorescence, lower the intensity of the excitation beam and use narrower bandpass emission filters. Also, avoid phenol red and high serum concentrations (>20%) in the medium.

Fluorophore bleaches too rapidly during acquisition	Decrease the excitation light intensity using either neutral density filters or by lowering the voltage to the Acousto-Optical Modulator and increase the gain on the detector side to collect light more efficiently. If this does not work try adding Oxyrase (Oxyrase Inc., Mansfield, Ohio), an oxygen scavenger, to the medium.
Cells round up during imaging	This could be due to gross photodamage to the cells from long periods of exposure to an intense excitation beam. To correct, decrease the intensity of excitation light. Alternatively, the cells may have depleted some nutrient in the medium due to remaining in coverslip chambers for too long. Replenishing the imaging media should help.
Cells are excited with a constant light intensity, but the fluorescence intensity varies over time	If this is not due to a biologically relevant process such as recruitment/degradation of fluorophore, then either the focus is shifting during acquisition or the laser power output is unstable. Maintain the focus either manually or by using autofocus software, which is available for some CLSMs. If laser output is a concern, then check it by exciting fluorescent beads and quantifying the emission over time. Fluctuating laser output may be due to the laser being operated at low power output. Increasing the power output to 50% may help. If this fails to correct the problem, then contact your confocal service representative.
Focus drifts	Try using relatively flat cells (i.e. COS-7) which may alleviate the problem. Try imaging with the pinhole partly or entirely open and use lower N.A. objectives. Be sure to pre-equilibrate the sample to the temperature of the objective, as this will prevent expansion/contraction of the coverslip/chamber during imaging. Make sure that the stage insert is mounted securely and that the sample is seated properly in the holder.
D values of two subsequent experiments of the same ROI in the same cell do not match	This suggests the area that has been photobleached has been damaged. Try using a different GFP variant that requires a lower (and less damaging) wavelength of laser light, such as YFP. Note that YFP may reversibly

	photobleach.
Slow recovery or lack of recovery after bleaching	Either the fluorophore is relatively immobile or there are discontinuities within the structure where the fluorophore is located. This is relevant for isolated membrane structures with little connection between membranes, such structures of the endocytic system (e.g lysosomes and endosomes).
Recovery is significantly faster than expected	A faster than expected D for a membrane protein may indicate that the protein is no longer assembled correctly or inserted in the membrane. This can be tested by performing immunofluorescence colocalization with a marker for the organelle of interest or performing immunoblotting to determine whether the GFP-chimera has become partially degraded. Another condition that may affect fluorescence recovery is loss of cell integrity or viability.
Nondiffusive behavior	A failure to fit recovery curve data by a simulation or to a least squares fit equation may suggest that the protein is not moving by simple diffusion. There are conditions that can not be described by equations for simple diffusion, anomalous diffusion and when there are multiple populations diffusing at different rates. **a)** Anomalous diffusion: Potential mechanisms of anomalous diffusion include binding and collisional interactions with mobile and immobile obstacles (Periasamy and Verkman, 1998) and recovery due to vesicular transport. **b)** The labeled protein in the FRAP experiments may alternate between multiple states, i.e. different sized oligomers or bound and unbound to a membrane or complex. These conditions pose significant problems to diffusion analysis. Gordon ***et al.*** (Gordon et al., 1995) describe the mathematical difficulties of separating multiple ***D*** coefficients. The main problem is that the investigator doesn't usually know the fraction

	of each population or the actual number of populations. It is worth noting that at least one group has successfully teased out multiple diffusing populations (Marguet et al., 1999). In this example, the group independently determined the ***D*** coefficients for the two populations and biochemically determined the relative percentages of each population.

SUPPORT PROTOCOL 1
Cell Transfection and Microscope Setup

Materials

Transfected eukaryotic cells expressing desired GFP chimeric protein

In general, most laboratories today use the enhanced GFP (EGFP) variant available from Clontech (Palo Alto, CA). Although spectral variants of GFP are available, EGFP is best suited for photobleaching experiments. See Commentary for more details.

Cell culture medium (i.e. DMEM with serum)
Imaging medium (Appendix)
Confocal Laser Scanning Microscope equipped with a filter set for GFP and a 488 nm excitation laser that is at least 10 mW and preferably 25 mW or more powerful laser
Imaging chamber (either a Lab-Tek chambered coverglass system or a siliconized rubber chamber described below)
Immersion oil (for oil microscope objectives)
Stage heater (for cells that grow at 37^{o}C)
A computer system capable of processing large image files (a 350 MHz or faster processor, a multiple gigabyte hard drive, and at least 128 MB RAM) equipped with image processing software (i.e. NIH Image or Metamorph (TM))

Transfection
Before performing a photobleaching protocol, the investigator must ensure that there is sufficient GFP fluorescence in the expressing cell to maintain a significant fluorescent signal relative to background noise after photobleaching. Most standard transfection protocols are sufficient to provide bright specimens. Stable transfectants express lower levels of protein. Transient transfectants usually express higher levels of proteins; this sometimes results in overexpression artifacts, such as protein aggregation or saturation of protein targeting machinery, which leads to inappropriate localization. Whichever transfection method is selected, the investigator needs several usable cells for each experiment. To enhance the probability of having multiple cells to choose from, it is recommended that the investigator select a high efficiency transfection method, such as electroporation or a lipid transfection reagent, such as FuGENE6 (Roche, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Adherent cells should be transiently transfected 16 to 36 hours prior to the experiment. The commercially available GFP expression vectors are under the control of a very strong promoter, the CMV promoter so cell toxicity or mislocalization of GFP-chimeras to other regions of the cell should always be a

concern. The optimal level of expression and timing of imaging should be determined empirically for each sample and condition.

Set up imaging system

1. Set up the confocal laser scanning microscope and its associated hardware.

2. It is assumed that the investigator is familiar with the basic operation of a confocal microscope. The investigator should understand both the concept and the operation of a pinhole, scan speed, zoom, detector gain, laser power, photobleach, and collection of a time series.

Set up the imaging chamber

3. When using an inverted microscope, adherent cells can be plated on Lab-Tek chambers (Nalgene, Naperville, IL). These chambers consist of wells with a cover glass bottom, which permits the use of high numerical aperture oil objectives for viewing. Prior to placing the cells on the microscope stage, the wells are filled to the rim with imaging media. This ensures that cells have sufficient nutrients during the imaging session. For experiments that will last longer than an hour, the top cover of the chamber should be sealed onto the chamber using petroleum jelly or silicon grease. These steps prevent both rapid evaporation of the media above the cells and decrease the alkalinization of the media by preventing room air from entering the chamber.

4. Alternatively, for upright and inverted microscopes, cells can be plated on glass coverslips. The coverslips can be inverted on rubber gaskets that have a hole punched in them (see ***UNIT 4.4*** diagram and description). These gaskets are mounted on glass slides with petroleum jelly or silicon grease. The hole is filled with imaging media and the coverslips are inverted and pressed onto the hole allowing the cells to face the media. The coverslip adheres to the gasket by capillary action. Excess liquid from the top of the coverslip is wicked away with absorbant tissue.

> ***Suspension cells can be grown in suspension and then adhered to coverslips or Lab-tek chambers by precoating the chamber or clean coverslip with a concentrated (5-10 mg/ml in PBS) solution of poly-lysine (Sigma, St. Louis, MO). Incubate for 15 minutes. Wash twice with PBS or dH_2O. The coating is usually good for up to one week. Suspension cells must be washed three times in PBS, before adhering to the poly-lysine-coated surface. The cells should incubate on the surface for two to five minutes. The nonadhering cells are washed away with PBS (wash twice). Finally, the cells are immersed in imaging medium.***

5. Cells are heated on the microscope stage using a stage heater, such as the Model ASI 400 Air Stream Stage Incubator (Nevtek, Burnsville, VA). A temperature probe, such as a Thermolyne Pryrometer (Carl Parmer, Vernon

Hills, IL), should be used to confirm that the proper temperature is maintained at the coverslip/chamber.

> ***In addition to the chambers described above, Bioptechs (Butler, PA) offers a completely closed chamber with built-in perfusion. This type of setup permits the maintenance of cells on the microscope stage for extended periods of time (greater than 24 hours) with minimal loss in viability. In addition, the investigator can perfuse drugs, dyes, and other reagents with controlled precision throughout the experiment. Thermal collars for objectives are another option for heating/maintaining the temperature of the chamber/coverslip. Thermal collars are available from Bioptechs.***

Data collection

Many laser scanning confocal microscopes provide the investigator with a number of options for data collection including image size (512x512, 1024x1024, pixels etc), range of data collection (8-bit or 12-bit), file formats, etc. It is worth determining the requirements for the user's image analysis software in advance. For example, some image analysis programs cannot process 12-bit images or only process "PGM" (portable graymap) image files, instead of "TIFF" (Tagged-Image File Format) files.

SUPPORT PROTOCOL 2
Inhomogeneous Diffusion Simulation

Before running the simulation on a Macintosh computer (note we collect all of our data on a PC system with the confocal microscope and process diffusion data using a Macintosh), a substantial amount of RAM memory (50-100 MegaBytes) should be allotted to the program. When running the simulation, other programs will run very slowly. Turning off other programs is suggested. Before running the simulation, determine the time interval between frames and the size of each pixel.

1. To run the simulation, the “tiff” files must be converted to a format recognized by the simulation program (“pgm”). “Tiff” files can be converted to “pgm” files with the Macintosh program "Graphic Converter" (http://www.lemkesoft.de/us_gcabout.html). (We recently discovered a bug in version 4.04 of the program available online. The program will create "pgm" files; however, the simulation program does not recognize these files. We have used version 4.01 without incident.) To run the simulation, the simulation program must be in the same folder as the “pgm” files. All files generated by the simulation will appear in this folder.

2. Open the first postbleach image and move the cursor to determine the coordinates of the smallest region that contains the whole cell and write down the coordinate numbers (x, y)(numbers should range between 0-511) for the upper

left corner of the box and the lower right corner of the box. Repeat for the photobleach ROI.

3. Start the simulation program. The program is designed to be user friendly. The first few steps are self-explanatory.

4. Enter the name of prebleach file (include full name, i.e. b009.pgm). If any colored saturation pixels are present in the images, the program will request that the user choose a channel (r, g, or b). Assuming the user has used GFP, enter "g" without quotation marks.

5. When asked "Is this is a FRAP?" and "Are you are supplying a series of post-bleach images, " answer "y" (without quotes) for each question.

6. The user is asked, "Do you want to supply a background value?" Usually the simulation determines an accurate number, though the user is free to change the value.

7. The program will request that the user enter the x, y coordinates of the ROI. The program is referring to the whole cell ROI, NOT the photobleach ROI. Enter each coordinate (first x and then y) separated by a space (no parentheses or commas), return and then enter the lower right hand coordinates, each separated by a space.

8. Next, the user is asked if he wants to monitor diffusion in individual rectangles. To use this option, open the "postbleach_grid.pnm" file and select grid rectangles of interest. The coordinates for each rectangle are determined by numbering the rectangles (0-9) across and down from the upper left corner of the grid. Each rectangle will correspond to "x y", entered with a single space and no punctuation. The user may opt for this method to measure diffusion differences in different regions of the cell. However, the rectangle grid generated by the program will not necessarily align with the photobleach ROI. In addition, ***D*** values will be different for different regions of the cell. This information can be useful, but the time required for equilibration of a region of the cell some distance from the photobleach ROI is often much longer than the equilibration time in the photobleach ROI.

To get ***D*** in the bleach box ROI, type "n" (without quotes) and return. Then enter the coordinates (x y) of the bleach box ROI that were determined using graphic converter or NIH Image. Enter a single space between coordinates with no punctuation.

9. For time, unless the user has used different times between frames, the user should type "y" (without punctuation) if the time intervals between postbleach frames are the same. Then, the user should enter the time between frames in seconds. Decimals (i.e. 0.798) are permitted.

10. The program will take 3-10 minutes to run depending on computer speed, the size of the cell ROI, and the number of images to be processed. The program becomes especially slow after the words "Starting inhomogeneous diffusion" appear. Finally, the program will state the effective diffusion constant for the photobleach ROI or the rectangles selected. **IMPORTANT**: At the end of the program, the user is asked, "Do you wish to plot the simulation data with a different D_eff (yes or no)?" The user must answer "y" or "n", without quotation marks and hit the return key or the program will not create the “experimental data” file. Without this file, the user can not compare the experimental recovery relative to the simulated recovery.

11. Compare the "sim" versus the "exp" data by simultaneously plotting both in a spreadsheet program. It is essential to plot the simulation data to determine whether the diffusion recovery has been accurately modeled. If the simulation misses the experimental data, the calculated diffusion rate is questionable, at best. The majority of the experimental data must overlap the simulation plot. A simulation that only overlaps with the initial steep rise or only with the later more gradual rise is not correct and these ***D*** values should not be used. It is important to use all of the recovery data because removing parts of the recovery can significantly alter the calculated ***D***. However, if the photobleach ROI fluorescence intensity has reached a genuine plateau and then rises or drops due to a focal shift or photobleaching, then the images after the plateau may be excluded from the analysis by simply removing them from the folder.

12. Sometimes the simulation will not be able to determine ***D***. If the simulation quits before stating that ***D*** can not be determined, the user may have entered incorrect coordinates (i.e. placing the photobleach ROI outside of the whole cell ROI. The user should repeat the simulation, with the correct coordinates (be sure to remove any files generated by the previous run of the simulation).

FIGURES

Figure 1. Photobleaching Techniques. (A) Example of FRAP. Cells expressing VSVG-GFP were incubated at 40^{o}C to retain VSVG-GFP in the ER under control conditions (Untreated) or in the presence of tunicamycin (Tunicamycin). FRAP revealed VSVG-GFP was highly mobile in ER membranes at 40^{o}C but was immobilized in the presence of tunicamycin. **(B)** Plot of fluorescence intensity in a ROI versus time after photobleaching a fluorescent protein. The prebleach (F_{pre}) is compared with the asymptote of the recovery (F_{∞}) to calculate the mobile and immobile fractions. Information from the recovery curve (from F_0 to F_{∞}) can be used to determine ***D*** of the fluorescent protein.

Figure 2. Defining ROIs and illustrating bleaching during imaging. (A) Example of ROIs, where A is the bleach ROI, B is the whole cell ROI, and C is the background ROI. **(B)** Plot of whole cell fluorescence (ROI B) versus time. The solid line represents an ideal plot in which the prebleach intensity of the cell does not change with time and after the photobleach there is a drop in mean cellular fluorescence. There is no further loss of fluorescence. The dashed line (– – –) represents a problematic plot of ROI B. The prebleach intensity steadily decreases with time, suggesting that the cell is being imaged at too high of a laser power resulting in photobleaching of the whole cell. The fluorescence loss continues after the photobleach of ROI A. The investigator must establish new imaging conditions.

Figure 3. Diffusion versus linear movement. Plot of distance versus time for a protein, such as a motor protein, that moves directionally at a linear rate of 4 μm/sec (open circles, dashed line) and a protein that diffuses with a diffusion coefficient of 4 μm^2/sec (filled squares, solid line). Note that a protein travels 4 μm in 1 second for the linear rate, but takes 4 seconds to travel the same distance by diffusion.

Figure 4. Fractional fluorescence. A plot of fractional fluorescence can be used to determine $t_{1/2}$, the time at which the fluorescence has recovered to 50% of the asymptote. The recovery data points (from F_0 to F_{∞} in Fig. 1B) have been transformed to a scale of 0-100%.

Figure 5. Example of FLIP. Protein fluorescence in a small area of the cell (box) is bleached repetitively. Loss of fluorescence in areas outside the box indicates that the fluorescent protein diffuses between the bleached and unbleached areas. Repetitive photobleaching of an ER GFP-tagged membrane protein reveals the continuity of the ER in a COS-7 cell. Image times are indicated in the lower right corners. The postbleach image was obtained immediately after the first photobleach. The cell was repeatedly photobleached in the same box every 40 seconds. After 18 minutes, the entire ER fluorescence was depleted indicating that all of the GFP-tagged protein was highly mobile and that the entire ER was continuous with the region in the bleach ROI.

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Table 1. Diffusion rates of GFP and GFP chimeras using FRAP.

Molecule	D_{eff} (μm^2/sec)	References
GFP in water	87	(Swaminathan et al., 1997)
GFP in cytoplasm	25	(Swaminathan et al., 1997)
GFP in the ER lumen	5-10	(Dayel et al., 1999)
Elastase-GFP in the ER lumen	0.5	(Subramanian and Meyer, 1997)
GFP in the mitochondrial matrix	20-30	(Partikian et al., 1998)
Nucleoplasm		
GFP-HMG-17	0.45	(Phair and Misteli, 2000)
GFP-SF2/ASF	0.24	(Phair and Misteli, 2000)
GFP-fibrillarin	0.53	(Phair and Misteli, 2000)
Endoplasmic Reticulum Membrane		
Lamin B Receptor-GFP (in ER)	0.35	(Ellenberg et al., 1997)
GFP-MHC Class I (murine)	0.46	(Marguet et al., 1999)
TAP1-GFP (murine)	0.12	(Marguet et al., 1999)
Galactosyltransferase-GFP (in ER)	0.48	(Nehls et al., 2000)
Signal Recognition Particle β subunit	0.26	(Nehls et al., 2000)
KDEL Receptor-GFP (+BFA)	0.43	(Cole et al., 1996)
VSVG-GFP tso45 (in ER+BFA 32^oC)	0.49	(Nehls et al., 2000)
VSVG-GFP tso45 (in ER 40^oC)	0.45	(Nehls et al., 2000)
Cytochrome P450-GFP	0.03-0.06	(Szczesna-Skorupa et al., 1998)
Golgi Apparatus Membrane		
Galactosyltransferase-GFP (in Golgi)	0.54	(Cole et al., 1996)
Mannosidase II-GFP	0.32	(Cole et al., 1996)
KDEL Receptor-GFP	0.46	(Cole et al., 1996)
Plasma Membrane		
E-cadherin-GFP	0.03-0.04	(Adams et al., 1998)
GFP-Ki-Ras (12V)	0.19	(Niv et al., 1999)
GFP-aquaporin (1 and 2)	0.009	(Umenishi et al., 2000)
Lutenizing Hormone Receptor-GFP	0.16	(Horvat et al., 1999)
Endosomal Membrane		
GFP-rab5	0.1	(Roberts et al., 1999)
E. coli Cytoplasm		
GFP	7.7	(Elowitz et al., 1999)
Maltose D-GFP	2.5	(Elowitz et al., 1999)