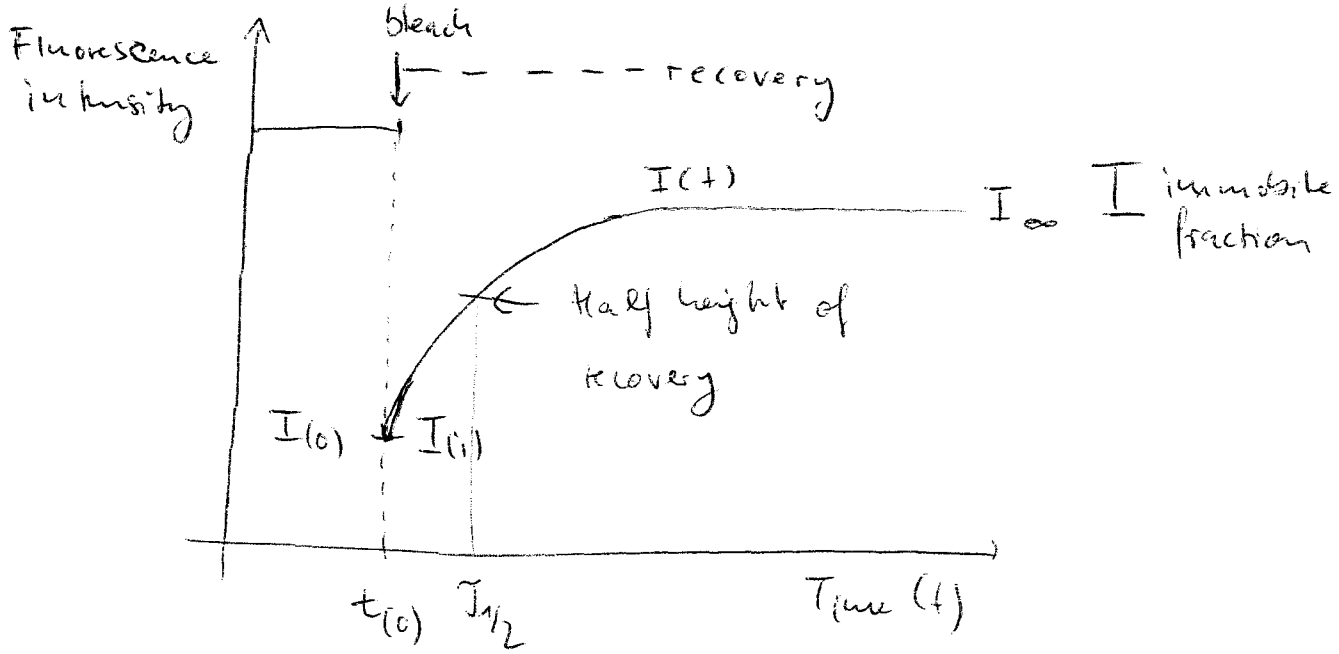
Fluorescent recovery after photobleaching (FRAP)

- measurement of diffusional mobility of membrane components, but also study of diffusion in cytoplasm is possible

- short pulse of intense laser light → destruction of fluorescence in a small area (micron size),
- Fluorescently - labeled lipids or proteins used

- Recovery of the fluorescence occurs by diffusional exchange between bleached and unbleached molecules, rate is characterized by the lateral diffusion constant D



Diffusion coefficient from $\tau_{1/2}$:

$\tau_D = \tau_{1/2} / \gamma$ $\quad$ γ = correction factor

$\tau_D = \omega_r^2 / 4D$ $\quad$ $\omega_r = e^{-2}$ – beam radius

(= half width of half height of spatial laser intensity)

79 Fluorescence Photobleaching Techniques

INTRODUCTION

Fluorescence recovery after photobleaching (FRAP) is also known as fluorescence photobleaching recovery, fluorescence photobleaching redistribution (FPR), and fluorescence microphotolysis. FRAP has been a major tool over the past two decades for measuring the diffusional mobility of membrane components labeled with fluorophores, with fluorescence antibodies, and, more recently, with GFP fusion proteins. FRAP is typically applied to measurements of protein or lipid components in cell membranes such as the plasma membrane; subcellular membranes such as the mitochondrial inner membrane; reconstituted membranes such as liposomes or planar bilayers. FRAP can even be used to study diffusion in the cytoplasm of the cell. FRAP is applicable to the study of most membranes and the general principles presented here can be adapted to such studies. In FRAP, a short pulse of intense laser light irreversibly destroys (photobleaches) fluorescence in a small micron-size area. Recovery of fluorescence into the area being photobleached occurs as a result of diffusional exchange between bleached and unbleached fluorescent molecules. The fraction of labeled proteins or lipids that can participate in this exchange is called the mobile fraction. The rate of this movement is characterized by the lateral diffusion constant D. The fraction of proteins that cannot move on the time scale of the experiment is called the immobile fraction (Fig. 79.1). The technique has been extensively reviewed (Axelrod et al. 1976; Jacobson et al. 1976; Kapitza and Jacobson 1986; Peters 1986; Petersen et al. 1986; Elson and Qian 1989; Wolf 1989). For more detailed and extensive descriptions and explanations, the reader is referred to these reviews.

Using the FRAP technique, it has been found that most membrane proteins do not have the continuous, unrestricted lateral diffusion characteristics of a random, two-dimensional fluid. Rather, there are many types of constraints to lateral diffusion of proteins within living membranes. These include interactions of membrane molecules with each other, with molecules of the cell cytoplasm, or with molecules of the extracellular matrix (Edidin 1992, 1994). For any given protein, D and the mobile fraction reflect a mixture of constraining effects of the cytoskeleton/cytoplasm, of bilayer viscosity, and of other membrane proteins (Zhang et al. 1993). It should also be remembered that D and mobile fraction are each an average value for the fluorescently labeled population being studied (Zhang et al. 1993). Knowledge of the physical constraints on mobility quantified using FRAP can provide important insights into the properties, interactions, and in some cases, the physiological functioning of proteins and lipids within cells.

GFP fusion proteins are excellent reagents for use in photobleaching studies, because GFP exhibits much less photodamage to its surrounding environment than do other fluorophores. This is likely due to the fact that the GFP chromophore is completely shielded from surrounding fluid by its dense, 11-stranded β-barrel protein structure (Ormo et al. 1996). Moreover, because GFP and GFP fusion proteins can

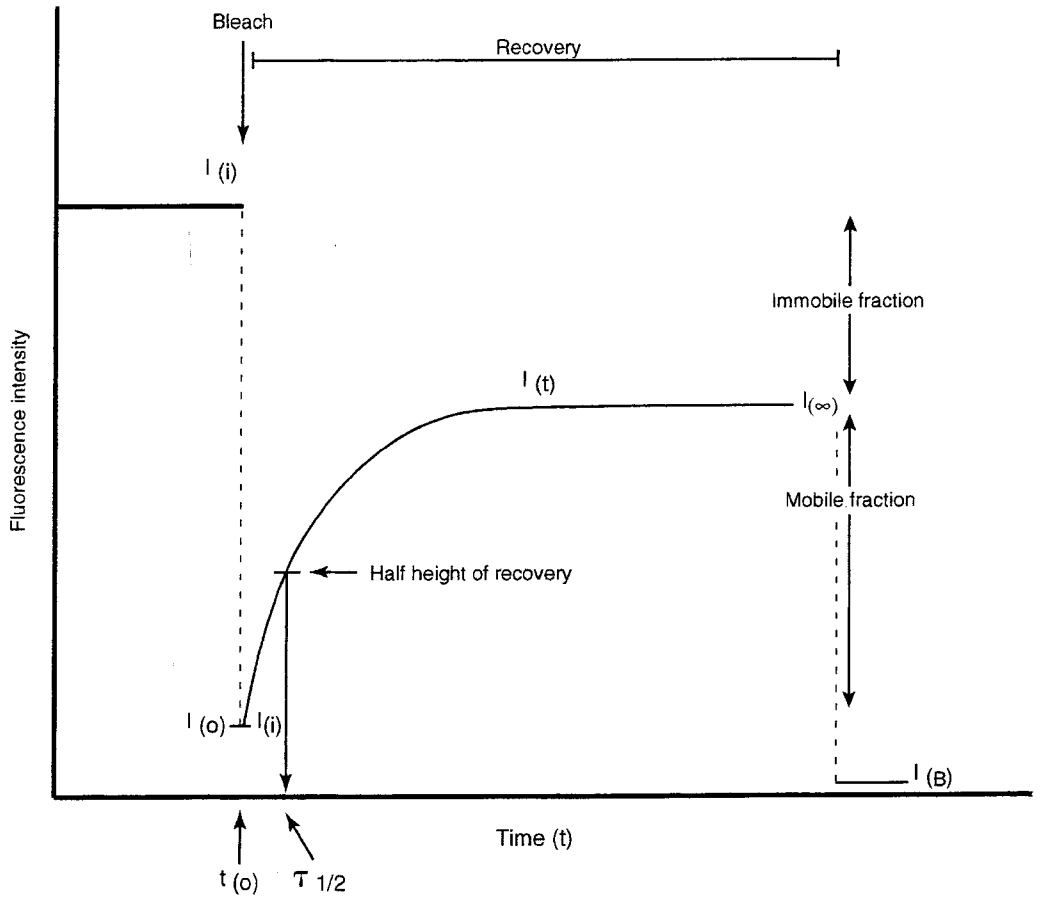


FIGURE 79.1
Parameters in a FRAP experiment. Fluorescent intensity inside a 1–3-μm box is measured as a function of time after bleaching with intense laser light. I_i is the fluorescence intensity inside the box before photobleaching, I_0 immediately after photobleaching, and I_∞ is fluorescence at the time the recovery is judged complete. The mobile fraction (mf) is the extent the initial fluorescence is regained. The immobile fraction is the percent of fluorescence that is not regained.

be expressed endogenously within cells, the mobility of GFP-tagged proteins within intracellular compartments can be probed using FRAP. This provides a powerful new tool for investigating fundamental properties of cells, including the viscosity of the cell cytoplasm (Swaminathan et al. 1997), the compartmental boundaries within cells (Cole et al. 1996), and the diffusional characteristics of intracellular membrane proteins (Ellenberg et al. 1997; Subramanian and Meyer 1997).

This chapter considers two major approaches to FRAP. One approach, the spot photobleaching technique, the original approach, will be termed conventional FRAP for this chapter. A number of variations on the original spot photobleaching technique, not presented here, have been developed and include pattern photobleaching (Smith and McConnell 1978), multipoint (Koppel 1979), video (Kapitza and Jacobson 1986), and total internal reflectance (Thompson et al. 1981). The second major approach utilizes the confocal microscope which is now usable for FRAP due to advances in confocal microscopes and the software to control them.

CONVENTIONAL FRAP INSTRUMENT

Although the concept underlying FRAP is rather simple, the instrumentation is somewhat complex. Most systems used by investigators are custom-built, and it is beyond the scope of this chapter to provide technical information on building a FRAP microscope/laser system. The heart of the instrument is an upright epifluorescence research microscope with appropriate dichroic mirrors and filters (e.g., for rhodamine or fluorescein) that is also equipped with phase-contrast or dark-field modes. An inverted microscope can also be used; however, in this configuration additional precautions to protect the operator from the laser beam must be implemented. An argon ion laser is capable of supplying approximately 5 μW of light energy for monitoring and up to about 50 mW for bleaching. Since there is a power loss due to the required optical components between the laser and the specimen, this leaves ~1 μW for monitoring and ~10 mW for bleaching at the membrane surface. A maximum output of 1 or 2 W at the laser at 488 nm and 514 nm wavelengths while operating in the TEM_{00} mode (transmission mode) is recommended. Such a laser may be rated at 3 W power for all emission lines collectively. Alternatively, a krypton laser can be used at somewhat different wavelengths. A red-sensitive, cooled photomultiplier tube (PMT) (with an amplifier/discriminator) operated in a photon-counting mode is used to detect the fluorescence signal (Fig. 79.2). The PMT must be protected from the bleaching pulse and full-field (Hg or Xe lamp) illumination by a shutter and/or by cutting the voltage applied to the first dynode of the PMT. The FRAP instrument (Fig. 79.2) also includes high-speed (millisecond) shutters to control the laser beam timing and a beam attenuation device to create the monitoring and bleaching beams. These two beam intensities are created using either a perfectly parallel neutral density filter (Jacobson et al. 1976), a beam-splitting device (Koppel 1979), or an acousto-optic modulator (Garland 1981). Other optical components are also required (cf. Petersen et al. 1986; Wolf 1989). One of these components is a biconvex (relay) lens used before the epifluorescence port of the microscope to focus the laser beam at an intermediate image plane (usually where the fluorescence field diaphragm is located) that in turn brings the beam into sharp focus at the specimen plane. A beam-steering device is used to precisely position the beam into the epifluorescence port, centering the beam with respect to the ocular cross hairs, and to align the beam so that it passes precisely perpendicular to the specimen plane. Various electronics and/or a microcomputer are necessary to precisely control the instrument timing and sequencing, and a multichannel analyzer is required to collect and store the data. The instrument (electronics) response time must be rapid (1–2 msec) to measure diffusion coefficients in the upper range of 10^{-7} cm^2/sec. It is advisable to have the instrument mounted on a vibrationally isolated optical bench to reduce vibrational interference and to maintain a secure alignment of the laser beam. The instrument should be operated in a darkened room or an area of a room partitioned off from interfering light. A few small 15 W red lights can be used to illuminate essential consoles.

The following sources are intended to help in acquiring the necessary instrumentation, hardware, and information to build a FRAP instrument. This is not intended to be an exhaustive list or a recommendation of one source over another. Spectra Physics and Coherent Laser are potential sources for lasers. The Newport Corpora-

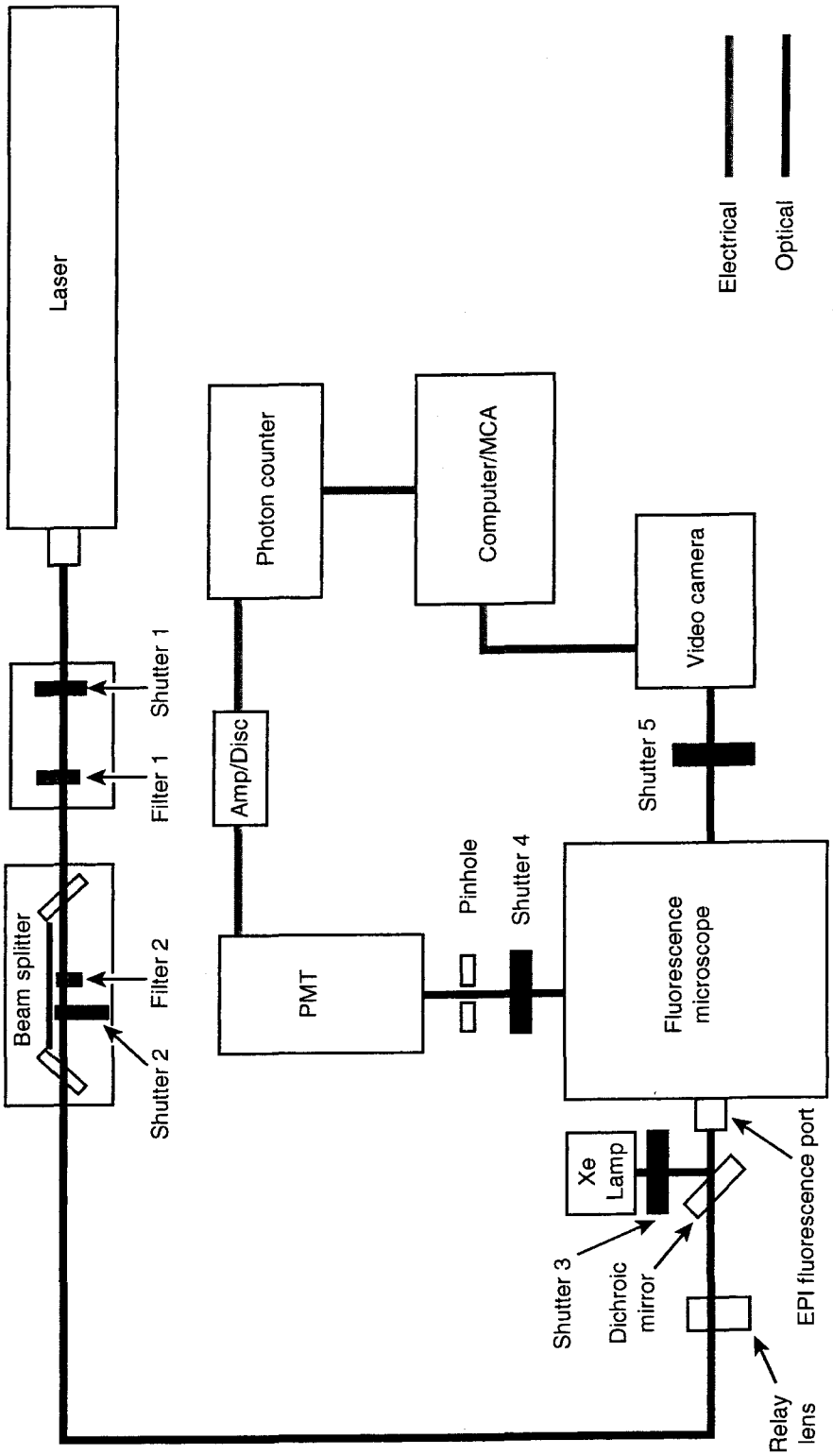

Laser
Shutter 1
Filter 1
Beam splitter
Filter 2
Shutter 2
Amp/Disc
Photon counter
Computer/MCA
Video camera
Shutter 5
PMT
Pinhole
Shutter 4
Fluorescence microscope
Xe Lamp
Shutter 3
Dichroic mirror
EPI fluorescence port
Relay lens
Electrical
Optical

FIGURE 79.2
General schematic of a FRAP instrument. Proceeding counterclockwise, in the *upper right* is the laser used to produce the excitation beam. Following the indicated optical path leads to shutter 1, sometimes called the beam flag, which is used to control the illumination of the sample by the laser beam. Next is filter 1, a perfectly parallel, neutral density filter that is optically flat and is used in systems where beam attenuation is done by a filter. When the solenoid-actuated filter is in the beam a low-power monitoring beam is produced; when out a bleaching beam is produced. If a beam splitter is used this filter remains out of the laser beam. The (Koppel) beam splitter is also used to produce superimposed bleaching and monitoring beams. When the laser beam hits the first glass, optical flat, a portion is reflected to produce lower power beams, one of which travels unobstructed to the second optical flat. The main, higher power, bleaching beam can be reduced in power to achieve a small intensity difference between bleaching and monitoring beams by (perfectly parallel) filter 2. Shutter 2 opens only to allow photobleaching to occur. (The spacing of the two beams is a function of the optical flats and must be considered in placing the shutter.) The adjustably positioned relay lens is used to focus the laser beam on a microscope intermediate image plane. The dichroic mirror permits the laser beam to pass through while permitting a Xe lamp with appropriate filters to illuminate the full field of the fluorescent sample. Shutter 3 permits brief user-controlled viewing under full-field illumination. The fluorescence microscope must have the appropriate dichroic filters, and so on. Shutter 4 blocks the Xe lamp or the high-power photobleaching beam from reaching the PMT. The changeable pinhole limits the depth of field of the fluorescence signal from the sample. Alternatively, the optical output of the microscope can be directed to a shuttered video camera. The electrical video signal from the camera then goes to a monitor and/or computer (with appropriate image processing software). From the PMT the electrical signal goes to a preamplifier/discriminator and then to a photon counter. The computer and/or MCA stores the data from the FRAP experiment. A computer or dedicated timer is used to control the timing and sequencing of the FRAP instrument components.

tion and Oriel Instruments are possible sources for optical hardware. Either IBM PC compatible or MacIntosh computers can be used with suitable interfaces for shutter drivers. A commercially built instrument has also been available.

SAMPLE PREPARATION FOR CONVENTIONAL FRAP

Sample preparations are described for living cells expressing GFP chimeras and for samples labeled with other fluorophores.

Sample Preparation Using GFP Chimeras

Living cells expressing GFP chimeras grown on no. 1 glass coverslips are mounted on depression slides in 200 µl of buffered growth medium and sealed with Valap (1 : 1 : 1 mixture of vaseline, lanolin, and paraffin wax). Alternatively, they can be mounted on top of a rubber chamber containing 100 µl of medium and sealed by pressing gently but firmly on the coverslip to remove all liquid on the edges, where the coverslip sits on top of the silicon rubber chamber. To make the silicon rubber chamber, cut out a small square or circle so that the coverslip will fit over it. Grease the back of the chamber slightly with inert silicon grease and press it down on a microscope slide. Fill chamber with medium and place the coverslip on top and press down carefully to make a good seal without air bubbles. Wipe excess fluid with a Kimwipe so that the outside of the coverslip and chamber is dry. Silicon rubber sheets can be ordered from Reiss Corporation (Blackstone, Virginia). Both techniques (i.e., depression slide and rubber chamber) allow viewing for extended time periods on a microscope stage.

The microscope stage can easily be heated to 37°C by covering the entire microscope with plastic and gently blowing in warmed air. Alternative temperature-controlled chambers for observing living cells are discussed in Chapter 75.

Sample Preparation Using Other Fluorochromes

The FRAP technique requires that the sample to be studied must have the diffusing component of interest fluorescently labeled and the whole membrane (e.g., cell) itself must be prevented from moving by attachment to an immobile substratum, such as a glass microscope slide or coverslip. Native biological membranes, derivatives of biological membranes, reconstituted membranes, single and multilamellar planar lipid bilayers, planar supported bilayers, as well as single and multilamellar vesicles, have all been used. Typically, fluorescent labels are highly specific immunofluorescent probes, fluorescent analogs of membrane molecules, for example, phospholipids, or native membrane molecules chemically labeled with a fluorescent group (Alecio et al. 1982; Vaz et al. 1982; Chazotte and Hackenbrock 1988; Chazotte et al. 1991; Gupte et al. 1991). It is critical that the fluorescent probe *not* be bleached by the low-power monitoring beam, but be irreversibly photobleached by the bleaching beam with first-order kinetics. Additionally, the probe should not be toxic for the system being studied. Rhodamine, fluorescein, Texas red, BODIPY (4,4-difluoro-4-bova-3a,4a-diaza-S-indacene), NBD (7-nitrobenz-2-oxa-1,3-dia-

zole), and carbocyanine fluorescent moieties are most frequently used in probes. Molecular Probes, with its extensive, referenced catalog of fluorescent probes, is one possible source.

It is critical that the membrane to be studied is itself *not* moving; that is, one should not attempt to measure lateral diffusion in the plasma membrane of a cell that is motile. A membrane, for example, mitochondrial inner membranes, may naturally attach to the glass substratum (Chazotte and Hackenbrock 1988) or it may be anchored by the use of polylysine-coated glass (Chazotte and Hackenbrock 1991). A 0.1% solution of 70–150 kD poly-L-lysine at 100 μg/ml can be made up in distilled, deionized water and stored at −70°C until used. Clean glass microscope slides or coverslips can be dipped in the polylysine solution, air-dried, and stored prior to use. It is advisable to test whether the polylysine will affect the diffusion measurements by testing a few membranes that have naturally stuck to the glass.

In some instances the native, biological membranes, such as the mitochondrial inner membrane, may be of insufficient diameter to easily make FRAP measurements. Generally a minimum 10-micron diameter membrane is desirable. Too small a diameter raises two potential problems: (1) both membranes (free and attached regions of, e.g., a vesicle) being bleached and monitored; and/or (2) too large a percent area being bleached relative to the total membrane surface area with the ensuing difficulties in analysis (Petersen and MacConnaughey 1981; Elson and Qian 1989; Chazotte and Hackenbrock 1991). In many studies it has been possible to fuse membranes (mitochondrial inner membranes) to a much larger size using calcium and low pH (Chazotte et al. 1985; Hackenbrock and Chazotte 1986) to facilitate and simplify FRAP measurements.

Depending on the fluorescent probe and membrane to be used, fluorescent labeling may be carried out before or after membrane attachment, but labeling should always be carried out in subdued light. No matter when the labeling is carried out it is important to *minimize background fluorescence* on the substratum and in solution. Relatedly, unless multibilayer membranes are to be specifically studied, it is preferable not to have membranes (e.g., cells) atop one another, as this complicates focusing the laser spot on the appropriate membrane. Immunofluorescent labeling with bivalent monoclonal or polyclonal antibodies must be carried out in saturating (excess) quantities to prevent antibody-induced cross-linking. The amount of antibody added is dependent on a number of factors such as the number of antigenic sites, antibody specificity, and the amount of membrane present. In plasma membranes one must be aware of whether a particular antibody can induce patch and/or cap formation of the antigen in the membrane (see, e.g., Jacobson et al. 1984). Alternatively, monovalent Fab fragments derived from IgG molecules by a papain proteolytic digestion may be used. Fluorescent analogs of membrane molecules, for example, DiI (1,1′-dioctadecyl-3,3,3′,3′,–tetramethylindocarbo-cyanine perchlorate) for phospholipid, or fluorescently labeled membrane molecules, for example, rhodamine–phosphatidylethanolamine, permit the ratio of fluorescent to nonfluorescent molecules to be controlled. Typically such probes are added in ethanolic solution (Jacobson et al. 1981; Chazotte and Hackenbrock 1988). It is desirable to have probe to unlabeled ratios between 1:1,000 and 1:10,000 to get both a sufficient signal level and a statistically sufficient population. However, these probes have the potential to be membrane perturbants and, therefore, high amounts of these probes are not advisable. Likewise, one should also be concerned

that the probe has adequate solubility in the membrane in general or membrane phase. Equal solubility in gel and liquid phases may be desirable, or one may make use of preferential solubility in gel or liquid phases (see, e.g., Vaz et al. 1982; Spink et al. 1990). Finally, caution should be exercised because label location may not be stable, as in the case of a living cell. Various receptors and parts of membranes can be transported or endocytosed into the cell as part of the normal function of the cell and affect the results.

INSTRUMENT SETUP FOR A CONVENTIONAL FRAP EXPERIMENT

Prior to the collection of any experimental data, the FRAP instrument must be appropriately aligned. Alignment is facilitated by mounting optics on commercially available precision mounts to allow fine control of position and orientation. The alignment should be checked prior to each experimental session on the instrument.

1. Prepare a thin (~0.25 μm) DiI film on a sealed microscope slide. This is one of the most useful and essential tools for alignment and calibration. DiI refers to a class of fluorescent lipid analogs such as $DiI_{C:18}$, 3′3′-dioctyldecylindocarbcyanine often used for FRAP.The slide is prepared as described below according to the method of Schneider and Webb (1981). The finished DiI film slide is placed on the microscope stage for alignment and calibration of the FRAP instrument.

   In the dark or subdued light, prepare a solution of 50 μl of DiI (C:16 or C:18) 1 mg/ml in ethanol, 250 μl of colloidion, and 750 μl of amyl acetate. Also prepare a covered, vertical slide chamber with a small amount of amyl acetate in the bottom to saturate the chamber. Place 200 μl of the solution on a horizontal glass microscope slide and let stand for 8 seconds. Stand slide vertically in an amyl acetate containing slide chamber for 30 minutes. Cover with coverslips, seal with paraffin, and protect from light.

2. Focus the laser beam at the sample plane using the biconvex relay lens before the EPI fluorescence port of the microscope (Fig. 79.2). The best focus is achieved by imaging the laser spot on a thin DiI film to achieve the brightest minimum spot size as viewed by eye through the microscope.

3. Ensure proper alignment of the beamsplitter assembly.

   If the beamsplitter assembly (Fig. 79.2 and Koppel 1979) is not aligned properly, the bleaching and monitoring beams will not be coincident at the sample and the diffusion measurement will be incorrect. If the bleaching and monitoring beams are misaligned by one beam radius, then the diffusion coefficient will be underestimated by a factor of 3.3. The simplest way to check this is to place the slide with the thin DiI film on the microscope stage, bring the (low power) monitoring beam's laser spot into bright, sharp focus by looking through the microscope, and adjusting the microscope focus. Then very briefly photobleach (1–3 msec) the slide and then again look through the microscope with the monitoring beam. If the beamsplitter is aligned, you should not see a fluorescent spot. If not, adjust the beamsplitter.

4. Position the beam so that it is perpendicular to the sample plane (microscope slide) to give a Gaussian shaped spot, that is, circular. If not, the FRAP measurement will be inaccurate.

   This alignment becomes even more critical as progressively higher magnification lenses are used. To check the alignment, use the DiI film slide on the microscope stage and look through the microscope. The laser spot should not move in the x-y sample plane as the microscope focus (z-axis) is changed. If the spot moves, remove the transmitted light condenser and place a piece of

white paper in the condensor's position marked with crosshairs centered (like the eyepiece reticule crosshairs). The goal is to have the fluorescent spot at the sample plane and the laser beam on the white paper in the same *x-y* position. The monitoring beam is positioned using the adjustment knobs on the beam steering device behind the microscope. It is usually easier to start with the beam in opposite quadrants at the sample compared to the white paper and to iteratively adjust the beam steering mirrors to have the beam in the same position in both places.

5. Install and align an appropriate diameter precision pinhole (e.g., 100 μm, 200 μm, 500 μm, 1000 μm) at the intermediate image plane before the PMT to limit the depth of field for signal collection to exclude the fluorescence outside of the sample focal plane (Fig. 79.2).

   This essentially creates a confocal effect in conventional FRAP so that fluorescence is *only* collected from *the membrane in focus.* This should be of concern, for example, when making measurements out at the thin leading lamellae of a cell's plasma membrane where the ventral and dorsal membrane surfaces can be close together, sometimes as close as 100–400 μm, in which case the pinhole cannot resolve the two membrane surfaces. With a smaller pinhole diameter the depth of field is narrower; on the other hand, the fluorescent signal to be measured is reduced. The depth of field will also vary with the objective lens selected. Profiles can be manually acquired and plotted for reference purposes. The pinhole alignment relative to the laser beam must be checked any time the laser beam position is adjusted. If the pinhole is misaligned, the fluorescence signal may be unusually low.

6. Select an objective lens suitable for fluoresence microscopy.

   Typically these may be 10×, 25×, 40×, 63×, or 100× magnification planapochromatic lenses. Phase objectives have been successfully used. Any lens used must correctly image the laser spot and must not move the laser beam position on the sample when changing between different lenses. A lower magnification lens will have a larger beam diameter and a lower intensity per unit area than a higher magnification lens. The expected D to be measured may influence the selection of the lens magnification. It is easier to resolve more rapid diffusion with a larger beam diameter. However, unless there are microdomains (Edidin 1992) in the membrane, the D should not vary with beam diameter. Typically a 40× lens may be used with an approximate 2-μm beam diameter. Beam diameters can range from ≤1 μm with a 100× lens to ~50 μm with low magnification lenses. Whatever lens is selected, it is *critical* that the beam diameter at the sample plane be precisely known in order to calculate D. Beam diameters for each lens are usually measured for each instrument and should be available for users to look up. If not, a DiI film slide is used to make the beam diameter measurements according to Schneider and Webb (1978) or by imaging using a linear detector such as a CCD camera.

NOTE

- *Beam diameter versus membrane area.* FRAP experiments typically assume that the beam diameter used is small relative to the total area of the membrane studied. If this condition is not satisfied, then the calculation of mobile fraction and/or D may be affected. This is another consideration in the selection of objective lens for experiments. In addition, in cases like the sperm cell plasma membrane, diffusion from region to region may be restricted; consequently there may be an insufficient amount of fluorophores to give a full recovery.

NEW SPECIMEN TESTING, GENERAL RULES, AND TROUBLESHOOTING FOR A CONVENTIONAL FRAP EXPERIMENT

Data acquisition can be a simple and routine process once the experimental conditions for a given system are worked out. However, for a new or relatively unknown specimen, sufficient controls and precautions must be exercised.

1. *Laser light power level:* The user should have three empirical goals in mind: *(a)* to have a measurable fluorescence signal; *(b)* to photobleach the spot at least 50% within one-tenth of the time for the half-time of recovery, i.e., $<0.1^* \tau_{1/2}$, otherwise D cannot be correctly calculated, since recovery will be significantly under way while bleaching is still occurring; *(c)* to avoid photobleaching with the (low power) monitoring beam. The fluorescence signal to be measured may be boosted by increasing the laser power, increasing the photomultiplier tube (PMT) voltage or, increasing (if possible) the fluorophore concentration. Changing the objective lens, fluorophore, sample labeling concentration, etc. may necessitate changing the laser power level.

2. *Bleaching/monitoring beam attenuation factor:* The selection of the attenuation factor between the bleaching and monitoring beam depends primarily on the fluorophore selected. Generally one wants a 10,000-fold (4.0 ND) attenuation for fluorophores such as DiI and fluorescein and a 1,000-fold attenuation for rhodamine. The attenuation factor can be adjusted on some FRAP instruments. For those instruments using the mechanical imposition of a neutral density filter in the beam to produce the monitoring beam (filter 1 in Fig. 79.2), one simply changes the perfectly parallel ND filter from, for example, ND 4 to ND 3. For those instruments using a Koppel beamsplitter arrangement (Fig. 79.2), one changes the ND factor from approximately 4 to 3 by inserting a perfectly parallel 1.0 ND filter (filter 2 in Fig. 79.2) in the shuttered bleaching beam to reduce the bleaching beam intensity and yield a net 3.0 ND factor difference between the bleaching and monitoring beams.

3. *Undesired photobleaching with monitoring beam:* One simple control to eliminate undesired photobleaching is to use the intended or a duplicate sample and monitor the fluorescent intensity for the full length of time required to acquire a FRAP curve, but without bleaching. The intensity should not progressively decrease. If it does decrease, either the laser power must be reduced or a pulsed interrogation mode should be used to limit the observation to a series of short periods during the recovery. Clearly, if during an experiment the intensity is decreasing during the prebleach period (Fig. 79.1), adjustments must be made before proceeding.

4. *Length of time to fully monitor recovery:* It is important that the total data collection time is at a minimum *ten times longer* (Axelrod et al 1976) than the expected half-time of recovery plus the time for monitoring prior to the photobleaching. For curve-fitting of the recovery, fifty times would be the optimal ratio (10 minimal) of τ_D to dwell time (see Gordon et al. 1994). Empirically, one can test by monitoring the recovery after photobleaching and ensuring that the recovery curve (Fig. 79.1) asymptotically levels off.

NOTES

- Never completely photobleach the sample, i.e., to zero intensity.
- *Signal averaging.* One way to improve the signal-to-noise ratio of the data is to sig-

nal average. This can be done by repeatedly analyzing the same spot on a membrane provided there is a very large reservoir of fluorophores. Otherwise the summing of the FRAP curves will show a progressive decrease in the intensity added. Alternatively, the recovery curve from separate membranes may be averaged. Signal averaging the same spot may not be a viable option if the recovery is not 100%, which is typical of many plasma membrane proteins. In the most difficult experimental cases when signal levels are very low, it is possible to get an estimate of D but no accurate information on the mobile fraction by signal averaging when recovery is less than 100%.

- *Photodamage.* Numerous control studies have been done to show that FRAP experiments do not introduce photodamage to a cell membrane (e.g., Jacobson et al. 1978) and that the laser beam does not raise the temperature of the membrane in the spot more than 0.03°C.

GENERAL SEQUENCE OF A CONVENTIONAL FRAP EXPERIMENT

The sequence and rapid timing for a FRAP experiment should be automated and controlled by a dedicated programmable timer control or a computer. Typically, instruments have times for prebleach monitoring; shutter activation, to allow time for any automatic mechanical repositioning before bleaching; bleaching; PMT delay, to prevent intense light from damaging the PMT, a minimum 25 msec; recovery monitoring; and baseline monitoring.

1. After determining the basic instrument settings, timings, and lens to be used (see above), place the specimen on the stage and examine under full illumination by transmitted or fluorescent light. Position the monitoring (low power) laser beam on the region of interest on the membrane surface. Turn off the full illumination and focus the laser beam to the brightest, sharpest spot.

   Focusing the monitoring beam is one of the most critical user-controlled sources of error. With care and practice, the beam can be focused with an uncertainty of 10%, which can give a 20% uncertainty in D.

2. Configure the FRAP instrument for data acquisition to the PMT.

   Make sure that all optical sliders on the microscope are in their proper position and recheck all time settings.

3. Begin the FRAP cycle and monitor the recovery on screen. The cycle should record an initial prebleach intensity $I_{(i)}$, the intensity immediately following photobleaching $I_{(0)}$, the recovery intensities $I_{(t)}$, the maximum recovery $I_{(\infty)}$, and the baseline intensity (dark current) $I_{(B)}$.

   Make sure the depth of bleach is greater than 50% and that the recovery is complete. The curve should roughly resemble Figure 79.1. Incomplete bleaching or recovery, as well a complete recovery over very rapidly, i.e., a very fast rise followed by a long flat asymptotic tail, are cause for new settings and repeating the process.

4. Recheck the membrane after completion of the FRAP cycle to ensure that the spot has not been displaced from the region of interest on the membrane surface and that the focus of the laser beam has not been lost during the experiment. If either has occurred, discard the data and find a new area for a new measurement.

NOTES

- To ensure that bleaching fluorescence does not damage the sample, it is useful to repeat FRAP on the same spot. *D* should remain the same, with the mobile fraction now close to 100%. This is because the immobile fraction was bleached in the prior bleaching episode. If *D* is changed or the mobile fraction is less than 100%, there should be concern about damage to the specimen.
- The FRAP measurement should be made by focusing the laser beam on the "free" membrane, that is, the one not directly attached to the glass slide, otherwise caution must be exercised, as the resultant *D* and mobile fraction may reflect the immobilizing effect of attachment rather than the membrane properties alone.
- Antioxidants are not recommended for conventional photobleaching measurement with fluorescent antibodies and fluorescent analogs because they may adversely affect the results. If necessary, they should be tested by comparison.
- A note of caution for GFP chimeras: Photobleaching is a poorly understood phenomenon at the chemical physics level. An important class of bleaching events are photodynamic, involving the combination of fluorophore, light, and oxygen. Virtually all fluorescent molecules in their excited state react with oxygen molecules to make free radicals, which can then damage cellular proteins. Although little photodamage has been reported so far in imaging studies with GFP chimeras in mammalian cells, if one is imaging with bright light for long periods, addition of antioxidants to the medium could be beneficial. Oxyrase (Oxyrase Inc., Mansfield, Ohio) (0.3 units/ml) and ascorbic acid (0.1–1.0 mg/ml) are two antioxidants that have been shown to reduce photodamage when added to the medium of cells for GFP chimera confocal microscopy measurements.

CALCULATION OF *D* AND MOBILE FRACTION IN CONVENTIONAL FRAP

The function of analysis is to fit the recovery curve to the diffusion equation. A sample recovery curve is shown in Figure 79.1. A number of methodologies have been published that accept different balances between accuracy, flexibility, and speed (for reviews, see Axelrod et al. 1976; Gordon et al. 1994; Petersen et al. 1986; and Wolf 1989).

1. The three-point fit method of Axelrod et al. (1976) is a simple method for determining the *D* and mobile fraction for single component diffusion without requiring a computer, although one can be programmed to do so. The three-point fit utilizes the prebleach initial, $I_{(i)}$, the first postbleach intensity, $I_{(0)}$ at $T_{(0)}$ and postbleach maximum recovery, $I_{(\infty)}$ fluorescence (arbitrary) intensities to calculate the half-height of recovery on the *Y*-axis. It is important that the baseline intensity $I_{(B)}$ be subtracted from all the data. The point at which the half-height recovery intersects the recovery curve indicates $t_{1/2}$ on the *X*-axis (Fig. 79.1). The depth of bleach relative to the *baseline* is calculated:

$$B = I_{(i)}/I_{(0)} \quad (1)$$

and the extent of bleaching (K) is defined in the following equation:

$$I_{(0)} = I_{(i)} [1\ 2\ e^{2K}]/K \quad (2)$$

The diffusion coefficient is calculated from $\tau_{1/2}$ using the following equation:

$$\tau_D = \tau_{1/2}/\gamma \tag{3}$$

where γ is a correction factor for the depth of bleach used in the three-point fit as described by Axelrod et al. (1976). Values for γ can be calculated iteratively for given values of K using Equation 2. Alternatively, values for γ can either be extrapolated from a table of values for γ as a function of K or from a nonlinear curve fit of the relationship (see NOTES below).

$$D = (\omega^2 \times \gamma)/(4 \times \tau_{1/2}) \tag{4}$$

where ω^2 is the beam radius at the focused spot. The mobile fraction (recovery) is calculated from the following equation:

$$R = (I_{(\infty)} - I_{(0)})/(I_{(i)} - I_{(0)}) \tag{5}$$

However, this approach gives no information about the goodness of the fit (only three points are used), whether there is only one diffusing component or more, and whether there is diffusion or flow.

2. The most accurate approach is to fit the recovery curve to the full series solution using a nonlinear least-squares method. This is also the most time-intensive and requires the computational power of a computer. Fortunately, the current microcomputers make this process rapid enough.

   Curve fitting permits the determination of the goodness of the fit of the recovery curve to a diffusion equation and permits the determination whether there is one- or two-component diffusion or flow and the respective coefficients. Two-component diffusion is a likely result for cell plasma membranes and should always be considered in analysis.

The full one-component model equation can be written:

$$I_{(t)} = \phi I_{(I)} f_{(t)} + (1 - \phi) I_{(0)} \tag{6}$$

where ϕ refers to the mobile fraction. The series expression, $f(t)$, is given by

$$f_{(t)} = \sum_{n=0}^{\infty} [(-K)^{-n}/n!](1 + n + 2nt/\tau_D)^{-1} \tag{7}$$

where τ_D is the characteristic time for diffusion, t is time in seconds, and the number of terms in equation 7 used for fitting can be as high as 30. It is important that the baseline intensity $I_{(B)}$ should be subtracted from all the data. See Gordon et al. (1994) for details on curve fitting one-diffusing component and two-diffusing components.

NOTES

- *Membrane sources of "error."* FRAP measurements of diffusion in membranes are predicated on the laser spot focusing on an infinite, single, planar membrane. The largest source of error is usually due to the beam diameter. Accurately knowing the beam diameter and carefully focusing the laser beam to the smallest, brightest spot on the membrane in an experiment are important in reducing potential error. Membranes that have a nonplanar (wavy) form, e.g., microvilli, theoretically could yield a maximum twofold underestimate in D (Aizenbud and Gershon

1982). When two or more membranes are closely opposed, D is subject to small variations and the mobile fraction becomes an apparent value; see Peterson and MacConnaughey (1982) and Chazotte and Hackenbock (1991). Extra caution is called for if the membrane surface being measured is attached to an immobile substratum; e.g., polylysine-coated glass slide, as D and mobile fraction may show the immobilizing effect of attachment to the substratum. If diffusion in the membrane is occurring in a limited area instead of quasi infinite, then the D may be overestimated; see Elson and Qian (1989) for details.

- *Two- versus three-dimensional diffusion.* The equations used are for two-dimensional diffusion in a membrane. They may be qualitatively applied to three-dimensional diffusion in, for example, the cell cytoplasm. Without knowing the shape and dimensions of the bleached volume element in three dimensions, an accurate D and mobile fraction cannot be calculated, but relative Ds may be measured.
- *Diffusion versus flow.* Axelrod et al. (1976) have shown that in contrast to diffusion, flow is a sigmoidal recovery which changes the shapes from that of the pure diffusion recovery curve (Fig. 79.1).
- *Gamma values to be used in three-point fitting of* D *derived from the bleaching parameter.* (Based on Axelrod et al. 1976.)

K	γ
1	1.08
1.5	1.12
2.0	1.16
2.5	1.2
3.0	1.27
3.5	1.33
4.0	1.37
4.5	1.40
5.0	1.43
5.5	1.46
6.0	1.49
7.0	1.57
8.0	1.65
9.0	1.73
10	1.8
15	2.05
20	2.2
30	2.41

- Linearization approaches to fit the data were developed to minimize computation time and provide a good estimate of D and mobile fraction for single component diffusion (Yguerabide et al. 1982). These approaches have a number of limitations (Peterson et al. 1986; Gordon et al. 1995), one of which is their inability to extract the parameters for two diffusing components even though a departure from linearity would indicate two components.

Qualitative and Quantitative FRAP Using A Confocal Microscope

Two basic experimental approaches, one for qualitative and the other for quantitative FRAP using the confocal microscope, are described. In qualitative mode the whole cell (or structure of interest, i.e., nucleus, Golgi, etc.) is imaged before the bleach and during recovery at high resolution with a comparatively long time interval between the recovery images (i.e., ~10 sec). This allows monitoring of the recovery in areas within and around the bleached zone with good morphological resolution and the overall effect of the photobleach on the cell. Qualitative experiments are a good way to set up laser intensities needed for imaging and photobleaching and to control for potential damage to the cell. They also allow one to estimate the mobile fraction of molecules. In quantitative mode the whole cell is imaged only before the bleach and after the recovery is complete. The recovery itself is imaged only within the bleached area, usually a strip across the cell with a width of 2–4 μm. This allows rapid measurement of fluorescence intensities within the bleached area (i.e., every ~0.5 sec) and monitors the initial fast recovery process accurately. This early recovery phase is crucial for the determination of the diffusion constant *D*.

A general description is provided of the routines that each experiment should contain. Most confocal microscope operating software allows programming of the mentioned functions within a command macro (i.e., Zeiss LSM 410/510; Bio-Rad MRC series; Leica TCS 4D) albeit always in a specialized macro language. Program codes for the Zeiss LSM 410 using an Omnichrome Series 43 Kr/Ar ion laser can be obtained via internet at http://dir.nichd.nih.gov/CBMB/pb2labob.htm.

QUALITATIVE FRAP

After the cell of interest has been identified using the confocal microscope, prepare the command macro that automatically changes settings quickly for the photobleaching routine. The macro will record the image of the cell before photobleaching, execute the bleach, and record an image after the photobleach and during recovery. An example of a qualitative FRAP experiment is shown in Figure 79.3.

Command macro operations

1. Set laser power, attenuation, neutral density filters, zoom, and averaging to imaging intensity levels that allow a high-resolution image of the whole cell or structure of interest.

   This has to be determined experimentally for every specimen depending on specimen size, brightness of label, and optical parameters. The imaging radiation should be 300- to 1000-fold lower than the maximum laser power available for the photobleach to ensure that bleaching does not occur during the monitoring step. This means using low power, higher attenuation, low zoom, and minimal averaging for imaging.

2. Scan the whole cell or structure of interest at imaging laser intensity. Save the image.

3. Define the region of interest for the photobleach (bleach ROI) at the highest possible zoom.

A high zoom will increase the time the laser prevails on the bleached region per line scan and thus increase the bleaching radiation per area. If the desired bleach area is larger than the whole field scanned at maximum zoom, lower the zoom until it just accommodates the bleach ROI. Some microscope software allows one to do the ROI definition interactively in a dialog window. Alternatively, the bleach ROI can be defined before the start of the macro; then the coordinates in the macro command to define it must be changed every time. An easy way around this is to define a conveniently sized ROI close to the center of the screen, mark it on the monitor surface, and position the cell prior to the experiment so that the bleach ROI hits the structure you intend to bleach.

4. Set laser power to maximum and attenuation to zero (bleaching intensity).

5. Scan the bleach ROI at bleaching intensity and bleaching zoom until fluorescence is reduced to background levels if the specimen is subsequently imaged at imaging intensity. Do not save the image.

   Ideally the bleach should be instantaneous, that is, one scan of the ROI without averaging. If that is not sufficient to reduce fluorescence levels to background intensity (at imaging laser settings), averaging and numbers of scans have to be increased accordingly. The required bleach intensity can be calibrated in a fixed specimen to exclude recovery of fluorescence. If a complete photobleach takes longer than 20 seconds, a more powerful laser may be required for the system.

6. Switch the laser power, attenuation, zoom, and averaging back to imaging intensity settings.

7. Take a series of whole cell scans at imaging intensity immediately after the photobleach until the recovery is judged complete. Save each image.

   Due to the large imaged area and the necessary averaging for good resolution, images will be taken every 5–30 seconds. The time for full recovery depends on the diffusion constant of the probe and the size of the bleached area. A good range is 1–10 minutes.

Qualitative FRAP allows one to monitor the effects of photobleach and recovery on the whole cell or structure of interest. It also allows estimates of the mobile fraction of molecules by comparing the ratio of fluorescent intensities before photobleaching and after recovery for two regions of interest, one within the bleaching zone and the other outside of it. For many intracellular organelles, the absolute fluorescence associated with the organelle is frequently lower after photobleaching than before photobleaching because a significant proportion of total fluorescence is lost in the photobleach. The above method corrects this error. Qualitative FRAP does not allow easy and accurate determination of diffusion constants (but see Ellenberg et al. 1997; Subramanian and Meyer 1997; N. Sciaky et al., in prep.). To determine D (and mobile fractions) it is easier to execute a quantitative FRAP experiment.

QUANTITATIVE FRAP

In contrast to qualitative FRAP, a quantitative experiment is designed to collect fluorescence intensities within the bleached area with high temporal resolution to allow accurate determination of the diffusion constant D. It does not provide morphological information of the whole cell during recovery.

After the cell of interest has been identified using the confocal microscope, prepare the command macro, which automatically changes settings quickly for the photobleaching routine. The macro will record the image of the whole cell before

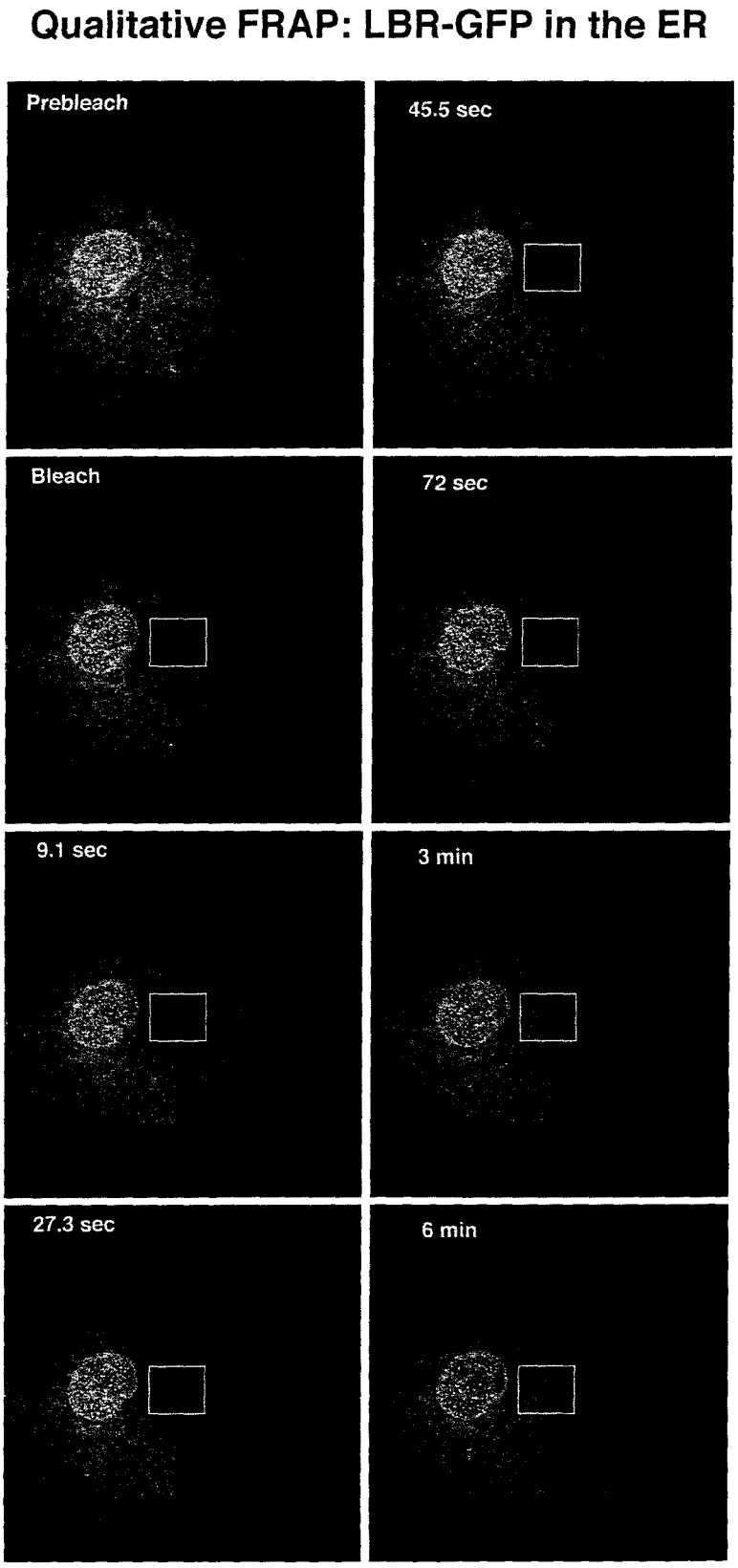


FIGURE 79.3
Example of qualitative FRAP experiment monitoring the diffusional mobility of GFP-tagged lamin B receptor in endoplasmic reticulum membranes. (Photo provided by J. Lippincott-Schwartz, National Institutes of Health.)

photobleaching, execute the bleach, record only the bleached area after the photobleach and during recovery, and record the whole cell after recovery.

An example of a quantitative FRAP experiment is shown in Figure 79.4.

Command macro operations

1. Set laser power, attenuation neutral density filters, zoom, and averaging to imaging intensity levels that allow a high-resolution image of the whole cell or structure of interest.

   This has to be determined for every specimen. See comments about qualitative FRAP.

2. Scan the whole cell or structure of interest at imaging laser intensity. Save the image.

   This image just provides a view of the overall prebleach morphology. Together with the image taken in step 9 after recovery is complete, it allows one to control for obvious photodamage and to calculate mobile fractions with the method described for qualitative FRAP.

3. Scan only the strip that is going to be bleached at imaging intensity and save the image.

   This measures the prebleach intensity in the bleach strip conveniently, although the information is contained in the first whole-cell image as well. To scan exactly the same ROI at imaging

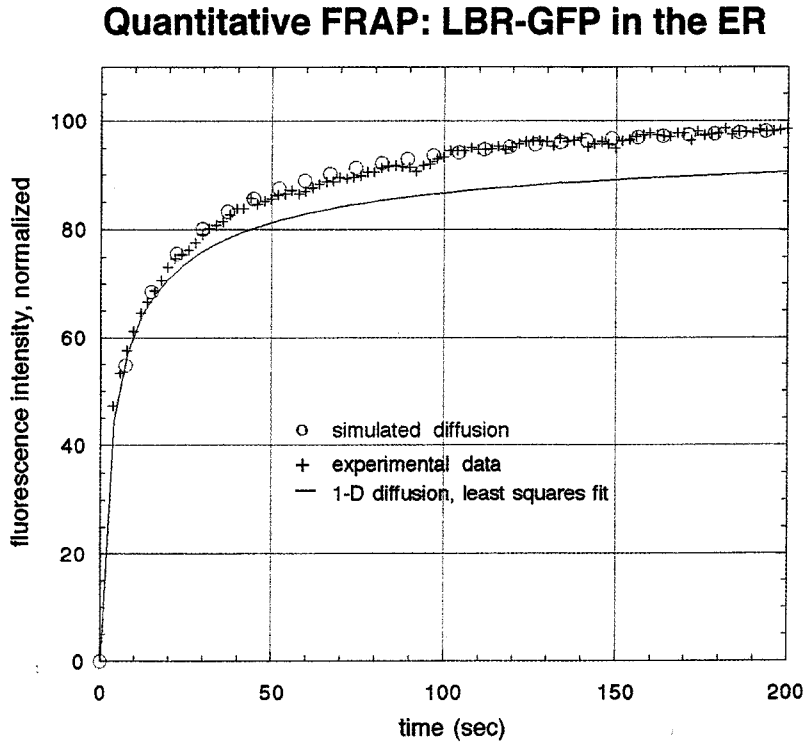


FIGURE 79.4
Example output from quantitative FRAP experiment that plots mean fluorescent intensities per area over time.

zoom that has been bleached at higher bleaching zoom, careful calibration in fixed material is necessary. Because this requires some time, it is useful to calibrate a number of different strips for the respective zooms, mark them on the monitor, and position the cell until the strip covers the desired area. The time for full recovery depends on the diffusion constant of the probe and the width of the bleached strip. A good range is 30 seconds to 5 minutes.

4. Define the region of interest for the photobleach (bleach ROI) at the highest possible zoom.

   For a quantitative experiment this should be a narrow strip of 2–4 μm all the way across the cell or the structure of interest. See also comments regarding zoom for qualitative FRAP.

5. Set laser power to maximum and attenuation to zero (bleaching intensity).

6. Scan the bleach ROI at bleaching intensity and zoom until fluorescence is reduced to background levels if specimen is imaged subsequently at imaging intensity. Do not save image.

   It is crucial that the cell is bleached all the way across and through its entire depth. The latter depends on the power and numerical aperture of the objective used. For structures with a significant depth, the bleach properties of each objective need to be calibrated in a fixed specimen. See also comments about qualitative FRAP.

7. Switch the laser power, attenuation, zoom, and averaging back to imaging intensity settings.

8. Take a series of scans of only the bleached ROI immediately after the photobleach at imaging intensity until the recovery is judged complete. Save each image in the series.

9. Scan the whole cell after recovery is complete. Save the image.

   This image allows the determination of the mobile fraction together with the image taken in step 2. It is also an important control for constant focus in the course of the experiment, obvious photodamage, and to assess overall loss of fluorescence due to the bleach. The latter should not exceed 20% and can be controlled by narrowing the width of the strip.

DETERMINATION OF DIFFUSION CONSTANT *D*

The main function of qualitative FRAP is to determine the diffusion constant D of the fluorescent probe. For this the images obtained within the bleached strip before the bleach and during recovery provide almost all the necessary information. The following steps for quantification and analysis can be automated as a batch program or command macro with most imaging programs (i.e., NIH Image, IP Lab Spectrum, Zeiss LSM software).

1. Background subtract all the images taken within the bleached strip.

2. Quantify the mean fluorescence intensity per area of the region within the bleached strip that contains fluorescent cellular material in the prebleached image.

Since the strip reaches all the way across the fluorescent material, it often encompasses nonfluorescent areas at the edges. To measure mean pixel value per area, the ROI can be narrowed down to the region that actually contained fluorescence before the bleach.

3. Correct the postbleach intensity for overall loss of fluorescence determined from total fluorescence in the whole-cell images taken at the beginning and end of the experiment.

   Overall loss of fluorescence is given by $(I_{\Sigma 0} - I_{\Sigma \infty})/I_{\Sigma 0}$ with $I_{\Sigma 0}$ = total cumulative pixel values in prebleach whole-cell image; $I_{\Sigma \infty}$ = total cumulative pixel values in postbleach whole-cell image. Due to this loss, postbleach fluorescence intensities in the strip can never reach 100% of the prebleach value and need to be corrected by multiplication with $I_{\Sigma 0}/I_{\Sigma \infty}$.

   If the cell has lost 10% of its overall intensity, complete recovery can never be 100% of the prebleach value; therefore, this needs to be normalized.

4. Plot the mean fluorescence intensities per area over time (see Fig. 79.4 for an example output).

5. Fit the experimental data to a formula for one-dimensional diffusion. A convenient empirical formula is:

$$I_{(t)} = I_{(\text{final})}(1 - (w^2(w^2 + 4\pi Dt)^{-1})^{1/2} \quad (8)$$

   where $I_{(t)}$ = fluorescence intensity as a function of time; zero of time t is taken as the midpoint of the bleach; $I_{(\text{final})}$ = final intensity reached after complete recovery; w = strip width, for example, 4 μm; and D = effective one-dimensional diffusion constant.

6. The diffusion constant D can be obtained directly from the above fit. The fitting can be performed by most spread sheet programs using a least-squares algorithm (i.e., Kaleidagraph, Abelbeck Software, Reading, Pennsylvania).

NOTES

- The above equation assumes one-dimensional recovery, since the membranes are bleached all across their length and entire depth. On average, the true motion occurs over a longer path than observed in a projected image of the cell and interpreted as one-dimensional diffusion. The real diffusion constant is therefore equal to or greater than the one extracted from the experiments. For some simple cases, this relation between the effective D in Equation 1 and the true D in convoluted membranes is given by Wey et al. (1981). D effective as measured with this method is generally underestimated with a maximum error of 1/3 (N. Sciaky et al., in prep.).
- To assess the effects of geometry, as well as the nonuniform fluorescence density in the cell, D calculated from Equation 1 can be checked against a numerical simulation that uses the prebleach intensity of the entire cell as input to simulate diffusive recovery into the bleached strip. A detailed description of such a simulation is beyond the scope of this chapter. The interested reader is referred to N. Sciaky et al. (in prep.).
- Mobile fractions can also be determined directly from the intensity plot instead of using the method described for qualitative FRAP. If the corrected prebleach in-

tensity is normalized to 100%, the final intensity reached in recovery is a direct measure for the mobile fraction (see Figs. 79.1 and 79.4).

FLUORESCENCE LOSS IN PHOTOBLEACHING USING A CONFOCAL MICROSCOPE

This technique provides a powerful means for examining the boundaries of a compartment, whether it be the endoplasmic reticulum, nucleus, or cytoplasm. FLIP measures the extent to which regions outside a photobleached box contribute to fluorescence recovery in a bleached site. Using this method, a region of interest is bleached repeatedly and fluorescence loss outside the region of interest is monitored over time. The extent to which areas outside the region of interest lose fluorescence over time describes the boundaries over which the fluorescent molecule is capable of diffusing.

Follow cell and microscope preparation described for FRAP using a confocal microscope.

Command macro operations

1. Set laser power, attenuation neutral density filters, zoom, and averaging to imaging intensity levels that allow a high-resolution image of the whole cell or structure of interest.

   This has to be determined for every specimen. See comments about qualitative FRAP.

2. Scan the whole cell (or structure of interest) at imaging laser intensity. Save the image.

3. Define the region of interest for the photobleach (bleach ROI) at the highest possible zoom.

   In contrast to FRAP, a FLIP experiment depends on depleting regions outside the bleached area of fluorescence. The bleach ROI should therefore cover a good portion (1/4–1/20) of the whole structure investigated. See also comments about qualitative FRAP.

4. Set laser power to maximum, attenuation to zero (bleaching intensity).

   See comments about qualitative FRAP.

5. Scan the bleach ROI at bleaching intensity and zoom for several times within a prolonged time period (i.e., 30 seconds). Do not save image.

   Bleaching for a long period will deplete not only the bleach ROI of fluorescence but also areas connected to it. To avoid excess photodamage the individual scans can be separated by pauses to allow exchange of fluorescent molecules into the bleached area that are then bleached in the next scan. Each individual scan should reduce fluorescence to background levels at imaging intensity settings within the shortest possible time, to optimize bleach length. See also comments about qualitative FRAP.

6. Switch the laser power, attenuation, zoom, and averaging back to imaging intensity settings.

7. Scan the whole cell (or structure) once at imaging intensity.

8. Save the image.

9. Repeat procedure from step 4.

   This loop should be executed until loss of fluorescence outside the photobleached area is no longer detected.

NOTES

Several controls should be performed to assess the FLIP results. These include FLIP of fixed cells to ensure fluorescence loss is not due to light leakage during FLIP or photobleaching during the monitoring step. It is also important to fix cells that have been FLIPed and immunolabel the structures that have been photobleached with antibodies. This is to confirm that structures inside the photobleached zone are not damaged by the repetitive bleaching episodes.

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