Scanning Microscopy

Volume 5 | Number 2 Article 7

2-17-1991

Fluorescence Recovery After Photobleaching Techniques to Measure Translational Mobility in Microscopic Samples

Ken Jacobson
Lineberger Cancer Research Center

Fen Zhang
Lineberger Cancer Research Center

Tsong-Tseh Tsay
University of North Carolina at Chapel Hill

Follow this and additional works at: https://digitalcommons.usu.edu/microscopy

 Part of the Biology Commons

Recommended Citation

Jacobson, Ken; Zhang, Fen; and Tsay, Tsong-Tseh (1991) "Fluorescence Recovery After Photobleaching Techniques to Measure Translational Mobility in Microscopic Samples," *Scanning Microscopy*: Vol. 5 : No. 2 , Article 7.
Available at: https://digitalcommons.usu.edu/microscopy/vol5/iss2/7

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Scanning Microscopy by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.

Scanning Microscopy, Vol. 5, No. 2, 1991 (Pages 357-362) 0891-7035/91$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING TECHNIQUES TO MEASURE TRANSLATIONAL MOBILITY IN MICROSCOPIC SAMPLES

Ken Jacobson,[1,2*] Fen Zhang[2] and Tsong-Tseh Tsay[1,3]

Laboratories for Cell Biology, [1]Department of Cell Biology and Anatomy,
[2]Lineberger Cancer Research Center and [3]Curriculum in Biomedical Engineering,
School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, N.C. 27599-7090

(Received for publication August 23, 1990, and in revised form February 17, 1991)

Abstract

The scope of photobleaching applications and the method itself are briefly reviewed. Two current applications in this laboratory are then outlined. First, the use of spatial Fourier transforms to analyze video photobleaching measurements is presented. This method extracts diffusion coefficients using all the image data and it does not require that the initial condition created by photobleaching be known. Second, the use of genetic engineering methods coupled with photobleaching analysis is discussed as means to uncover the structural determinants of membrane protein lateral mobility.

Key words: Photobleaching Methods, Fluorescence, Lateral Mobility, Biomembranes, Translational Diffusion.

*Address for correspondence:
Ken Jacobson
Department of Cell Biology and Anatomy
CB#7090, 108 Taylor Hall
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7090
Telephone Number: (919) 966-5703

Introduction

The fluorescence recovery after photobleaching (FRAP) technique was developed by biophysicists to measure the rate of translational motion of membrane components in the plane of model or biomembranes. This technique has also been named the fluorescence photobleaching recovery (FPR) method, or the fluorescence microphotolysis (FM) method. Scientists investigating the visual receptor membrane invented the photobleaching technique (Poo and Cone, 1974; Liebman and Entine, 1974) to measure the lateral diffusion of rhodopsin, using its optical absorption bands. Peters et al., (1974) performed similar measurements on the diffusion of fluorescein-labeled red cell membrane macromolecules, employing the fluorescence of the exogenous dye to detect the concentration of labeled components. This was the first fluorescence recovery after photobleaching (FRAP) measurement. Several groups subsequently engineered more sophisticated versions of the FRAP microscope (Jacobson et al., 1976; 1977; Koppel et al., 1976).

The uses of photobleaching technology are now widespread in cell and membrane biology. Apart from extensive measurements on artificial and plasma membranes (Peters, 1981; Jacobson et al., 1987), measurements have been made on intracellular organelle membranes such as the inner mitochondrial membrane (Hackenbrock et al, 1986), the Golgi (Cooper et al, 1990) and the nuclear membrane (Puddington et al, 1983). In addition, measurements on proteins and other molecules moving through the cytoplasm of living cells have assisted in our understanding of the cytoplasmic matrix (Jacobson and Wojcieszyn, 1984; Hou et al., 1990).

Photobleaching "marks" on fluorescent labelled membrane and cytoskeletal structures and following the movement of the marks has been invaluable in the study of various cell motility phenomena. In these cases circular marks, lines, or grids are bleached onto the specimen and transport of the mark is followed by low light level video technology before the mark is erased due to diffusion. In this way the dynamics of actin in microfilament bundles (Wang, 1985) and tubulin in micro-

tubules (Salmon et al., 1984) as well as the movement of the plasma membrane in locomoting cells has been investigated (Lee et al., 1990).

Photobleaching methods can be conveniently employed to measure molecular and macromolecular diffusion in solutions (Barisas and Leuther, 1979) and are also useful in studying dynamics in polymer films (Smith, 1982) and solutions (Wang et al., 1985).

Methods

The simplest version of the technique can be termed spot photobleaching. Briefly, this version of the method is based on photobleaching a small circular region on the surface of single cells bearing fluorescent molecules to destroy the emission from that region. Subsequently, the recovery of fluorescence due to the diffusion or flow of unbleached fluorophores from the surrounding area into the irradiated area is measured. When only isotropic lateral diffusion occurs, the recovery kinetics, characterized by the time, $t_{1/2}$, to obtain 50% of full recovery, are related to D, the diffusion coefficient by $D = w_s^2 \gamma/4t_{1/2}$, where w_s is the $1/e^2$ radius of the Gaussian profile laser beam used for both photobleaching and measuring fluorescence, and γ is a parameter that depends on the extent of photobleaching. The mobile fraction of the measured population of probe molecules is obtained from the degree to which the final fluorescence level approaches the pre-bleach fluorescence value. In this simple version, an ion laser is used to excite the fluorescence observed in a fluorescence microscope equipped for incident light excitation. The laser beam is focused to a waist on the secondary image plane of the objective by a weak biconvex lens and the objective further focuses the light to a small spot on the specimen (Jacobson et al, 1977). The $1/e^2$ spot diameter ($2\ w_s$) is a function of the objective power and can be adjusted, typically, from $\leq 1\ \mu m$ to as large as $50\ \mu m$.

A number of investigators have developed variations on the spot photobleaching techniques. Koppel (1979) introduced a multipoint analysis of the basic FRAP experiment by monitoring recovery at several points within the bleached and surrounding area. This approach allows diffusion and flow to be readily recognized.

Pattern photobleaching employs the bleaching of periodic patterns on the specimen (Smith and McConnell, 1978). The lateral transport of the observed fluorophores causes the gradual decay of these bleached patterns over time. The evaluation of this decay may be done in a very direct fashion by photographing the decaying pattern (via an image intensifier) and calculating the time constant for the decrease in the amplitude of the bleached pattern. If the bleached pattern consists of parallel stripes of constant width, scanning across this pattern immediately after bleaching will give a 'square wave' of defined period. The 'square wave' may be decomposed into a sum of sinusoidal functions (Fourier decomposition), of different spatial frequencies. A short time after bleaching (given by one tenth the product of the diffusion coefficient and the period of the bleached pattern), it is sufficient to follow only the decay of the first harmonic in the Fourier series. This amplitude decays as a simple exponential function, characterized by a time constant which contains the diffusion coefficient. Bleaching a two-dimensional pattern (a 'checkerboard' pattern) followed by a two- dimensional Fourier transform analysis of the decaying bleached patterns allows detection of anisotropic lateral motion, i.e. different diffusion coefficients along different directions in the plane (Smith et al., 1979). Patterns have usually been produced by inserting a Ronchi ruling in the path of the laser at the rear focal plane of the microscope, but they have also been generated by the interference of two coherent laser beams for fringe pattern photobleaching (Davoust et al., 1982). Also, Lanni and Ware (1982) demonstrated that scanning the Ronchi ruling across the path of the monitoring beam during the recovery phase could be used to improve the signal to noise ratio (S/N) of the measurement.

Imaging methods combined with photobleaching can yield detailed information on more complicated lateral transport processes. In spot photobleaching, fluorescence detection is normally done by a photomultiplier, so that unless the laser beam or stage is scanned, no spatial information on the recovery process can be obtained. In the "video-FRAP" technique, the photomultiplier is replaced by a very sensitive video camera, which is calibrated to operate as a time-resolved (30-ms-resolution) spatial photometer. Thus, spatial information on the fluorescence redistribution process is obtained.

Below, we outline two recent photobleaching applications from our laboratory, one illustrating a technical advance in the analysis of video-FRAP data and the other illustrating an important application of spot photobleaching to membrane biology.

Analysis of Video-FRAP Data Using Spatial Fourier Transforms

As pointed out above, in video-FRAP, a series of images is acquired of the spatial character of recovery using a low- light-level video camera which allows direct detection of anisotropic diffusion and flow (Kapitza et al, 1985). To utilize all of the available data to determine the transport coefficients, the two-dimensional Fourier transform is taken of the images after photobleaching (Jacobson et al., 1987). The change in the transform between two time points reflects the action of diffusion during the interim; in particular, the evolution of each spatial frequency is predicted by the diffusion equation. When the transforms at the two time points are divided, all information concerning the initial distribution of bleached fluorophores is removed, and the remaining information gives the diffusion coefficients directly. It has as been demonstrated (Tsay et al., 1990 and Tsay and Jacobson, submitted) that in computer simulated diffusional recovery after photobleaching that the

Table 1.A: Effect of Image Signal to Noise Ratio on Precision of Extracted Diffusion Coefficients by Fourier Transform Analysis[a].

Input Diffusion Coefficient (2.3×10^{-9} cm^2/sec)				
S/N	∞	30	15	7.5
D ($\times 10^9$ cm^2/sec)	2.29 ± 0	2.28 ± 0.26	2.28 ± 0.52	2.36 ± 1.26

[a] A computer simulation of a fluorescence recovery after photobleaching measurement. S/N varied by increasing Gaussian noise superimposed on a uniform signal level of 150 grey levels.

Table 1.B: Experimental Results Comparing Fourier Transform Analysis of Video FRAP Data to Conventional Spot Photobleaching.

	Diffusion Coefficient[a] ($\times 10^9$ cm^2/sec)		
Temperature (°C)	0	10	24
video FRAP	1.06 ± 0.19	1.90 ± 0.73	2.52 ± 0.36
spot FRAP	1.24 ± 0.14	1.86 ± 0.40	2.25 ± 0.32

[a] Measurements performed on a 0.96 μM solution of Rhodamine-succinyl Con A (Vector Labs, Burlingame, CA) in 99.5% glycerol at the indicated temperatures.

Fourier analysis of the simulated images will return the same diffusion coefficient as employed to produce the simulated recovery (Table 1.A). Furthermore, our work has shown that diffusion coefficients extracted from the Fourier analysis of video FRAP data agree with those obtained from conventional spot FRAP. This is shown for Con A-glycerol solutions (Table 1.B). One drawback of this analysis is its requirement for a large S/N in the image in order to obtain reasonable precision as shown in Table 1.A. (Note in Table 1 we have azimuthally averaged the results from similar spatial frequencies in transform space). Thus, this type of analysis may eventually find more use in studies of polymer films where fluorescent probe density can be made greater (up to the point of self quenching) in order to obtain brighter fluorescence images.

Application of FRAP to the Lateral Diffusion of Plasma Membrane Proteins

One of the major applications of FRAP technique is the measurement of the lateral mobility of protein and lipid components of the plasma membrane (Peters, 1981). Membrane protein lateral mobility is related to the protein's biological function (Axelrod, 1983; Chazotte and Hackenbrock, 1988), and also reveals the interactions of diffusants with both intra- and extramembrane components. The combination of molecular biology techniques to create mutant membrane proteins and FRAP analysis is being applied to understand these interactions.

It has been known for some time that many membrane proteins exhibit much lower lateral mobility in plasma membrane than in artificial membranes (Vaz et al., 1984; Jacobson et al., 1987). Thus far, several conclusions have been reached in regard to which domain of the membrane protein (ecto-, intramembrane, or cytoplasmic) is responsible for this reduced mobility. The ectodomain of certain single spanning membrane proteins [e.g., the vesicular stomatitis virus G glycoprotein (VSV G), Table 2] appears to play a dominant role in determining their lateral mobility presumably via interactions of the ectodomain with structures on the cell surface. These interactions lead to low lateral diffusion coefficients (1×10^{-10} cm^2/sec $\leq D \leq 5 \times 10^{-10}$ cm^2/sec). Alterations in the ectodomain of such proteins can affect their diffusion. For example, mutants of Type I major histocompatibility antigens with reduced or completely deleted glycosylation sites exhibited increased lateral mobility (Wier and Edidin, 1988) indicating that glycosylation in the ectodomain contributes to reducing lateral mobility in some cases. One further indication of the importance of the ectodomain is the fact that a chimeric mutant designated G-Thy, having the ectodomain from a slowly diffusing protein (i.e., VSV G) linked to the glycosylphosphatidylinositol (GPI) anchor from the fast

diffusing Thy-1 lipid-linked exhibits D values similar to VSV-G glycoprotein (see Table 2). Thus, the type of membrane anchor, be it a membrane spanning peptide with attached cytoplasmic domain or a glycolipid "tail", appears not to be a major determinant of the lateral mobility of this class of proteins. This is consistent with earlier findings that truncation of the cytoplasmic domains of VSV G (Scullion et al., 1987), the epidermal growth factor receptor (Livneh et al., 1986) and the major histocompatibility Type I antigens (Edidin and Zuniga, 1984) had little effect on their lateral mobility which also suggests that interactions of cell surface structures with other domains in the protein are crucial to restricting lateral diffusion.

Certainly, the cytoplasmic domain plays an important role in determining lateral mobility of some proteins. For example, the interaction of cytoplasmic domain of the major erythrocyte membrane protein, Band 3, with the cytoskeleton significantly reduces its lateral mobility (Golan and Veatch, 1980; Sheetz et al., 1980). The disruption of this interaction increases the Band 3 lateral diffusion coefficient more than 40 fold.

On the other hand, proteins which are normally GPI linked, such as placental alkaline phosphatase (PLAP), continue to exhibit D values more like their GPI linked "parent", even when their normal GPI anchor is substituted with the membrane spanning and cytoplasmic domain from VSV G (PLAP-G, see Table 2). In this case, the lateral diffusion coefficient appears to be determined by the movement of the membrane anchor through the plasma membrane bilayer. Thus, factors which affect "membrane fluidity" (lipid composition and phase structure, membrane protein concentration, etc.) will modulate the lateral mobility of this type of membrane protein.

Although the picture is not yet complete, we believe that data such as this will begin to give us a more detailed understanding of the dynamic structure of the plasma membrane and the rules which govern lateral mobility of its constituents.

Acknowledgements

This work was supported by NIH GM 41402.

References

Axelrod D. (1983) Lateral Motion of Membrane Proteins and Biological Function. J. Membr. Biol. 75:1-10.

Barisas BG, Leuther MD. (1979) Fluorescence Photobleaching Recovery Measurement of Protein Absolute Diffusion Constants. Biophys. Chem. 10:221-229.

Chazotte B, Hackenbrock C. (1988) The Multicollisional, Obstructed, Long-Range Diffusional Nature of Mitochondrial Electron Transport. J. Biol. Chem. 263: 14359-14367.

Cooper MS, Cornell-Bell A, Chernjavsky A, Dani J, Smith SJ. (1990) Tubulovesicular Processes Emerge from Trans-Golgi Cisternae, Extend Along Microtubules and Interlink Adjacent Trans-Golgi Elements into the Reticulum. Cell 61:135-145.

Davoust J, Devaux P, Leger L. (1982) Fringe Pattern Photobleaching, A New Method for the Measurement of Transport-Coefficients of Biological Macromolecules. EMBO J. 1:1233-1238.

Edidin M, Zuniga M. (1984) Lateral Diffusion of Wild-type and Mutant L^d Antigens in L cells. J. Cell Biol. 99:2333-2335.

Golan D, Veatch W. (1980) Lateral Mobility of Band 3 in the Human Erythrocyte Membrane Studied by Fluorescence Photobleaching Recovery: Evidence for Control by Cytoskeletal Interactions. Proc. Natl. Acad. Sci. 77:2537-2541.

Hackenbrock CR, Chazotte B, Gupte SS. (1986) The Random Collision Model and a Critical Assessment of Diffusion and Collision in Mitochondrial Electron Transport. J. Bioenergetics Biomemb. 18:331-368.

Hou L, Lanni F, Luby-Phelps K. (1990) Tracer Diffusion in F-Actin and Ficoll mixtures; Toward a Model for Cytoplasm. Biophys. J. 58:31-43.

Jacobson K, Wu E-S, Poste G. (1976) Measurement of the Translational Mobility of Con-Canavalin A in Glycerol-Saline Solutions and on the Cell Surface by Fluorescence Recovery After Photobleaching. Biochim. Biophys. Acta 433:215-222.

Jacobson K, Derzko Z, Wu E-S, Hou Y, Poste G. (1977) Measurement of the Lateral Mobility of Cell Surface Components in Single, Living Cells by Fluorescence Recovery After Photobleaching. J. Supramol. Struct. 5:565-576.

Jacobson K, Wojeceszyn J. (1984) The Translational Mobility of Substances within the Cytoplasmic Matrix. Proc. Natl. Acad. Sci. USA 81:6747-6751.

Jacobson K, Ishihara A, Inman R. (1987) Lateral Diffusion of Proteins in Membranes. Ann. Rev. Physiol. 49:163-175.

Kapitza H, McGregor G, Jacobson K. (1985) Direct Measurement of Lateral Transport in Membranes by Using Time-Resolved Spatial Photometry. Proc. Natl. Acad. Sci. USA. 82:4122-4126.

Koppel D, Axelrod D, Schlessinger J, Elson E, Webb W. (1976) Dynamics of Fluorescence Marker Concentration as a Probe of Mobility. Biophys. J. 16:1315-1329.

Lanni F, Ware BR. (1982) Modulation Detection of Fluorescence Photobleaching Recovery. Rev. Sci. Instrum. 53:905-908.

Lee J, Gustafsson M, Magnusson K-E, Jacobson K. (1990) The Direction of Membrane Lipid Flow Locomoting Polymorphonuclear Leucocytes. Science 247:1229-1233.

Liebman PA, Entine G. (1974) Lateral Diffusion of Visual Pigment in Photoreceptor Disk Membranes. Science 185:457-459.

Livneh E, Benveniste M, Prywes R, Felder S, Kam Z, Schlessinger J. (1986) Large Deletions in the Cytoplasmic Kinase Domain of the Epidermal Growth

Table 2: Lateral Mobility of Transmembrane and GPI linked Proteins and their Chimeras Expressed on the Surface of Transfected COS cells[a].

	PROTEINS[b]				
	VSV G	Thy-1	G-Thy	PLAP	PLAPG
Lateral diffusion coefficient ($X\ 10^{10}$ cm^2/sec)	3.8 ± 2.1	27 ± 10	5.9 ± 2.1	24 ± 6	12 ± 4

[a] from Zhang F, Crise B, Su B, Hou Y, Rose JK, Bothwell A, Jacobson K. Lateral Diffusion of Membrane Spanning and Glycosylphosphatidylinositol Linked Proteins: Toward Establishing Rules Governing the Lateral Mobility of Membrane Proteins, submitted to J. Cell Biol.

[b] Explanation of protein designations: VSV G, vesicular stomatitis virus G glycoprotein; PLAP, human placental alkaline phosphatase; G-Thy, chimeric construct with VSV G ectodomain and Thy-1 GPI linked moiety; PLAP-G, chimeric construct with PLAP ectodomain and VSV G transmembrane and cytoplasmic domains.

Factor Receptor do not Affect its Lateral Mobility. J. Cell. Biol. 103:327-331.

Peters R, Peters J, Tews K, Bahr W. (1974) A Microfluorimetric Study of Translational Diffusion in Erythrocyte Membranes. Biochim. Biophys. Acta 367: 282-294.

Peters R. (1981) Translational Diffusion in the Plasma Membrane of Single Cells as Studied by Fluorescence Microphotolysis. Cell Biol. Int. Repts. 5:733-760.

Poo MM, Cone R. (1974) Lateral Diffusion of Rhodopsin in the Photo-Receptor Membrane. Nature 247:438-441.

Puddington L, Lyles D, Jacobson K, Parce W. (1983) Lateral Diffusion of the Vesicular Stomatitis Virus Glycoprotein is Significantly Slower in the Nuclear Membrane of Infected Cells than it is in the Plasma Membrane. Biophys. J. 41:203a. (Abstract).

Salmon ED, Leslie RJ, Saxton WM, Karow ML, McIntosh JR. (1984) Rapid Rate of Tubulin Dislocation from Microtubules in the Mitotic Spindle in vivo Measured by Blocking Polymerization with Colchicine. J. Cell Biol. 105:69-75.

Scullion BF, Hou Y, Puddington L, Rose JK, Jacobson K. (1987) Effects of Mutations in Three Domains of the Vesicular Stomatitis Viral Glycoprotein on its Lateral Diffusion in the Plasma Membrane. J. Cell Biol. 105:69-75.

Sheetz M, Schindler M, Koppel D. (1980) Lateral Mobility of Integral Membrane Proteins is Increased in Sphenocytic Erythrocytes. Nature 285:510-512.

Smith B, McConnell H. (1978) Determination of Molecular Motion in Membranes Using Periodic Pattern Photobleaching. Proc. Acad. Sci. USA 75:2759-2763.

Smith B, Clark W, McConnell H. (1979) Anisotropic Molecular Motion on Cell Surfaces. Proc. Natl. Acad. Sci. USA. 66:5641-5644.

Smith B. (1982) Measurement of Diffusion in Polymer Films by Fluorescence Redistribution After Pattern Photobleaching. Macromolecules 15:469-472.

Tsay T-T, Inman RT, Jacobson K. (1990) Fourier Transform Image Processing to Extract Transport Coefficients from Video FRAP Measurements. Biophys. J. 57:376a (Abstract).

Vaz W, Goodsaid-Zaluondo F, Jacobson K. (1984) Lateral Diffusion of Lipids and Proteins in Bilayer Membranes. FEBS LETT. 174:199-207.

Wang Y-L. (1985) Exchange of Actin Subunits at the Leading Edge of Living Fibroblasts: Possible Role of Treadmilling. J. Cell Biol. 101:597-602.

Wier M, Edidin M. (1988) Constraint of the Translational Diffusion of a Membrane Glycoprotein by its External Domains. Science 242:412-414.

Discussion with Reviewers

K. Luby Phelps: At what S/N was the comparison in Table 1.B performed?
Reviewer III: The quantity S/N is not clearly defined in the paper. There is no mention about the origin of the noises. Why is S/N superimposed on a uniform signal of 150 grey level, when the signal level varies during the recovery?
Authors: The issue of signal to noise is more complicated and these issues are discussed in a submission to the Biophysical Journal.

K. Luby Phelps: Are there functional reasons why different transmembrane proteins employ different modes of controlling their lateral mobility?
Authors: Probably there are but they are not well understood at this juncture.

Reviewer III: The authors correctly pointed out that the major advantage of video-FRAP is the capability to

detect anisotropic diffusions and flows. Nevertheless, their analysis presented was performed on isotropic systems. In isotropic systems the mathematical approach employed in video-FRAP analysis is identical to that used by Axelrod et al. for a spot-FRAP (Biophys. J), except in the latter case the diffusion coefficient is determined from the time dependence of the integrated intensity. Thus the good agreement of D values obtained by two FRAP methods is expected. Perhaps, the authors can provide a quantitative comparison (for instance, from the S/N points-of-view) of the accuracies between two frap methods under the same excitation and bleaching conditions.

Authors: Of course, the point was to provide a check on the new analysis by comparing it to the results obtained by a known analysis in the spatial domain. As mentioned above, the S/N issues are more complex and treated in a submission to the Biophysical Journal.