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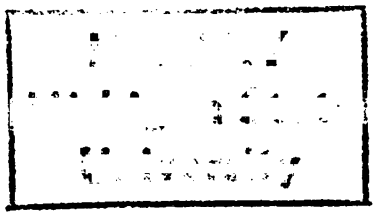

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DEVELOPMENT OF LASER PHOTOBLEACHING SYSTEM
FOR USE WITH UNIQUE MICROSCOPY TECHNIQUES

presented by

Mark N. Melkerson

has been accepted towards fulfillment
of the requirements for

Masters of Science degree in Mechanical Engineering

John F. McDonald
Major professor

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DEVELOPMENT OF LASER PHOTOBLEACHING SYSTEM FOR USE WITH UNIQUE MICROSCOPY TECHNIQUES

by

Mark N. Melkerson

A Thesis

Submitted to
Michigan State University
in Partial Fulfillment of the Requirements
for the Degree of

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1986

427-636X

ABSTRACT

DEVELOPMENT OF LASER PHOTOBLEACHING SYSTEM FOR USE WITH UNIQUE MICROSCOPY TECHNIQUES

By

Mark N. Melkerson

An experimental laser photobleaching system has been developed. The system is functional and preliminary results have been obtained. The laser system was applied to four microscopy techniques. Two of the techniques were designed to select single bilayer vesicles "in situ" prior to their use in the quantitative determination of membrane characteristics. The developed fluorescence contrast technique and comparison technique for selection of single bilayer vesicles were unsuccessful. The other two techniques were applications of fluorescence recovery after photobleaching (FRAP) for the determination of lateral diffusion coefficients of fluorescently labeled molecules present in membranes. The spot technique predicted lateral diffusion coefficients for rhodamine (4.6×10^{-6}M) in water ranging from (0.2 to 1.36×10^{-6}) cm^2/sec. These lateral diffusion coefficients agree in magnitude with published values (1.2×10^{-6} cm^2/sec). The multi-point technique was unsuccessful in predicting lateral diffusion coefficients for rhodamine (4.6×10^{-6}M) in water.

Dedicated to my Dad, my Mom, and my Uncle Walt

ACKNOWLEDGMENTS

I would like to express my appreciation to the following people whose help was invaluable in the completion of my thesis: Leonard J. Eisele -- for his help with the design and machining of the system fixtures; Ronald K. Kraus -- for his help with the system instrumentation; Robert E. Rose -- for his help with the heat exchanger and the electrical work; Ronald L. Haas -- for his help with the laser; John F. Holland -- for his help with the laser system design and software; Melvin S. Schindler -- for his help with fluorescence recovery theory and application; Carol J. Bishop -- for her help with the word processing of the thesis; Gerna J. Rubenstein -- for her help with the editing of the thesis; Lyla J. Melkerson-Watson -- for her help with the photography; and John J. McGrath -- for his support and advice.

I would especially like to thank Serge Weiss for his work with the quantization applications of the laser photobleaching system.

One last note of thanks goes to Meridian Instruments Incorporated, Okemos, Michigan for their help with the laser system design and controlling software.

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NOMENCLATURE

A	-attenuation factor with respect to total output power
C_0	-initial concentration of fluorescently labeled molecules
C_K	-concentration of fluorescently labeled molecules
D	-lateral diffusion coefficient
$F_K(t)$	-fluorescence intensity
$f_K(t)$	-normalized fluorescence intensity
$I(r)$	-bleaching intensity
K	-amount of bleaching
M	-immobile fraction
m	-superscript denoting a measured value
n	-summation index
P_n	-Legendre Polynomial of order n
P_o	-total laser power
q	-quantum efficiency (absorption*emission*detection)
r	-position
T	-bleaching time
t	-time
V_o	-bulk flow velocity
w	-effective beam diameter (e^{-2} height, 86%)
α	-extent of bleaching ($0 < \alpha < 1$)
$\partial/\partial x$	-partial derivative with respect to x
γ_i	-ration of $\tau_{1/2}/\tau_i$ where i = D,F
ρ	-effective beam radius (e^{-2} height, 86%)
τ_D	-characteristic time for diffusion
τ_F	-characteristic time for flow

1.0 Introduction

1.1 Motivation

The development and research described in this thesis were conducted at the Bioengineering Transport Processes (BTP) laboratory of Michigan State University. The project was to adapt and develop the experimental instrumentation needed to to study membrane changes related to freezing injury and to determine the number of molecular layers present in the membranes of models of biological cells. This development and research were part of a study relating the transport properties of cell membranes to model cell membranes during freezing and thawing.

The number of molecular layers and changes in membranes affect the behavior of biological cells and models during freezing. As water in the environment surrounding a cell freezes solutes separate from the crystal structure of the ice. The increase in ice surrounding the cells causes the solutes to become more concentrated in the remaining liquid. The increased solute concentration in the "local" environment causes the cell to attempt to regain an equilibrium between the chemical potential inside and outside of the cell. The dynamic response for such a situation is governed by the permeability characteristics of the cell or cell models.

The permeability of a membrane to water depends upon the amount of resistance encountered in crossing the membrane during freezing or thawing. An increase in the number molecular layers present in a membrane decreases its permeability to water across the membrane due to the increased resistance from each layer (42). Changes in mobility of

molecules or lesions in membranes also affect permeability. Leakage eliminates the resistance to crossing the membrane and quickly restores equilibrium. Effects on permeability of water across membranes, hence freezing and thawing of cells, due to changes in mobility of molecules from changes of phase in membranes described in (47).

From these effects on permeability of membranes it is apparent that the incorporation of this additional information on the number of molecular layers and molecular mobility into models of freezing and thawing would better estimate the actual processes.

1.2 Background

The models used in this study are liposomes and bilayer lipid membranes (BLMs). Liposomes and BLMs are comprised of a double layer (bilayer) of phospholipids. (See Figure 1.1.) Phospholipids form these bilayers because of their hydrophobic "tail" portions of the molecule and hydrophilic "head" groups of the molecule. (See Figure 1.1.)

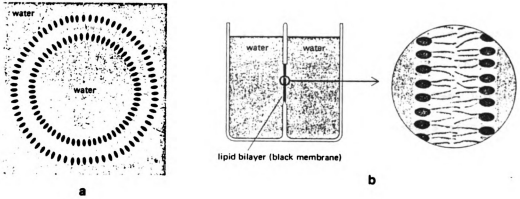

Fig. 1.1 Membrane structure of a) a liposome b) a BLM, reproduced from (46).

There are many techniques for determining the number of bilayers and the lateral diffusion coefficients in the phospholipid membranes of liposomes. The determination of the number of bilayers (lamellea) in liposomes is usually accomplished by qualitative means. The most widely used technique is visual. It uses phase contrast microscopy to choose liposomes with the faintest (least visible) membranes. The results from this visual technique for choosing liposomes varies greatly depending on the observer, the light intensity used, and the magnification used.

Liposomes and BLMs can be considered reasonable models for the study of membrane characteristics in biological cells for three reasons. First, phospholipids comprise 50 to 75 percent of cell membranes. Secondly, liposomes are generally spherical and trap solvents within themselves much like a biological cell. Finally the phospholipids making up liposomes and BLMs are extracted from biological materials such as egg yolk.

One quantitative technique for determining the number of bilayers in liposomes was developed by Servuss and Boroske (27,38). It also uses phase contrast. It eliminates the subjective "visual selection" but still deals with the contrast intensity of the membrane. The contrast intensity of liposomes is measured by a photometer system, normalized and then plotted versus the liposome radius. The results of their work is shown in Figure 1.2. Servuss and Boroske proposed that each grouping was a different number of lamellea (bilayers)in liposomes. The grouping with the lowest normalized define the unilamellar liposomes. This technique provided some indication that quantization of the number

of bilayers was possible. But to determine the actual number of bilayers present in any liposome the differences in the normalized intensities need to be more distinct.

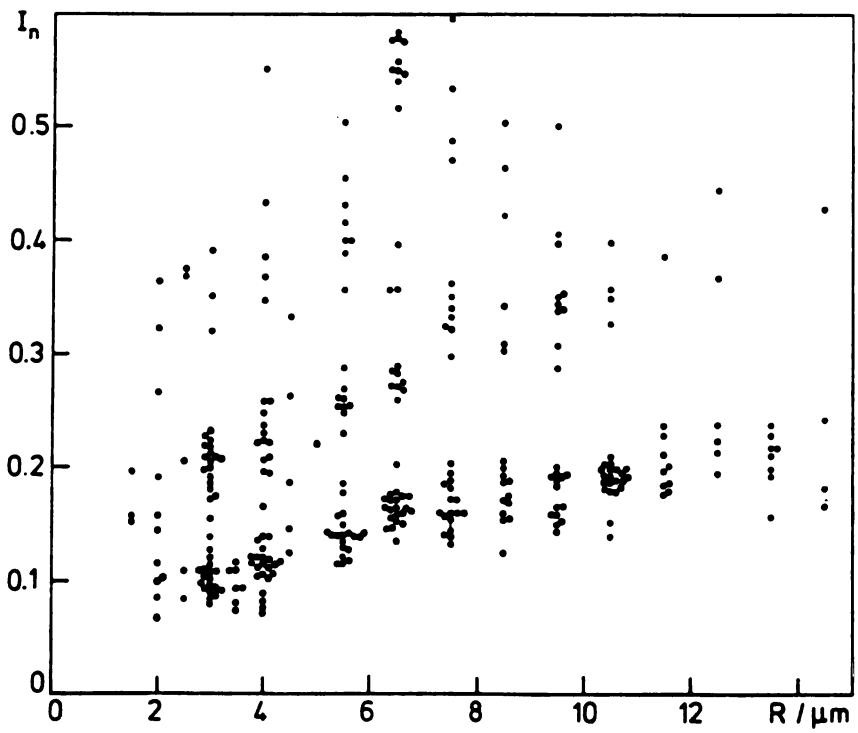

Fig. 1.2 Distribution of normalized phase contrast intensities versus radius for liposomes reproduced from (27).

Indications that a fluorescence technique for determining the number of lamellea in liposomes could be possible came from observations of others. Chan and Webb (28) used fluorescently labeled phosolipid multilayers to measure increased emission intensities for increasing numbers of lipid layers. Tamm and McConnell (35) have determined that fluorescent intensity doubles when a second fluorescently labeled

monolayer is applied to a single fluorescently labeled monolayer. Fahey and Webb (6) stated that the number of bilayers in an unilamellar fluorescently labeled liposome chosen by visual techniques could be determined by comparing it to a similarly labeled BLM. These observations suggested that a fluorescence method to measure the number of bilayers in liposome membranes could be developed.

Among the fluorescence methods for studying membrane characteristics there are two which determine lateral diffusion coefficients: Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Recovery After Photobleaching (FRAP)(1). The FCS method is based on a statistical determination of the motion of fluorescently labeled molecules in membranes. This method was rejected for this study because the associated analysis requires an extensive background in statistical probability. The FRAP method is based on the fluorescence intensity recovery due to the diffusion of fluorescently labeled molecules in a membrane. This method was chosen because the associated analysis is based on the diffusion equation.

The FRAP method for determining the lateral diffusion coefficients of molecules can be outlined as follows (1). Fluorescently labeled samples with a low molecular concentration of fluorescent probe are observed on a microscope. A focused low intensity beam (at the proper wavelength) is used to excite the fluorescing material to a higher energy level. The fluorescent material emits energy at a longer wavelength as it returns to its ground energy level . This emitted energy is detected by a photometer and recorded. This initial intensity can be related to the initial concentration of fluorescently labeled molecules in the sample excited by the focused beam. The fluorescent

sample is then excited by a brief high energy pulse at 1000 - 10000 times greater than the observing intensity excitation. This pulse eliminates the ability of the probe to emit energy. Unable to emit energy the spot under the focused beam appears black. This condition of blackness is called "photobleaching". The black region emits almost no energy initially. It begins to emit energy as non-bleached fluorescent molecules diffuse into the region under the focused beam at the initial observing intensity. The affected fluorescent molecules diffuse out of the region at the same time. This recovery of the fluorescence emission intensity is monitored and recorded. Ideally the fluorescence intensity should recover to its initial level. The lateral diffusion coefficients of molecules can be determined from these fluorescence recoveries.

Lateral diffusion coefficients can be determined by using any of the four general FRAP techniques. These FRAP techniques are called the spot, multi-point, normal-mode, and periodic pattern techniques. The spot technique (1) is the earliest of the four. This technique is workable but involves some complex mathematical analysis due to the possibility of bulk motion of the sample. Bulk motion introduces a non-homogeneous term involving the velocity of the sample to the diffusion equation. Many researchers have avoided this problem by affixing the cells in place or using sealed samples of fluorescent solutions. This leaves the velocity term zero. The zero velocity term reduces the problem to just a simple diffusion equation for the concentration of fluorescently labeled molecules. This simplification does not work for systems where the velocity term was not zero.

The other three FRAP techniques (multi-point, normal-mode, and periodic pattern) are attempts to account for the bulk motion possibility. The multi-point technique (2) accounts for the bulk motion possibility by monitoring the fluorescent recovery at a number points including the bleached spot. If the membrane does move, the shift in intensity can be detected while still tracking the recovery. The normal-mode FRAP technique (44) monitors the fluorescent intensity along a line which passes through the entire cell or liposome. In this technique a spot on the very edge of the membrane, where the line of excitation is normal to the surface, is bleached. The recovery of points along this line are monitored and any bulk motion can be accounted for. In the periodic pattern technique (15) many lines are bleached. The recovery of these lines across the plane of the sample are monitored to account for the possibility of bulk motion.

1.3 Objectives

There were three main objectives for the study described in this thesis: a) produce an operational laser photobleaching system operational, b) verify some photobleaching techniques, and c) develop fluorescence technique for the quantization of the number of bilayers in liposomes.

Producing an operational laser photobleaching system operational required a decision on which FRAP technique to use. The multi-point FRAP technique has been selected as the system model for two reasons. It allows for the determination of lateral diffusion coefficients with the existence of bulk flow of the sample. 2) It can also perform the spot FRAP technique with only minor software modifications. Other

factors con-tributing to the decision were: a) the microscope used by Koppel (2) was identical to the one in BTP laboratory, b) a system using the normal-mode FRAP technique existed at MSU (45), and c) budget limitations.

Meeting this first objective required: a) gaining experience on an existing FRAP laser system (48), b) ordering all required equipment, c) writing necessary software, and d) interfacing the equipment and software.

The objective of verifying a photobleaching technique was to show that a laser photobleaching system could perform the determination of lateral diffusion coefficients using the spot and multi-point FRAP techniques. Verification beyond establishing that the system was functional would be left to others.

The last major objective of this study was to develop fluorescence microscopy techniques to determine the number of bilayers in liposomes. This determination was to include investigating: a) the phase contrast technique used by Servuss and Boroske (27,38), b) designing a "fluorescence contrast" technique and c) designing a technique for a fluorescence comparison of liposomes to a known phospholipid single bilayer.

These three major objectives outlined the initial goals of this study. As in most developmental and experimental work some modifications of these goals were made. The remainder of this study presents an analysis of FRAP theory, a description of the laser photobleaching system and applications, results, conclusion, and suggestions for future work.

2.0 Analysis

In this section the theoretical and experiment bases for the Fluorescence Recovery After Photobleaching (FRAP) microscopy technique are presented. The majority of the content of this presentation is extracted from a detailed analysis by Axelrod, et al. (1). Their analysis has been used by many researchers using FRAP systems. (2,9 , 18,19). Also the presentation includes the solution of the diffusion equation required by the FRAP technique used in this study.

2.1 Theoretical

FRAP theory is based on the lateral transport (diffusion and/or convective flow) of bleached and non-bleached fluorescently labeled molecules into and out of a defined region of a membrane. Fluorescently labeled molecules allow the researcher a convenient method for measuring the concentration at a given position and time. Fluorescence and concentration of a spherical membrane are related by the following relationship (1)

$$F_K(t) = (q/A) \int I(r)\, C_K(r,t)\, d^2r \qquad (2.1)$$

The equation is valid for time $t \geq 0$, with zero denoting time just after bleaching. The term q is the product of all the quantum efficiencies of light absorption, emission, and detection ($0 < q < 1$). The attenuation factor of the beam during observation with respect to the total intensity is represented by the term A. The bleaching intensity is denoted by I(r). (1,5,10). The concentration $C_K(r,t)$ of fluorescently labeled

molecules at position r and time t is the solution to an equation describing the lateral transport of fluorescently labeled molecules. The subscript K denotes a dependence on the amount of bleaching, $K = \alpha I(0)T$. Here α is a constant characterizing the extent of bleaching ($0<\alpha<1$) and T is bleaching time. Integrating the product of the bleaching intensity and concentration over a defined region gives the total possible fluorescence. The total fluorescence multiplied by the quantum efficiency and attenuation factor gives the predicted fluorescence at time, t, and location, r, of a spherical membrane.

The application of the fluorescence equation 2.1 requires an expression for both bleaching intensity and concentration. The bleaching intensity I(r) for properly aligned lasers has been shown to have a Gaussian profile (1,5,10). An expression for the bleaching intensity is given by

$$I(r) = \frac{2P_o}{\pi w^2} \exp\left(-2r^2/w^2\right) \qquad (2.2)$$

Here w, the effective laser beam size, is the half width containing 86.5% of the beam (e^{-2} height). P_o is the total laser power. (1,5). The first group of terms represents the total power applied per area. The last term describes the exponential nature of the intensity with increasing distance ($r \geq w$) from beam application position.

Obtaining an expression for the concentration C(r,t) involves solving an equation describing the lateral transport of a single type of fluorophore. This is expressed by the equation

$$\frac{\partial C}{\partial t} = D\nabla^2 C - V_o \frac{\partial C}{\partial x} \qquad (2.3)$$

D is the lateral diffusion coefficient, and V_o is the uniform flow velocity in the x direction. (1). The boundary condition imposed on this equation is that the concentration at large distance from the bleached area but still on the membrane is finite, $C(\infty,t) = C_o$, where C_o is initial concentration of fluorescently labeled molecules. The initial condition depends on the assumption that photobleaching a fluorophore to a nonfluorescent one is an irreversible first-order reaction with rate constant $\alpha I(r)$ (1/sec) with no chemical regeneration. (1,6,11). The initial condition is given by

$$C(r,o) = C_o \exp[-\alpha\, TI(r)] \qquad (2.4)$$

In this equation α is a constant characterizing the extent of bleaching and T is bleaching interval (1).

Methods for solving the lateral transport problem vary greatly among researchers. (1,12,13,14). Many researchers other than Axelrod, et al. only solve the concentration equation for the simplified case where the flow is assumed to be zero. (12,13,15). The differential equation remaining after the assumption $V_o = 0$ is simply the diffusion equation:

$$\frac{\partial C}{\partial t} = D\nabla^2 C \qquad (2.5)$$

Since the case $V_o = 0$ is also a solution presented by Axelrod, et al. the equation (2.5) was solved here as a verification of their analysis. The diffusion equation is solved for a spherical coordinate system using separation of variables. The solution is presented in terms of Legendre polynomials. The solution obtained for the diffusion equation is the following:

$$\frac{C}{C_o} = 1/2(1 + \cos\theta_o) + \sum_{n=1}^{\infty} \frac{(2n + 1)}{2(n + 1)} [\cos\theta_o P_n(\cos\theta_o)$$

$$- P_{n-1}(\cos\theta_o)] \; P_n(\cos\theta) e^{-n(n+1)(D/\rho^2)t} \qquad (2.6)$$

Here (ρ^2/D) is the characteristic time for diffusion. The term θ_o describes the circular bleached region and ρ is the membrane radius. The expressions P_n are Legendre polynomials of degree n. For mathematical expressions for these polynomials see (16,17). A detailed analysis of the solution of the lateral transport equation for the case of diffusion only can be found in Appendix D. Identical solutions of the diffusion equation can be found in many references (12,13,14).

Expressions obtained for bleaching intensity and concentration allow for the calculation of the theoretical fluorescence. Again this presentation refers to the solutions presented by Axelrod as do most other researchers working with FRAP systems (2-4,6-9,18,19).

Axelrod presents solutions of the fluorescence equation 2.1 for three possible modes of transport. These modes of transport are diffusion only with $V_o = 0$, flow only with $D = 0$, and combined diffusion and flow. In all cases the solution assumes that the beam has a Gaussian profile. Only the solutions for each mode are presented. For derivations see reference (1).

Diffusion Only ($V_o=0$)

The fluorescence is given by

$$F_K(t) = (q\ P_o C_o/A) \sum_{n=0}^{\infty} (-K)^n/n!]\ [1 + n(1 + 2t/\tau_D)]^{-1} \qquad (2.7)$$

In the equation $\tau_D = w^2/4D$ is the characteristic diffusion time, and $K = \alpha I(0)T$ is the amount of bleaching (a non-dimensional energy density).

Uniform Flow Only (D=0)

The expression for fluorescence in this case is the following:

$$F_K(t) = (q\ P_o C_o/A) \sum_{n=0}^{\infty} (-K)^n/(n+1)!\ \exp[-2n/(n+1)](t/\tau_F)^2 \qquad (2.8)$$

Here, $\tau_F = w/V_o$ is the characteristic time for uniform flow.

Combined flow and diffusion

The fluorescence is shown to be expressed as:

$$F_K(t) = (q\ P_o C_o/A) \sum_{n=0}^{\infty} \frac{(-K)^n \exp\{-2\,(t/\tau_F)^2\, n/[1 + n\,(1 + 2t/\tau_D)]}{n!\ [1 + n(1 + 2t/\tau_D)]} \qquad (2.9)$$

where $\tau_F = w/V_o$ and $\tau_o = w^2/4D$. In all cases $qP_o(C_o/A \equiv F_K t)$ describes the fluorescence before bleaching.

In all three cases it is difficult to use or plot typical results for the fluorescence equation 2.1 without introducing a method to normalize them. A convenient way to normalize the expressions for fluorescence is to use the following

$$f_K(t) = \frac{F_K(t) - F_K(0)}{F_K(\infty) - F_K(0)} \qquad (2.10)$$

Here, $F_K(0)$ is the fluorescence immediately after bleaching and $F_K(\infty)$ is the fluorescence at very long times after bleaching (1). The term for fluorescence immediately after bleaching is expressed by

$$F_K(0) = (q P_o C_o / A) \bar{K}^{-1} (1 - e^{-K}) \qquad (2.11)$$

also from (1). The fluorescence at long times approaches the fluorescence before bleaching and is approximated by the following

$$F_K(\infty) \tilde{=} F_K(-) = q P_o C_o / A \qquad (2.12)$$

In both $F_K(0)$ and $F_K(\infty)$ the beam profile is Gaussian (1). Typical plots of normalized fluorescence for each mode of transport can be seen in Figure 2.1, reproduced from (1).

It is the normalized fluorescence equation (2.10) that allows for the determination of the transport parameters, D and V_o. The methods of solution are presented as part of the experimental analysis.

2.2 Experimental

Up to this point only the theoretical basis for FRAP systems has been discussed. This theoretical basis was presented to develop avenues for calculating the transport parameters, D (lateral diffusion coefficient) and V_o (bulk flow velocity), from measured fluorescence time histories. The experimental theory describes the collection and manipulation of fluorescence time histories. It is this experimental theory which was used as a guideline for the development of a laser photobleaching system. Again the experimental theory presented is from Axelrod, et al.(1), which has been used by many others. (3,4,6, 7, 18,19).

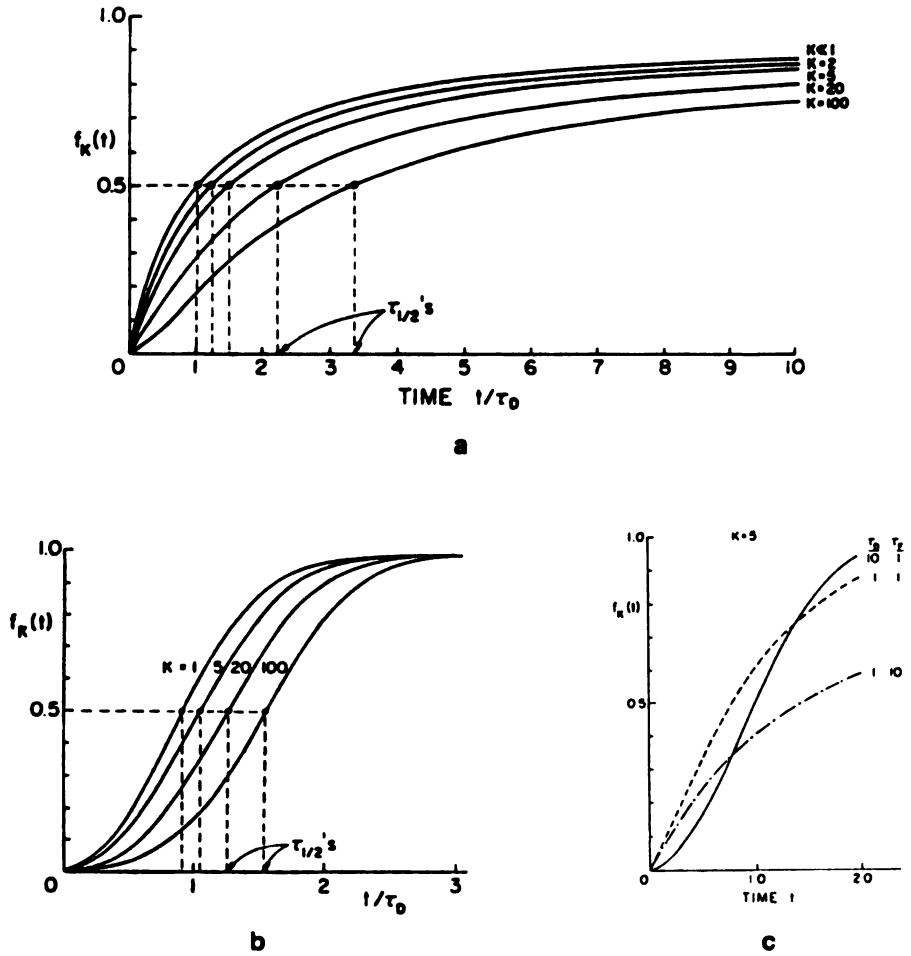

Fig. 2.1 Normalized fluorescence recovery. a) diffusion only, b) flow only, c) combined diffusion and flow; reproduced from (1).

As stated earlier, the normalized fluorescence equation (2.10) allows the experimental determination of the transport parameters, D and V_o. The parameters may be obtained by using measured fluorescence intensities instead of the theoretical fluorescence intensity expression terms contained in equation (2.10). Rewriting Equation (2.10) in terms of measured values leaves

$$f_K^m(t) = \frac{F_K^m(t) - F_K^m(0)}{F_K^m(\infty) - F_K^m(0)} \qquad (2.13)$$

The superscript m denotes measured intensities. The subscript K denotes a dependence of each term on the amount of bleaching (non-dimensional energy density, $K = -\alpha I(0)T$) (1).

Values for the diffusion and the flow velocity can be determined using either of two methods presented by Axelrod, et. al (1). The first is essentially a three point fit of the normalized data. This method is most useful when $F_K(\infty)$ can be measured or estimated and the nature of transport is known. The first point to determine is the time $\tau_{1/2}$, the time when $f_K^m(t) = 0.5$. Solving for this time requires an iterative process to find the time from the fluorescence intensity history $F_K^m(t)$ which provides the value 0.5 for Equation 2.13. The next step is to find the value for the non-dimensional energy density (amount of bleaching) K. The value of K is found by determining $F_K^m(0)/F_K^m(-)$ and solving for K in equation (2.11), where $F_K^m(-)$ is the fluorescence before bleaching. The third point of the data fit is to calculate the lateral transport coefficients by using one of the following (1):

Diffusion coefficient: $$D = (w^2/4\tau_{1/2})\gamma_D \qquad (2.14)$$

Flow velocity: $$V_o = (w/\tau_{1/2})\gamma_F \qquad (2.15)$$

In both equations, w is the effective laser beam width. The constants γ_D and γ_F are given by $\gamma_D = \tau_{1/2}/\tau_D$ and $\gamma_F = \tau_{1/2}/\tau_F$, where τ_D and τ_F are characteristic times for diffusion and flow. The values for γ_D and γ_F are found using the value for K determined in step 2 and Figure 2.2 reproduced from Axelrod, et al. (1).

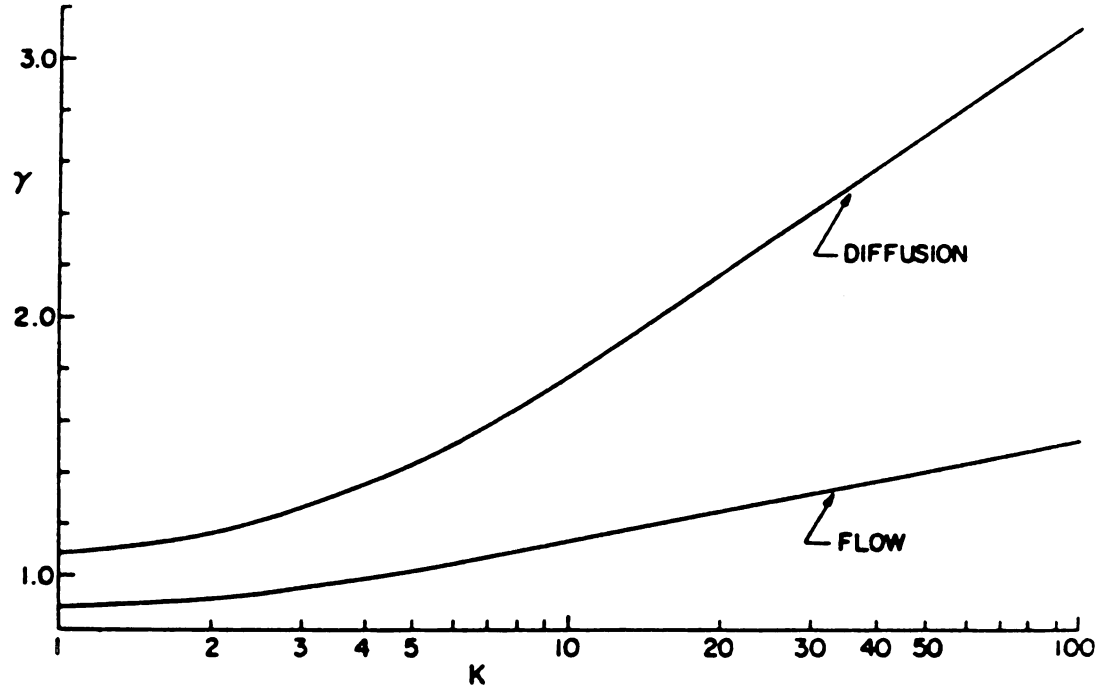

Figure 2.2 Factor $\gamma(\gamma_D = \tau_{1/2}/\tau_D$ or $\gamma_F = \tau_{1/2}/\tau_D)$ versus K for a Gaussian beam; reproduced from (1).

This first experimental solution method also allows for the calculation of the immobile fraction of the fluorophore. If some of the fluorophore is immobile fluorescence at large times, $F_K^m(\infty)$ will be less than the fluorescence before bleaching $F_K^m(0)$. Thus the mobile fraction is given by (1) as

$$m = \frac{\left[F_K^m(\infty) - F_K^m(0)\right]}{\left[F_K^m(-) - F_K^m(0)\right]} . \qquad (2.16)$$

The second experimental solution method presented by Axelrod et al. (1) to determine the transport parameters is a graphical method. It is based upon the observation that plots of $[F_K^m(t) - F_K^m(0)]$ vs. t and $f_K(t)$ vs. t/τ are different by two multiplicative factors: an intensity scale and time scale. The multiplicative factors when plotted in log-log form show up as orthogonal displacements along the intensity and time axes. Superposition of the two plots determines the displacements of the intensity and time scales. These displacements give the amount of fluorescence recovery and the characteristic time of experiment.

The superposition method for determining the characteristic time and mobile fraction is described only briefly here. See (Axelrod) et al. (1) for further details. The characteristic time is found by superpositioning the point $t/\tau = 1$ of the theoretical log-log plot of $f_K(t)$ vs. t/τ with the time axis of the log-log plot of the experimental $[F_K^m(t) - F_K^m(0)]$ vs. t. The displacement of these log-log plots along the time axes gives the experimental characteristic time τ. The transport parameters of D or V_o can then be calculated from this

characteristic time. Likewise, the superposition of the point $f_K(t) = 1$ on the log-log plots with $[F_K^m(\infty) - F_K^m(0)]$ gives the amount of recovery $F_K^m(\infty) - F_K^m(0)$. It is from this amount of recovery that the mobile fraction can be calculated.

Figure 2.3, reproduced from (1), illustrates this graphical method using the superposition of experimental data and the theoretical values of $f_K(t)$.

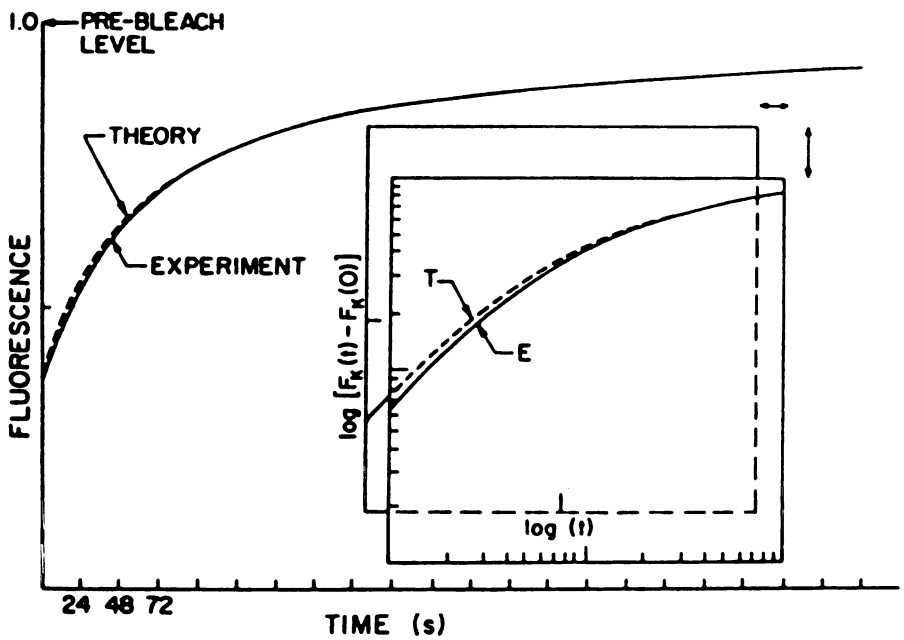

Fig. 2.3 A graphic illustration of the log-log superposition method; reproduced from (1).

Besides providing percent recovery and characteristic time this second method of solution allows for the determination of the mode of transport. The characteristic time found is compared to the theoretical expressions for characteristic time for diffusion ($\tau_D = w^2/4D$) and flow ($\tau_F = w/V_o$). If the mode of transport is that of flow the characteristic time will be proportional to w, the effective laser beam size.

If the mode is diffusion then it will be proportional to w^2. For mixtures of diffusion and flow, small w tends to make diffusion dominant and large w tends to make flow dominant. The theoretical analysis and experimental analysis of Axelrod et al. (1) as presented above supplied the guidelines needed for the development of the laser photobleaching system.

3.0 FRAP Laser System

3.1 Introduction

The main objective of this work was the development of a laser photobleaching system. In this section the laser photobleaching system will be briefly described as a whole. Its individual components will be described in detail. After the description of all the components, a presentation of the laser photobleaching system operation and characterstics will be made.

The laser photobleaching system which has been developed can be seen as a block diagram in Figure 3.1.

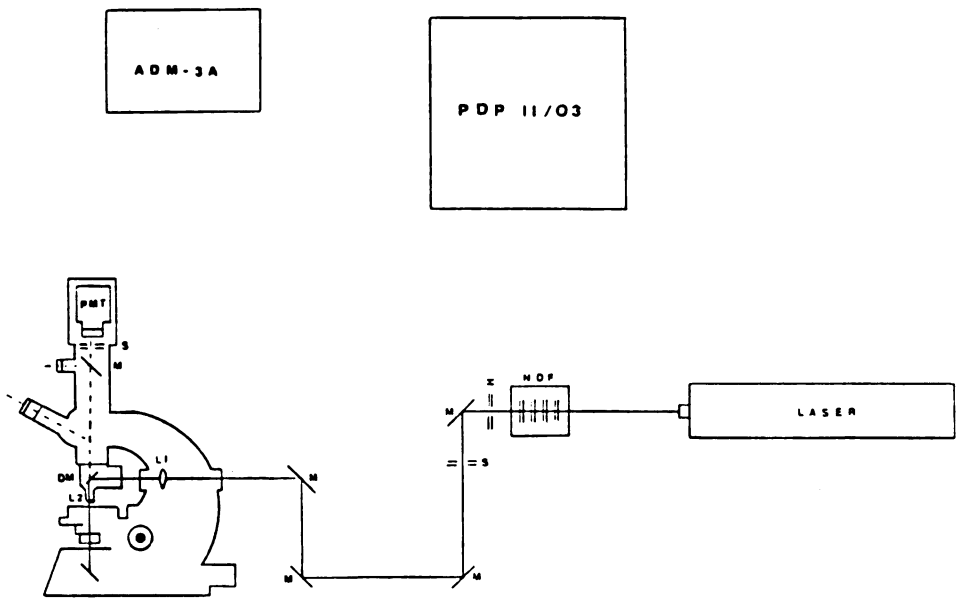

Fig. 3.1 Components of the laser photobleaching system

The system consists of two major divisions: system hardware and the other the system software. The hardware division consists of energy generation, beam manipulation, and light detection. The software division contains two operating systems. One is for control only. The other is for data acquisition and control of the laser photobleaching system.

The energy generation system (hardware) produces the necessary means to excite fluorescent probes. The laser provides the proper wavelength needed to excite a particular fluorophore. The power supply regulates the current to the laser. The purpose of the heat exchanger is to cool the plasma tube of the laser and power supply and to prevent damage due to contaminates.

The beam manipulation system includes all components dealing with the optical hardware. The optical hardware deals with considerations of attenuation, direction, filtration, fixation, position, protection, and reduction. The attenuation grouping controls intensity levels needed for observation and bleaching. The direction system determines the path of the laser beam into the microscope and then to the sample. The filtration system eliminates any unwanted wavelengths being passed along the optical path of the beam. The fixation of the laser system assures precise alignment of the optical path of the laser and microscope. The positioning group includes the hardware required to select the desired location for bleaching and observing. The protection system protects both the sensitive photodetection equipment and the operators of system. The reduction system allows the desired beam diameter to be selectable at the membrane surface.

The light detection system is the most sensitive of the laser photobleaching hardware. This system collects and relays fluorescence intensities. It is this collected and relayed data on which FRAP theory is based.

The software division of the laser photobleaching system consists of a control only system and the other a data acquisition and control system. Both are menu-driven operating systems. These operating systems are interactive "user friendly" programs and subroutines which allow the reasearcher to select experimental parameters. The control portion of both operating systems are a set of subroutines which control the instrumentation of the system in real-time. Subroutines which collect and store experimental data in real-time form the data acquisition portion of the second operating system.

3.2 Hardware

In this section each hardware grouping, will be described in greater detail.

The energy generation group contains three components. The first component is a Spectra-Physics argon laser model 164-05. The second is the Spectra-Physics power supply model 265 for the laser. The last component is a heat exchanger similar to that developed by Edwards (20).

The heat exchanger is the only portion of the energy generation system designed and built as part of the system development. This component has a recirculating cooling loop which includes a pump, the

laser, a heat exchanger, a filter, a deionizer, and a reservoir. See Figure 3.2. The outside loop of this heat exchanger is an open loop which uses filtered tap water.

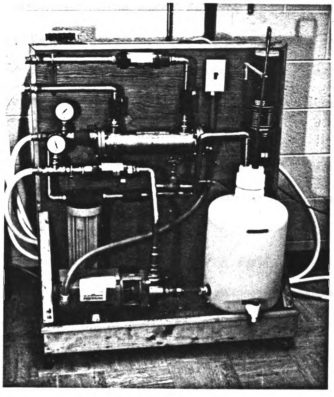

Figure 3.2 Heat Exchanger.

The pump of the heat exchanging unit is a close coupled turbine pump (Burks pump model 5CT5M). It provides a water flow rate of 4.5 gallons per minute at a pressure of 70 psi.

The water from the laser is cooled with a shell and tube heat exchanger (Basco model 500). It is a single pass, counterflow unit. The recirculating cooling water for the laser and its power supply passes

through the shell side of the unit. The filtered top water flows through the tube side removing the heat generated by the laser and power supply.

The recirculating cooling water of the heat exchanger unit is both filtered and deionized. The water filters are Ameteck Plymouth Supreme model PS-S1 filters. These filters surpass the 25 μm limit for particles set by Spectra-Physics. The deionization is handled by a Barnstead Cartridge deionizer. It not only deionizes the water, it also removes oxygen, scale , and other contaminant.

The heat exchanger components are connected with general pumping fixtures and polyvinyl tubing. The exchanger is connected to the laser and power supply with 5/8" garden hose.

The heat exchanger water flow rate and water pressure are both monitored and adjustable. The flow rate is measured by Hedland flow meters in both loops of the heat exchanger. The flow rate (minimum 2.5 gal/min) is adjusted by opening or closing water valves in each loop. The pressure is monitored before and after the laser by 100 psi gauges from U.S. Gauge. The pressure is adjusted to the required operating range (30-50 psi) by opening or closing a valve which bleeds off water from the pump.

Water temperatures throughout the heat exchanger can be monitored. Thermocouples and a ten channel Omega Digicator Box can determine temperature in five locations. These locations include inlet and outlet to the laser, inlet and outlet to the heat exchanger for the tap water, and the reservoir.

The heat exchanger assures proper cooling of the laser and power supply. It also prevents damage from contaminants, excessive pressure, and other sources.

The remaining portion of the energy generation system provides a flexibility to the laser photobleaching system. The model 164-05 laser has prism selectable wavelengths from 351.1-1090 nm. This range of wavelengths allows the use of many different fluorescent probes. The power supply can generate a measured maximum of 4W total output across all wavelengths. This output capacity implies that the laser can emit enough power at multiple wavelength settings to perform FRAP experiments using many different fluorescent probes.

The beam manipulation system contains many different groups of equipment which control the delivery of the beam to the membrane sample. The first task performed by the manipulation system is attenuation of the laser beam. It is accomplished either by adjusting the laser power supply or by placing reflecting neutral density filters (Melles Griot) into the beam path. Attenuation using the power supply is limited to controlling power or current requirements of the laser. Therefore the power supply is only used to set the maximum level from which the beam will be further attenuated using the neutral density filters.

The laser intensity is controlled by positioning different combinations of neutral density filters in the beam path. The optical densities of the filters used in the positioning unit are 0.3, 1.0, 2.0 and 3.0. The percentages of the beam transmitted by these filters are 50%, 10%, 1% and 0.1% respectively. Further attenuation is obtained by

adding optical densities of neutral density filters combined in series. Transmittance percentages are found by multiplying corresponding percentages since $T = 10^{-D}$.

The neutral density filters are positioned by a solenoid activated mechanism. See Figure 3.3. The positioning unit is activated by applying a voltage to an appropriate solenoid. This pulls the filter out of the beam path. The positioning unit has four such mechanisms. They permit 16 different filter combinations or 14 different intensity levels. The intensity levels for the laser at a wavelength of 514.5 nm producing 500 mW of power are listed in Table 3.1.

TABLE 3.1: LASER SYSTEM INTENSITY LEVELS

INTENSITY LEVEL	POWER TRANSMITTED
Observation Levels	
1	250 mW
2	500 mW
3	2.5 μW
4	5 μW
5	25 μW
6	50 μW
Bleaching Levels	
7	250 μW
8	500 μW
9	2.5 mW
10	5 mW
11	25 mW
12	50 mW
13	250 mW
14	500 mW

*Based on maximum output of 500 mW at wavelength of 514.5 nm.

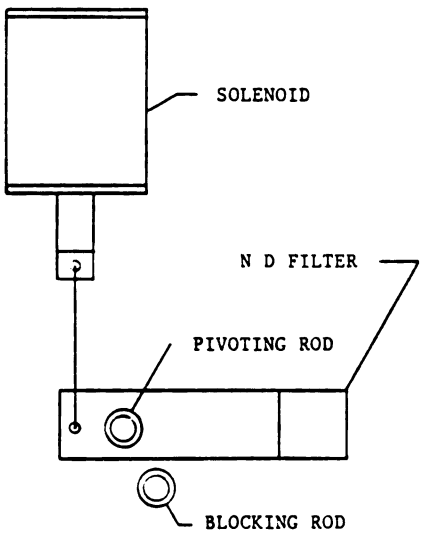

Fig. 3.3: Neutral Density Filter Positioning Unit Solenoid Mechanism

The neutral density filter positioning unit was designed for computer control. The concept used in designing the unit was to make use of the transistor transitor logic (TTL) capability of a parallel line unit (DEC DRV11) in an LSI-11/02 computer. The parallel line unit can apply a high logic level (≈5V) or a low logic level (≈0V) voltage to any of 16 output channels. The use of TTL capability to control the filter position keeps the four D/A channels open for other equipment requiring variable voltages as inputs. The use of the parallel line unit also provides the response needed for real time to position the filters for observing and bleaching.

The electronic circuit used to control the solenoids of the neutral density filter positioning unit was designed by Joseph Peplinski an undergraduate working in the BTP laboratory. Each circuit requires a separate TTL channel from the DRV11 to control the application of a

voltage to a solenoid. Applying a high logic level (≈5V) activates a solenoid to pull a filter out of the path. Each of the circuits acts independently allowing any combination of the four solenoids to be activated.

The interfacing of the neutral density filter positioning unit and the parallel line unit of the computer was a joint effort. The original interfacing of the neutral density filter positioning unit was done by Joe Peplinski. His interfacing box splits the 16 output channels into two portions, a 10 line ribbon cable for the positioning unit and a 6 line ribbon cable for other laboratory equipment. The 10 line ribbon cable connects directly to the positioning unit.

The addition of computer controlled shutters required that the 10 TTL channels be split to complete the interfacing. The Electronic and Computer Services (Department of Engineering Research, Michigan State University) built the signal splitting board and connecting cables. The signal splitting board allowed ten TTL channels on a ribbon cable to be split to service the neutral density filters, the laser shutter, and the photomultiplier shutter. The connecting cables permit the matching of the electrical connectors for each of those units.

The task of directing the beam was left to three different types of mirrors. The mirrors used and their optical path can be seen in Figure 3.4. The first mirror used to direct the beam from the laser to the Ziess Universal microscope is a planar front-surfaced mirror (Optics for Research MU-51). These two inch diameter mirrors are capable of

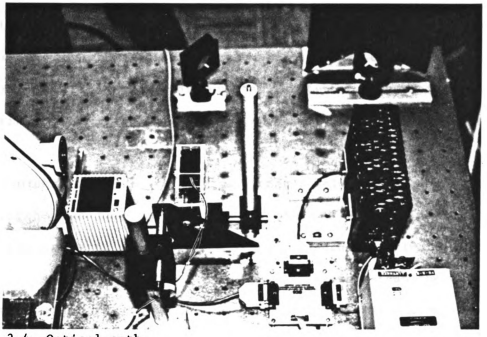

Figure 3.4 Optical path.

reflecting over the spectral range of 200 nm to 2 μm, with reflectance of 80-90%. Three of these planar mirrors manipulate the beam through three 90 degree direction changes. The scanning mirror, a 5 mm diameter mirror (General Scanning M2-0505-00) mounted on a servo-driven motor, directs the beam into the microscope. It allows the beam to scan across a given region of the sample membrane. The third type of mirror, is the dichroic mirror. These mirrors are contained in the epi-illumation attachment of the Ziess microscope. These dichroic mirrors are selectable for the wavelength being used. They come as part of filter sets which will be described later. The dichroic mirror is used to re-direct the beam to the membrane sample.

Filtration of the beam is performed in the epi-illumination attachment of the microscope by three filter sets. The filter sets (Ziess) used are set #487702 for ultra-violet excitation, set #487709 for blue-excitation, and set #487714 for green-excitation. The fourth

position of the epi-illuminator contains only a dichroic mirror for the visible light range. The filter sets consist of an excitation filter, a dichroic mirror, and at least one barrier filter. Operating the laser at a single wavelength (514.5 nm) eliminates the need for an excitation filter.

The dichroic mirrors reflect wavelengths below a cutoff while longer wavelengths are transmitted through the reflective coating. This filtering property of dichroic mirrors is useful for fluorescence work because emission is always at a longer wavelength than the wavelength required for excitation. The laser beam with an excitation wavelength shorter than the cutoff is reflected down to the membrane sample by the dichroic mirror. Emitted light from the sample membrane at any wavelength longer than the cutoff passes through the dichroic mirror to the light detection system.

Barrier filters are placed in the optical path after the dichroic mirrors and before the light detection unit. These filters prevent the unwanted reflections of excitation wavelengths from reaching the light detection unit. Thus only the intensity of emission from the fluorescencing sample will be measured.

The fixation group of the equipment prevents misallignment of the laser beam optical path and the microscope optical path. Each piece of equipment is affixed to a Micro-G model 71-151 vibration damping optical table. The optical table has air activated supports which must be leveled so the table is supported equally. The optical surface has pre-tapped holes with 2 inch centers for mounting equipment to the table. Mounting equipment to the table insures that it is on the same reference plane in space.

The mounting fixtures required for each piece of the equipment were machined by Leonard J. Eisele, who also aided in their design. The laser is held in place by two mounting plates which permit movement in the x-y plane of the optical table. The neutral density filter unit is mounted with two supports. These were designed to allow movement in three dimensions. The planar mirrors are fixed to the optical table by mounting brackets which allow adjustment in the x-y plane of the table. The scanning mirror mounts permit rotation about the z axis as well as three dimensional movement with respect to the table. The final mounting fixture allows for rotation about the z axis and facilitates three dimensional adjustment of the microscope.

The positioning of the beam depends on the mounting fixtures of the equipment. Having the equipment affixed to the optical table provides a fixed reference frame. It is from this reference frame that the optical path of the laser beam can be precisely matched to the optical path of the microscope. See Appendix C for detailed positioning procedures.

The optical path of the laser beam is kept parallel or normal to the reference plane by using the planar mirrors. The planar mirrors are positioned using adjustable mirror mounts (Oriel #1750).

The scanning mirror system is the most critical component for positioning the beam. The scanning mirror redirects the beam into the microscope and controls the position of the beam on the membrane sample. Its position is controlled by a galvanometer (Frequency Control Products model ALS 200 PS) with a resonant frequency of 200 Hz at a maximum of 15 degrees of deflection. The positioning galvanometer is rated at a sensitivity of 20 mA/degree and a repeatability of 0.05%. The galvanometer is externally controlled by the digital/analog (D/A)

channel A from an LSI-11/02 computer. The controlling electronics for the scanning mirror system were developed in conjunction with Electronics and Computer Services (ECS, of the Department of Engineering Research, Michigan State University).

The laser photobleaching system includes many features which protect the laser and its operators. The first protection feature is that the laser can be shut down from various sources. These sources include the wall circuit breaker, power supply key, and power supply circuit breakers. Simply turning off the heat exchanger pump is also a protective feature, but is an extreme emergency measure. A second operator safety feature is that the beam can be blocked or attenuated. Blockage of the beam is accomplished by manipulating (Vincent Associates model 2GL) shutters located at the laser and at the photomultiplier. These shutters are controlled by a Uniblitz electronic control unit (Vincent Associates model 325B). This control unit can open or close two shutters either manually or under computer control. The beam is attenuated by the neutral density filters either manually or under computer control. A final protective feature is shielding which blocks stray reflections of laser beam.

The laser itself is protected by the power supply. The power supply shuts down if the key is not in place and turned on. It also reacts to an insufficient supply of cooling water, and improper current and voltage levels. In addition, the power supply sounds warning signals for high cooling water temperature and low gas pressure.

One of the most critical tasks of the beam manipulation system is the reduction of the beam. The beam has to be reduced from 1.25 mm to approximately 1 micrometer when the sample is in focus on the micro-

scope. The beam is reduced by inserting a convex lens (40 mm focal length (Optics for Research #LL-25-40) in the beam path so that its focal point closely matches the focal point of the focusing objective (Ziess 25X Neofluor). A diffraction limited 1.5 micrometer spot is produced when a sample is in focus, due to an image of a point seen by the objective. "Spot" diameters of the laser beam measured to be 1-4 micrometers are obtained by changing the manification power of the objectives.

The last hardware system controls light detection. This light detection system consists of two parts, a photometer (Ziess MPM 01K) and a measuring amplifier support box (Electronic and Computer Services, MSU).

The photometer is comprised of a photomultiplier tube, an electronics housing, a mounting housing, and a protective shutter. The photomultiplier tube (Hamamatsu/Ziess # R928HA) detects light energy and converts it into a proportional current. The electronics of the photometer changes this proportional current into a voltage, 0-1.6V dc. This voltage is then used as an input for the amplifying support box.

The measuring amplifier support box was built according to requirements supplied by Ziess. The support box supplies power for the photomultiplier tube, PMT electronics, and hinged mirror control of the photometer housing. This box amplifies the output voltage supplied by the PMT electronics, to a 0-10V dc scale. The support box also controls the gain of the photomultiplier tube with both a fine gain (0-10x) and a decade gain (1x, 10x, 100x, 1000x). It also supplies various output

signals on a 0-10V dc scale on digital display, banana plug, and BNC port. The latter two allow for strip chart or A/D records of the output from the photomultiplier tube.

The mounting housing for the photometer contains selectable pinhole stops and a hinged viewing mirror. The pinhole stops (Ziess 4T1380) have diameters ranging from 50 μm to 12 mm. These diameters correspond to 1.6 μm to .38 mm measured diameters at the sample membrane using a 20x objective. The hinged viewing mirror selects the path of the emitted light of the sample. One position of the mirror sends the emitted light to an observing eyepiece another position sends the emission to the photomultiplier tube. The position of this mirror is controlled by a manual foot pedal switch.

3.3 Software

As mentioned earlier the software of the lasers/photobleaching system developed here consists of two operating systems. In this section, the capabilities and development of each of these systems is described in detail.

The laser photobleaching can be controlled by two different operating systems, written in FORTRAN IV. The first operating system controls the laser system only. The control only system is used for aligning, initializing, and testing the laser photobleaching system.

The run image of control only system is named LOPERA.SAV. This run image is composed of the programs and subroutines contained on the following files: LOPERA.FOR, MPOSIT.FOR, FPLACE.FOR, CLOCIN.FOR, DTACHA.FOR. The actual contents of these files are reprinted in Appendix B.

The control only operating system is based on programs and subroutines contained in the five files listed above. This system is menu driven by the program LOPERA contained on LOPERA.FOR. The program LOPERA allows for the selection of intensity levels, shutter conditions, miscellaneous options, or exiting.

The subroutines FPLACE, TRANSV, SHUTTR, and OCTREP in the file FPLACE.FOR control intensity level selection and shutter conditions. The subroutine FPLACE called by LOPERA allows selection of the six observing intensity levels and eight bleaching intensity levels presented in table 3.1. The subroutine TRANSV called by FPLACE determines the percentage of the beam transmitted for the chosen intensity levels. The subroutine OCTREP also called by FPLACE determines the octal values needed to activate the proper TTL (transistor tranistor logic) channels of the parallel line unit to remove the neutral density filters from the beam path. The subroutine SHUTTR allows the user to select from four shutter conditions. The shutter conditions are 1) both open, 2) both closed, 3) only the laser shutter opened, and 4) only the PMT shutter opened. SHUTTR also provides octal values needed to activate the TTL channels of the parallel line unit for the chosen shutter condition .

The subroutines MPOSIT, CONVRT, and MOVEM of the MPOSIT.FOR file select mirror settings. The subroutine MPOSIT called from LOPERA selects operational modes for the scanning mirror. One mode changes the mirror position until the desired position is reached. The other mode scans the sample over a given range for a specified number of passes at a selectable rate. The scanning mode of the subroutine MPOSIT is supported by the subroutine CONVRT. The subroutine CONVRT converts desired deflection voltages corresponding to a certain mirror position

into integer equivalents for the D/A (digital/analog) converter. The analog signal produced is directed out of channel A of the D/A board. This signal is used as the external input to the scanning mirror control box.

The subroutine MOVEM is used in the position mode of the subroutine MPOSIT. The subroutine MOVEM increases or decreases the D/A output of channel A to the scanning mirror control box until the desired beam location is reached.

The files CLOCIN.FOR and DTACHA.FOR must be linked with the file MPOSIT to control the mirror positioning or scanning. The subroutine CLOCIN called by MPOSIT selects real-time clock settings to control the rate of scanning. These settings include operating frequency, mode of operation, and duration of operation.

The subroutine DTACHA called by MPOSIT is actually an Interrupt Service Routine (ISR) An interrupt service routine allows the computer to interrupt a running program temporarily. It then completes a task and resumes the running program at the point of interruption. For more information about ISRs consult (21-24). The tasks performed by the ISR DTACHA include outputting desired voltage from D/A channel A and counting the number of scans.

The second operating system for the laser photobleaching system is designed for real-time data acquisition and control. The name of the run image for this acquisition and control operating system is LASCON. SAV. This run image consists of the programs and subroutines found in these files: LASCON.FOR, INSHUT.FOR, EXPRUN.FOR, LASET.FOR, CPARAM.FOR, LPARAM.FOR, NAMEF.FOR, SETIME.FOR, LTIME.FOR, ATDC13.FOR, STNDBY.FOR, and HELPME.FOR. The contents of these files may be found in Appendix B.

The program LASCON, contained in the LASCON.FOR file, is the interactive menu of the acquisition and control system. This program contains and defines most of the initial and default variables. LASCON allows the user to change parameters, list parameters, name output files, run FRAP experiments, adjust laser system, go to a stand by mode, ask for help, or exit the program.

The subroutine CPARAM, in CPARAM.FOR file, permits changes of the parameters of the output file name, the intensity levels, the shutter conditions, and the experimental time settings.

The subroutine NAMEF, contained in NAMEF.FOR, can be called by LASCON or CPARAM. It allows the output file name to be varied from FTN10.DAT to FTN99.DAT. The default output file name is FTN10.DAT.

The subroutine INSHUT, called by CPARAM, is contained in INSHUT.FOR. This subroutine permits the user to select the observing intensity level, bleaching intensity level, and shutter conditions. The subroutine INSHUT calls the subroutines TRANSV, OCTREP, and SHUTTR. INSHUT also allows the user to select one of six observing intensities and one of eight bleaching intensities presented in Table 3.1 . The subroutine TRANSV, called by INSHUT matches the chosen intensity level with the fraction of the beam transmitted to the sample. The transmission fraction is written into the output file and to the screen. The subroutine OCTREP determines the octal values which the TTL channels of the computer parallel line unit need to activate the neutral density filters. The SHUTTR subroutine allows the selection of the four possible shutter conditions and provides the octal values needed to activate these shutters.

The selection of the experimental times is handled by the SETIME subroutine called under CPARAM. SETIME permits the user to select length of bleaching, length of experimental run, and the time interval between the collection of data. The bleaching and run times set the parameters for the line time clock the computer's . The collection time interval specifies the of real time clock parameters. These clock parameters define values needed to prepare the programs interrupt service routines. For more information on interrupt service routines refer to (21-24).

LASCON program lists the existing parameters when the subroutine LPARAM is called. This subroutine is contained in the file LPARAM.FOR. LPARAM is also called by the subroutine CPARAM.

The subroutine STNDBY called by LASCON puts the laser photobleaching system into a "safe" mode. This "safe" mode lets the user leave the system unattended without having to power down the laser photobleaching system. In this mode the laser is attenuated to its lowest level and the photomultiplier shutter is closed. The subroutine STNDBY is contained in the file STNDBY.FOR.

The system user can get help when using the subroutine HELPME. This subroutine gives information on each of the menu selections in the LASCON program. The subroutine HELPME is contained in the file HELPME.FOR.

The initial set up of the laser is completed by using the LASET subroutine of LASCON. LASET allows for focusing the beam, centering the beam, verifying intensity choices, checking shutter conditions, setting PMT controls, and checking strip chart recorder settings. This subroutine is contained on the file LASET.FOR.

The execution of a FRAP experiment is performed by the subroutine EXPRUN. This subroutine controls all the system hardware and collects the recovery intensities using the parameters set by the user or the default values. EXPRUN also performs the necessary housekeeping chores of opening files, closing files, and writing data into files.

The equipment control and data acquisition in EXPRUN is performed by two interrupt service routines (ISRs). Further information on ISRs can be found in (21-24). The two ISRs used in EXPRUN are LTIME and ATDC13.

The ISR LTIME controls both the bleach time and the run time of the FRAP experiments. The other, ISR ATDC13, controls sampling of data and mirror positioning. ATDC13 uses A/D channel 13 to take data. This ISR also uses the D/A channel of the computer to position the scanning mirror.

The subroutine EXPRUN continuously checks for a (done) flag while the ISRs are functioning. Once the run time is completed and the flag is detected, EXPRUN completes the file management. It then offers the user the choice of running another experiment with the same parameters or returning to the main menu of LASCON.

Both operating systems, programs and subroutines, may be reviewed in Appendix B.

3.4 Operational Characteristics

In this section characteristics and response of the hardware and software of the laser photobleaching system are presented.

The response of the energy generation system and the other systems hardware is dependent on the operational settings. The following operational settings remain the same throughout the characterization of

the laser photobleaching system. The power supply is in the current control mode. The current is set to 30A. The magnetic field control is set to its maximum. The laser is set to 514.5 nm in the single line mode. The heat exchanger provides a minimum flow rate of 2.5 gal/min at 40 psi to the power supply and the laser. At these settings the laser produces 500 mW of power when operating at peak output. See Appendix A for peaking laser output.

The first component of the beam manipulation system which is characterized is the attenuation by the neutral density filters (NDFs). With the laser producing 500 mW of power, the NDFs provide intensities ranging from 25 μW to 500 mW. For each intensity level within the range of a calorimeter type power meter (Scientech #36-0001), the predicted intensities are compared with experimental intensities. The results of these comparisons are listed in Table 3.2. The intensities for level 8 and 9 have asterisks because estimated errors reflect fluctuations of the power meter.

TABLE 3.2: INTENSITY COMPARISONS

INTENSITY LEVEL	PREDICTED INTENSITY (W)	EXPERIMENTAL INTENSITY (W)
8	0.50 mW	0.5 ± 0.5 mW*
9	2.50 mW	2.26 ± 0.1 mW*
10	5.00 mW	4.95 ± 0.05 mW
11	25.00 mW	26.0 ± 0.15 mW
12	50.00 mW	51.5 ± 0.5 mW
13	250.00 mW	263.0 ± 1.5 mW
14	500.00 mW	505. ± 5 mW

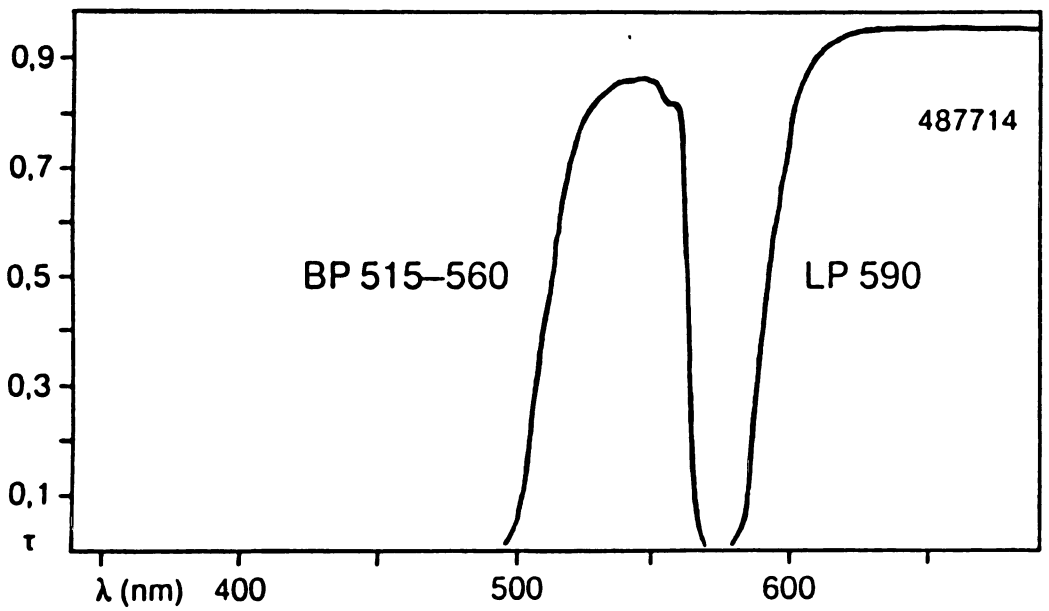

Fig. 3.5: Fluorescence response curve for green excitation, reproduced from (41).

The mechanical response of the neutral density filter positioning unit is measured using the photomultiplier system and an oscilloscope. The mechanical response time for removing a filter from the beam path is found to be 4 ± 1 msec. Replacing a filter yields a slightly slower response time, 6 ± 1 msec.

The filtration system is not characterized experimentally. The fluorescence response curve reproduced from (41) for the green excitation filter set (Ziess, #487714) used in most of the applications of this laser photobleaching system can be viewed in Figure 3.5.

Theonly characterized response of the protection system is the response time of the shutters. The laser and PMT shutter opening and closing times are measured with a photo-diode system and an oscilloscope. The opening and closing times are found to be 1.5 ± .2 msec.

The results of the beam positioning are qualitative in some cases and quantitative in others. Qualitative results come from the alignment of the beam optical path with the optical path of the microscope. Any misalignment of optical paths results in multiple reflections and a non-symmetrical emission spot at the sample. Only when the optical paths of the beam and of the microscope match is a symmetrical (assumed Gaussian) spot seen. Viewing the beam with only a dichroic mirror gives symmetric interference patterns. A single secondary reflection is seen when aligned properly.

Quantitative results come from the position of the scanning mirror. Using a video tape recording of the emission spot from a fluorescently coated microscope slide, a total beam excursion limit of 11.0 ± 0.5 μm

is measured. This excursion limit corresponds to ± 5.6 μm movement from a centered position. Eleven locations are used to define the beam position over 10 μm of the sample. The point where the beam is centered in the microscope is defined as position six. Each change in position corresponds to a 1 μm position change on the sample.

The results from the beam reduction are also determined with a video tape recording of the emission spot of a fluorescently coated microscope slide using a color camera. Table 3.3 shows the measured emission spot diameters for the objectives available in our laboratory. The errors reflect the resolution possible from the television monitor for each objective.

TABLE 3.3 BEAM REDUCTION RESULTS

OBJECTIVE			EMISSION SPOT DIAMETER
Ziess	Neopluar	10X	3.0 ± 0.5 μm
Ziess	Neofluar	25X	1.5 ± 0.4 μm
Ziess	Neofluar	63X	1 ± 0.2 μm
Leitz	Hoffman Modulation	20X	2.0 ± 0.5 μm

It should also be noted that a faint symmetric emission pattern was seen surrounding the emission spots except at intensities below level 6. A higher intensity or a shorter effective focal length of the objective increases the intensity of this symmetrical emission pattern.

The operational characteristics for each operating system are presented separately. The control only operating system (run image LOPERA.SAV) is characterized first. Then the operational response of

the data acquisition and control operating system (run image LASCON.SAV) is presented.

The operational characteristics of the control only system software are dependent on the laser photobleaching system's hardware and the computer system hardware. The response of the control only operating system interaction with the hardware has already been presented as part of the hardware response. Only operational characteristics of the control-only system software with the computer system hardware are addressed here.

The subroutine FPLACE controls the neutral density filters and shutters (FPLACE) of the control only system. It uses the parallel line unit (DRV11). This subroutine selects which TTL channels are to be activated. The channel is activated by setting one of 16 bits of the DRV11 output buffer. The bit numbers used and their corresponding functions are listed in Table 3.

TABLE 3 DRV11 OUTPUT BUFFER CONFIGURATION

BIT #	FUNCTION
4	PMT shutter closed when set
5	Laser shutter closed when set
12	Filter #1 (Optical Density 0.3) removed from path when set
13	Filter #2 (Optical Density 1.0) removed from path when set
14	Filter #3 (Optical Density 2.0) removed from path when set
15	Filter #4 (optical Density 3.0) removed from path when set

The subroutine MPOSIT controls the mirror positioning for the control only operating system. It uses the digital to analog (D/A) converter. The first positioning option increases or decreases the digital value supplied to the D/A converter moving the mirrors about a center of deflection. This center of deflection is the digital value of 3071_{10} (5777_8) corresponding to + 2.53 V. The minimum deflection of the mirror is at a digital value of 4095_{10} (7777_8) corresponding to 0V. Its maximum is at the digital value $0_{10}(0_8)$ or +5.0V. The reason for these odd values is that the scanning mirror responds to an input range of -5.0 to 5.0 V and the D/A converter operates over a range from 0 to 10V. The D/A output signals are also inverted, so that the digital value 4095_{10} (7777_8) supplies 0V and $0_{10}(0_8)$ supplies +10V.

The scanning option of the subroutine MPOSIT of the control only system uses the real-time clock for the initiation of the interrupt service routine DTACHA. The real-time clock is limited by software to two selectable frequencies, 60 Hz and 100 Hz. The DTACHA ISR positions the mirror using the D/A converter within the user selectable range of 0 - 5.12V DC. The number of cycles run is left to the operator discretion.

Some of the operational characteristics of the data acquisition and control system software are identical to those of the control only system software. Other characteristics display only minor differences between the two operating systems. Still other responses are unique to the acquisition and control operating system.

Identical operating characteristics are found in controlling the neutral density filters and the shutters. These characteristics are identical because the subroutine INSHUT is in fact the subroutine FPLACE organized somewhat differently.

Minor differences between the two operating systems characteristics are seen in the positioning of the mirror. In the control only operating system the mirror position in the manual mode is user selectable. The acquisition and control operating system manual positioning in the subroutine LASET restricts the mirror to one of only eleven positions. These eleven positions correspond to a diffraction limited scanning range of ±5 μm from the center position at the sample membrane corresponding to [digital values from 3046_{10} (5746_8) to 3096_{10} (6030_8)]. In both cases positioning of the mirror is defined by sending a single digital value to the D/A converter and applying voltage to the scanning mirror positioning control unit.

In both operating systems the scanning portion of the mirror positioning is accomplished using the D/A converter channel A in interrupt service routines. Again the acquisition and control operating system uses the same eleven defined points while the control only system scans the entire range set by the operator. The final minor difference stems from the scanning rate. It is dependent on the data collection rate in the acquisition and control operating system, while being user selectable in the control only system.

The unique operating responses of the acquisition and control operating system are in timing and data acquistion. The fastest rate that data could be collected is once every 5 msec. Attempts to collect

at faster rates cause a computer system failure. Other data sampling rates are user selectable: 8 msec, 10 msec, 100 msec and 1 sec. All rates are compatible with 1 kHz real-time clock frequency.

Other unique operational characteristics of the acquisition and control operating system involve timing of controlled events. The bleach times are user selectable : 250 msec, 500 msec, 1 sec, and 2 sec. These bleaching times corresponded to those used by other researchers (1) (2) (15) (18) (19). The experimental run times are calculated from the maximum allowable space in the program temporary storage arrays and the software selectable sampling rate for data.

Once the operational characteristics of the hardware and software for this laser photobleaching system had been determined, application of the system began.

4.0 Applications of the System

The laser photobleaching system was applied to four microscopy techniques. Two of these techniques employed the system to quantize the number of lamellae (bilayers) in liposomes. The other two techniques were used to verify the FRAP capabilities of the system. In the following sections the quantization techniques, the FRAP techniques, and the results of these applications are presented.

4.1 Quantization Techniques

It was proposed to use the laser photobleaching system in developing a fluorescence microscopy technique for the quantization of bilayers in liposomes "in situ". Two microscopy techniques were evaluated. One method was a fluorescence version of a phase contrast technique (27,38) for the determination of lamellae in liposomes. The other method compared fluorescently labeled liposomes to fluorescently labeled single bilayer standards.

The fluorescence contrast technique was attempted at the suggestion of John J. McGrath, Ph.D. This study began by evaluating a phase contrast technique (27) to quantize the number of bilayers in liposomes. The work continued by fluorescently labeling liposome membranes and then observing the emission differences at low intensity excitation. These observations were to form the basis for the proposed fluorescence contrast method.

The fluorescence contrast technique to quantize the number of bilayers varied slightly from the phase contrast technique (27) in liposome composition and detection response. The liposomes used in the

phase contrast technique were composed entirely of EPC and hydrated in water. The liposomes of the fluorescence contrast technique were prepared from EPC (99.9% pure egg phosphatidylcholine, Leon Labs), diI (3.3'-dioctaderylindotricarbocynanine, Molecular Probes) and hydrated in distilled water. The fluorescent probe, diI, was in a 1:1000 mole ratio with EPC to avoid possible variations in membrane properties.

The difference in the intensity response of the two contrast techniques is that they are inverted with respect to each other. The fluorescence contrast technique has a response that is above the background intensity level within the liposome membrane and at the greatest intensity at the membrane. The phase contrast response is at the background intensity within the liposome membrane and has the lowest intensity at the membrane. Refer to Figure 4.1 to compare the detection response of these two techniques from typical experimental records. There is only a minor difference in procedure between the fluorescence contrast and phase contrast techniques. This difference is in the movement of the liposome past the detection point. The fluorescence contrast technique allows the liposome to float through the detection point (laser beam). Both responses in Figure 4.1 are from liposomes floating through the detection point. The phase contrast method (27) moved the liposomes on a step motor controlled microscope stage, with 0.5 μm resolution, through the detection point.

The analytic method for the fluorescence contrast technique for quantization of lamellea is based on that used by Servuss and Boroske (27,38). Their analysis method takes the difference in intensity at the

membrane, ΔI, and normalizes it with respect to the maximum intensity, I_o. These intensities are depicted for both techniques in Figure 4.1. The normalized intensity ($IN = \Delta I/I_o$) is then plotted versus liposome

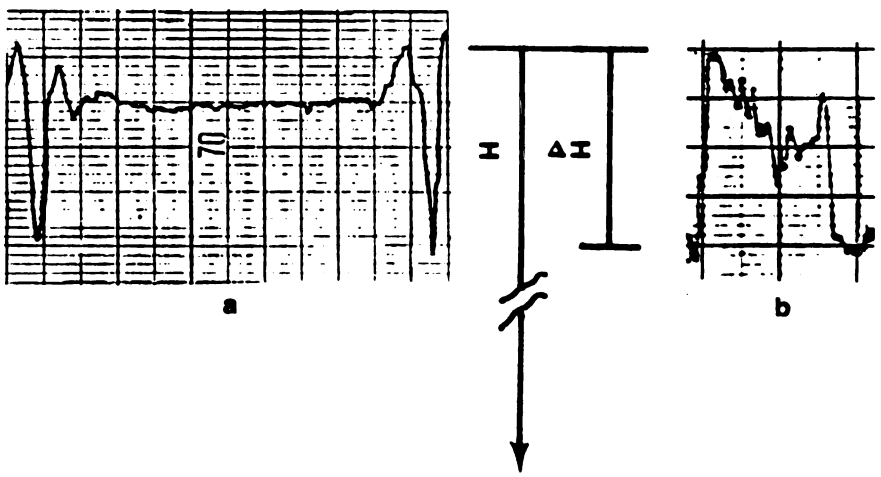

Fig. 4.1 Intensity detection of a) phase contrast b) fluorescent contrast from float passed method

radius. This plot of normalized intensities shows groupings corresponding to different numbers of lamellea Figure 1.2, reproduced from (27) shows this data.

The development of the comparison technique for quantization of lamellea in liposomes was a joint effort. The possibility of such a comparison technique was conceived by John J. McGrath, Ph.D., associate professor of the Mechanical Engineering Department at M.S.U. The experimental equipment, parameters and procedures used to realize this concept were developed as part of this study. The experiments for this comparison technique were conducted by Serge Weiss, an undergraduate student completing an independent study in the field.

The development of this comparison technique required first that experimental parameters and procedures be defined. The first experimental parameters defined the criterion of the comparison method. A major requirement for the method was its ability to be used "in situ" prior to quantitative determination of membrane characteristics. The method also required flexibility for use with a diffusion chamber (50), a computer controlled freezing stage (51), and a microscope slide. Further the comparison technique needed to be easily applied, quick, yet accurate.

The next parameters to be determined were the standards to be used in the comparison technique. The two standards used in the comparison technique were a known single bilayer standard and a normalizing standard. The known single bilayer standard had to consist of a verifiable single bilayer. The normalizing standard was to allow for

intensity comparisons between the liposomes and the known single bilayer standard.

The known standard selected was the bilayer lipid membrane (BLM). This selection was made because of its widespread use (31, 32, 34, 35, 36). BLMs were also chosen because a technique already existed to verify that they consisted of a single bilayer. This technique, described in (31), stated that a BLM observed to thin to "black" (non-passage of light through a membrane due to diffraction) can be shown to consist of a single bilayer.

The normalizing standard chosen was fluorescent polystyrene microspheres (Duke Scientific). The microspheres were considered initially because they were small (9.5 μm diameter) and said by the manufacturer to be inert. It was two other manufacturers' claims that determined factor the choice to use these microspheres as the normalizing standard. The claim that the microspheres would provide a constant fluorescent intensity when excited. They also claimed that the intensity from microsphere to microsphere would be uniform.

The last group of experimental parameters dealt with the composition of the liposomes and BLMs. The composition of each system had to have the same molecular ratio of constituents. The major constituent of the liposomes and BLMs was EPC (99.9% pure Leon Labs). Cholesterol was included as a component of each system since it helped form more stable BLMs. The last constituent was the fluorescent probe diI (Molecular Probes). This probe was chosen because of its use by others in both liposomes and BLMs (1, 2, 6, 8, 19, 32).

The actual compositions used in the comparison technique were dependent on the BLM preparation solution. The "recipe" for the BLM preparation solution is based on one regularly used in Tien's laboratory from (31). This BLM preparation solution consisted of 6.7% EPC, 1.1% cholesterol, and 0.0067% diI (percentages from weight of solute/ weight of solvent). The solvents used in the BLM preparation solution were hexane and octane. The diI percentage was kept small (1:1000, diI:EOC molecular ratio) to prevent possible membrane property changes due to its presence. The liposome membrane reflected these component ratios for similitude.

The comparison technique for quantizing the number of bilayers in liposomes consists of three procedures. The first of these procedures is the forming and transferring of BLMs. The next procedure is the collection of normalized intensities of BLMs as the known single bilayer standard. The final procedure is the acquisition of normalized intensities of liposomes.

The formation and transferring procedure of BLMs was derived from methods used in Tien's laboratory (31). The BLM formation apparatus used in this procedure is pictured in Figure 4.2. The formation apparatus consists of a formation tank, a light source, a 2.5X binoculars, a BLM formation stage, ring stands, and supports. The use and description of each component is described as the procedure to form BLMs is presented.

BLMs require an aqueous environment to be formed. The construction of an 8" x 6" x 3" formation tank meets this requirement. This tank is large enough to provide easy access to the BLM formation stage.

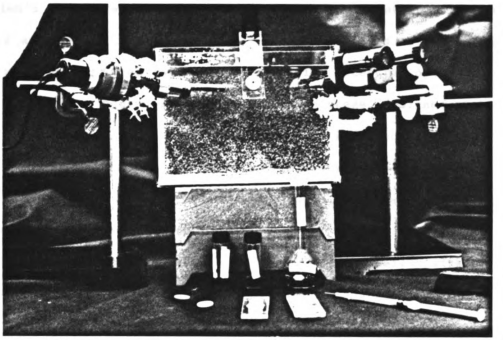

Fig. 4.2 BLM formation apparatus.

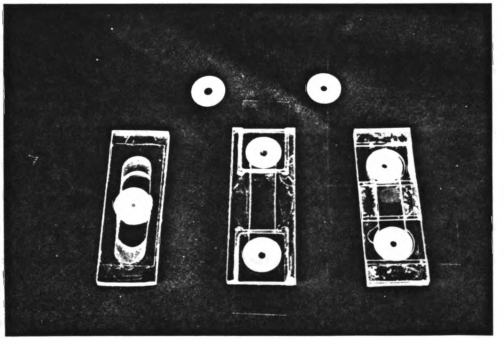

Fig. 4.3 BLM formation stages.

Tien's procedure calls for the BLM prepartion solution to be applied across an opening in a teflon cup. For the purposes of this study the cup has been replaced by a teflon disk with a central opening. These teflon disks are housed in the BLM formation stage, designed as part of this work. Figure (4.3). The formation of BLMs requires the ability to seal off the BLM for its removal from the tank and placement on the microscope stage. The formation stage could be sealed by sliding 22mm square coverslips over the teflon disks.

Allowing the membrane to thin to "black" on the teflon disk verifies that the BLM is a single bilayer for use as the known single bilayer standard. For further description of this portion of the experimental procedure refer to (31) and (39). Once the BLMs thinned to "black", coverslips were slid into place sealing off the BLM in the formation stage.

The final step in the formation and transferring procedure is to transfer the BLM held in a vertical orientation in the tank to the microscope stage. The sealed formation stage has to be raised from the tank. The stage must then be released and placed in a horizontal orientation. Once in the horizontal orientation procedure it is transported to the microscope stage.

The next procedure of the comparison technique is the collection of normalized intensities of BLMs. The formed and transferred BLM is supported in one side of the BLM formation chamber. In the other end of the chamber fluorescent microspheres are sealed into the opening of another teflon disk with coverslips. The microspheres, the normalizing standards, are sealed in this location to assure the microspheres and BLM were at the same focal plane.

The collection of normalized BLM intensities follows these steps. The laser's excitation intensity is adjusted until the microspheres produced a voltage of 9.0V on the photomultiplier display for a preselected gain. Once adjusted to this voltage the formation stage is moved to the BLM portion of the chamber. Several emission "normalized" intensity readings of the BLM are then taken using the photomultiplier excited at the same intensity of the laser beam. This portion of the procedure is repeated until a statistically significant number of the BLM emission intensities are recorded. It is from this statistical sample that a mean intensity value for a single bilayer standard can be determined.

The final procedure in the comparison technique is to apply the laser photobleaching system and find normalized intensities for liposomes. The laser intensity is attenuated in the same manner using the fluorescent microspheres as the normalizing intensity standards to get 9.0V on the photomultiplier display at a preselected gain. The liposomes, sealed into a teflon disk, replace a BLM in one end of the BLM formation stage. This replacement assures that no stage dependence has been introduced in the procedure. Once again the formation stage is moved from the microspheres (the normalizing standard) end to the liposome end where "normalized" emission intensities can be taken.

The intensity readings are taken from multilamellar and unilamellar liposomes (distinguished by qualitative visual selection). The intensity readings for liposomes normalized by the intensity of the microspheres (9.0V) can then be compared to the mean intensity of the similarly normalized single bilayer standard. Those intensities within two standard deviations (95% confidence level) are classified as a single

bilayer. Intensities greater than this mean single bilayer intensity are classified as multi-layered. Hopefully further information on the number of layers in these multi-layered liposomes can also be produced by the comparison technique.

4.2 FRAP Techniques

The laser photobleaching system has multiple application capabilities. Besides its quantization capabilities, the system can perform both single point and multi point FRAP techniques. In this section, the experiments conducted to verify the laser photobleaching FRAP capabilities are presented.

The experiments verifying the single point technique follow experiments presented by Axelrod et al. (1). The laser photobleaching system tests its single point FRAP capabilities on the diffusional recovery of aqueous solutions of rhodamine 6G (Sigma Chemical).

The parameters defined for the verification experiments follow those presented by Axelrod et al. (1). The aqueous solutions of rhodamine 6G for the experiments have a concentration 4.6×10^{-6}M. The solvents are distilled water and glycerol : distilled water (1:1 by volume). These aqueous solutions are sealed into thin layers on microscope slides using (24 mm x 50 mm) coverslips and silicon grease instead of the rectangular sealed container 100 μm thick used by Axelrod (1).

The next parameters needed for the single-point FRAP verification experiments were settings for the laser photobleaching system. The diameters for the beam used in verification experiments are 1.5 - 20 μm.

These diameters are function of the distance between the beam focusing lens (+ 40 mm focal length) and the microscope objective (25 x magnification, 7.1 mm effective focal length). The bleaching intensities need in these experiments are 5 mW and 500μw (bleaching intensity level 10 and 8 respectively). The photomultiplier gain selectors were adjusted to display 5.0 volts for the fluorescence emission intensity of a sample with the laser attenuated to 5 μW or 0.5μw (observing intensity level 4 or 2).

The procedure for these experiments followed earlier FRAP theory. The sealed aqueous solutions of rhodamine 6G were focused on the microscope stage using phase contrast. The beam attenuated to an observing intensity level was focused to the desired spot diameter of 2.0 μm. The pinhole stop was then selected to be slightly larger than the emission spot diameter. The photomultiplier measuring amplifier gains were then selected to produce 5.0V on the display. The FRAP experiment was then run with the desired time intervals. The aqueous solution of rhodamine 6G was bleached using a bleaching level (level10). The recovery due to the diffusion of fluorescently labeled molecules into the bleached spot was monitored by the photomultiplier system. The time history of the recovery was recorded by the data acquisition portion of the program. The diffusion coefficient was obtained from these collected time histories .

The results of the verification of the spot FRAP technique are presented in the next section.

The ability of the laser photobleaching system to perform the multi-point FRAP technique (37) was briefly examined. Verification experiments were designed to give preliminary information on the system

characteristics using the multi-point technique. The system characteristics were gained from applying the multi-point FRAP technique to the aqueous solutions of rhodamine 6G from the spot technique.

The experimental parameters used to examine the characteristics of the multi-point FRAP technique closely followed those of the spot technique described in (1). The same aqueous solutions of rhodamine 6G (4.6×10^{-6} M) were used, as was the same system of sealed microscope slides. A bleaching intensity of 5 mW (level 10) for 1 second was used in these experiments. The observation intensity was 5 μW (level 4) . Data was collected at 5 msec intervals, implying the intensity for each point was measured every 55 msec. The wavelength used in these experiments was 514.5 μm. The emission spot of the beam was focused to 2.0 μm.

The experimental procedure for the multi-point FRAP experiments was nearly identical to the spot technique used by Axelrod (1). The laser photobleaching system was preset with the experimental parameters given above. The sealed sample was placed on the microscope and focused. The sample was then bleached with a high intensity beam (5 mW, level 10) for 1 second. The response at all eleven points was monitored by the system using 5 μW (level 4). These monitored values were collected using A/D channel 13 and written into data files. It was from these recovery intensity data files that the operational characteristics of the multi-point technique were determined.

4.3 Application Results

Both qualitative and quantitative results were determined for the four microscopy techniques to which the laser photobleaching system was applied. The results of the two techniques used to quantize the number

of lamellea in liposomes are presented first. The findings of the application of the two FRAP techniques follows.

The development of the fluorescence contrast technique required the verification of the published data using the phase contrast technique (27). The normalized intensities as afunction of radius for the phase contrast experiments are plotted in Figure 4.4. Figure 4.4 was reproduced from (27) and the results from this study were superimposed on to this figure. The normalized intensities for the phase contrast verification experiments are plotted with zerosn Figure 4.4. Both multilamellar and unilamellar liposomes were chosen for these experiments by a visual selection technique. The results agreed with those measured by Servuss and Boroske (27).

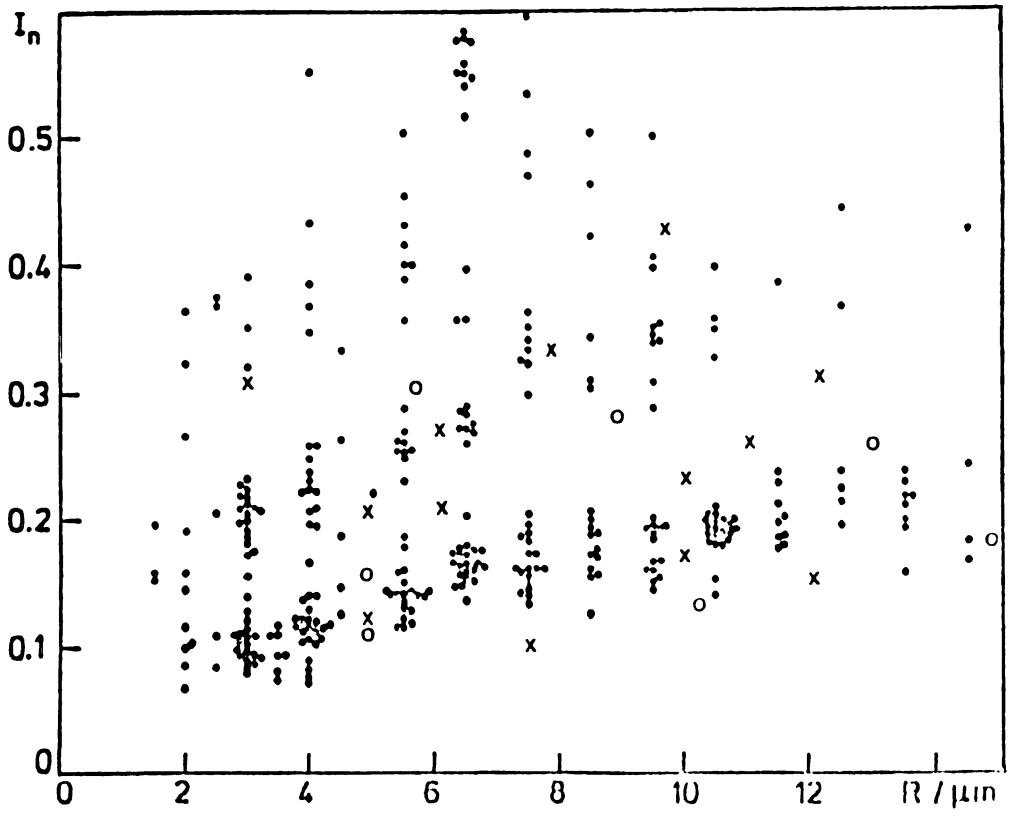

Fig. 4.4 Phase contrast and fluorescent contrast results: x-fluorescent contrast data; o-phase contrast data; ·-Servuss & Boroske phase contrast data; background reproduced from (27).

The normalized intensities as a function of radius for the fluorescent contrast were plotted with X's on Figure 4.4: Again both multilamellar and unilamellar liposomes were selected by a visual selection technique under phase contrast. Although fluorescent contrast intensities cannot be compared closely to the phase contrast intensities, similar trends were seen.

The comparison technique to quantize the number of bilayers in liposomes was a two-stage project. The results of the stages of the development of the technique are presented here in both qualitative and quantitative form.

The initial stage of the comparison technique was the determination of procedures to form BLMs. Using a formation stage designed by R.A. Callow and a hexane-based BLM preparation solution BLMs were able to be "formed" (observed to thin to a "black" state). These BLMs seemed stable for approximately 10-15 minutes in a vertical orientation. All attempts to tranfer the thinned to "black" BLMs to a horizontal orientation on the microscope failed. In each attempt the presence of the fluorescently labeled BLM was determined by exciting the opening in the teflon disks with a mercury lamp source. At this time it was believed that the BLM ruptured due to the shearing forces of the water during removal. The formation stage was redesigned to seal the BLM from the rest of the external water.

The final stage of the comparison technique experiments were designated as an undergraduate independent study project conducted by Serge Weiss. Under direct supervision he conducted formation and transportation experiments, single bilayer standard characterization

experiments, normalizing standard characterization experiments, and liposome characterization experiments. The major results of this independent study project are reproduced and presented as part of this study. For complete results of this independent study refer to (39).

The formation and transportation experiments conducted in (39) can be summarized by the following results. BLMs formed using the hexane based solution in conjunction with the redesigned stage could be formed in 10-15 minutes. These BLMs were stable in the vertical position for 10-15 minutes. The formation stage could be sealed after the BLMs thinned without rupture. Attempts at removing the BLM from the formation tank and placing in a horizontal orientation were not successful.

Further formation and transportation experiments were conducted in (39) after the BLM formation chamber was redesigned, the preparation solution was modified, and the formation tank was replaced. BLMs formed using the octane based solution with cholesterol added could be formed in 10-20 minutes. These BLMs were stable in the vertical position for up to 30 minutes. Sealing, removing, and placing a thinned BLM in a horizontal orientation was successful no more than 5% of the time. Attempts at transporting the thinned BLMs to the microscope to measure their intensities failed (39).

Other methods were tried to form BLMs so that their intensities could be measured. One method sealed the BLM formation chamber before thinning was complete. This method failed since the membrane could not be observed to thin to black. Forming a BLM in a horizontal orientation on the microscope was also attempted. These attempts failed because proper orientation of the light source and binoculars needed to

visualize the diffraction pattern were unobtainable on the microscope. The last method tried was varying the diameter of the aperture over which the BLMs were formed. Six different apertures diameters were investigated (0.125-0.203 inches). Results showed that stability increased with decreasing diameter of the aperture. The time required to form the BLM also increased when aperture diameter decreases. The optimal aperture diameter for BLM formation in 10-20 minutes with increased stability was found to be 0.156 inches (39).

The inability to successfully transport a thinned BLM forced the abandonment of the single bilayer standard characterization experiments. The independent study project continued with the normalizing standard characterization experiments.

The findings from the normalized standard characterization experiments were different than what was expected. The first group of experiments were to determine the reproducibility of a mean intensity for the microspheres. Results of these experiments are reproduced from (39) in Table 4.1.

TABLE 4.1. MEAN FLUORESCENT INTENSITIES OF MICROSPHERES

Group I		Group II	
RUN	INTENSITY	RUN	INTENSITY
1	2.65 ± 0.25 V	1	3.10 ± 0.50 V
2	2.30 ± 0.20 V	2	5.50 ± 0.25 V
3	1.65 ± 0.20 V	3	4.75 ± 0.75 V
4	1.90 ± 0.20 V	4	2.95 ± 0.20 V
5	2.20 ± 0.20 V	5	4.05 ± 0.25 V
6	0.60 ± 0.10 V	6	4.75 ± 0.25 V
7	1.20 ± 0.15 V		
8	0.50 ± 0.20 V		
9	0.25 ± 0.10 V		

These experiments showed the inability of the microspheres to reproduce a constant mean intensity value to be used as a normalizing standard. The microspheres were exposed to laser excitation 5-10 seconds before intensity readings were taken for these experiments. The voltages listed represent the emission intensities detected by the photomultiplier. The errors presented represent the fluctuation of the analog output from the photomultiplier measuring amplifier box. Values in Table 4.1 were determined by exciting the microspheres with 16.7μW (obtained at intensity level 6 with an additional neutral density filter). The two groups of values represent 2 different samples of microspheres at different photomultiplier gains.

Further characterizations of the normalizing standards were determined in long exposure experiments. In these experiments the time histories of the intensities of the microspheres were recorded by the strip chart recorder. A typical response reproduced from data supplied by (39) can be seen in Figure 4.5. These experiments were approximately 10 minutes in length. The microspheres were excited by 16.7 μW at 514.5 nm. The microsphere intensities decreased by approximately 50% during the experiments as shown in Figure 4.5. Also evident from these long exposure experiments records were noise to signal ratios on the order of 1:5 in all cases (39). The noise in all cases was of a high frequency nature.

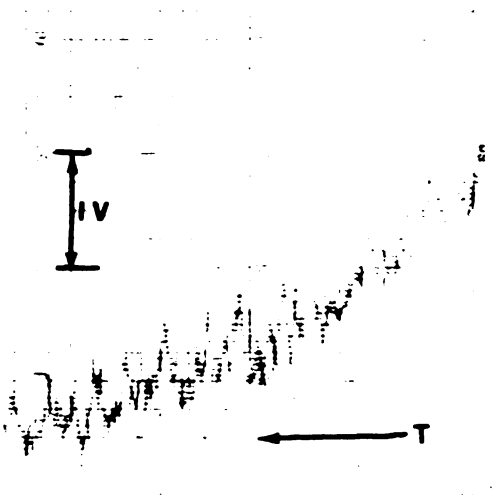

Fig. 4.5 Long term exposure response of fluorescent microspheres reproduced from data supplied by (39).

Still other characterizations of the "normalizing standard" (microspheres) helped determine the laser photobleaching system dependence on the focusing of samples. In the first focusing experiments many different microspheres were taken in and out of focus. Their intensities deviated only 10% from a mean in focus intensity (39)when an excitation intensity of 16.7 μW was used. In other focusing experiments intensity readings were taken at equal distances above and below a microsphere focal plane. These experiments showed less than a 5% difference in mean intensity levels for above and below the sample focal plane (39).

The final experiments conducted as part of the independent study project were liposome characterization experiments. Long exposure time characterizations of liposomes also showed a decrease of intensity. These decreases of intensity were typically 10-30% for the 10 minute

length of the experiment (39). Even more evident were the large noise to signal ratios, 1:4 in most cases (39). Typical long term liposome response can be seen in Figure 4.6, reproduced from (39). Another point to be made about Figure 4.6 is how erratic the high frequency noise of the signal was.

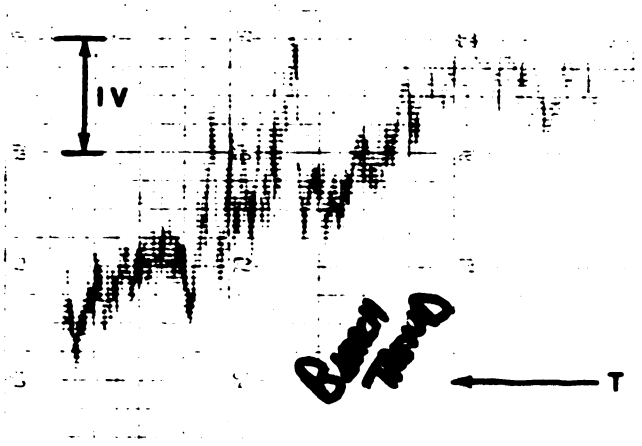

Fig. 4.6 Long term exposure response of liposomes reproduced from data supplied by (39).

The major results of this study came from the remaining two applications of the laser photobleaching system. These two applications were the spot FRAP technique and the multi-point FRAP technique. The results from the multi-point technique provided only preliminary information. The spot technique confirmed FRAP capabilities of the the laser photobleaching system.

The preliminary information gained by the multi-point technique application covered many areas. The laser system with a 55 m sec cycling time was unable to monitor the diffusion of rhodamine solution with calculated 8 msec characteristic time for diffusion for 2 μm diameter focused beam . In the quantitative multi-point experiments it was found that the emission intensities for the eleven locations (±5 μm from the center point) decreased the farther the beam was from the optical center. In the qualitative multi-point experiments it was found that the emission "spot" became more asymmetrical the farther the beam was from the optical center. These multi-point experiments also revealed that an average of many initial intensities should be taken instead of only one initial intensity for a sample.

With the experience and information gained from the multi-point experiments some successful applications of the spot FRAP technique were obtained. Of the sixteen spot-technique experiments conducted, twelve produced some type of recovery. The remaining four experiments showed no change in detected intensities. The unchanging intensity was due to not supplying enough energy to bleach the sample (intensity level 7). Of the twelve experiments displaying some type of recovery seven displayed an "expected" fluorescence versus time exponential recoveries. A typical "expected" recovery for an experiment can be seen in Figure 4.7. The other five experiments surpassed the initial intensities measured for the sample then in time returned to a value slightly less than the initial intensity. A typical response of an "abnormal" recovery can be seen in Figure 4.7.

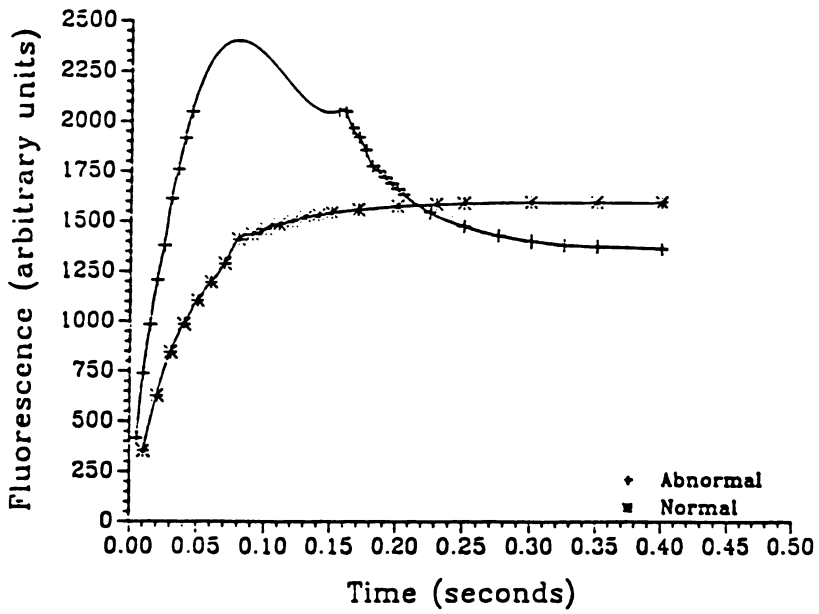

Fig. 4.7 Typical recovery responses for spot - FRAP technique

The diffusion coefficient for 4.6 x 10^{-6} M rhodamine in water was calculated by two methods from the spot technique recovery data. The first method was a three point fitting method used by (1). This three point method predicted a mean diffusion coefficient for the seven exponential recoveries to be (1.22 ± .68) x 10^{-6} cm^2/sec. The error of the calculated mean diffusion coefficient reflects the mean of standard deviation from the seven recoveries. The diffusional coefficients calculated using the three point method can be seen in Table 4.2. The errors listed reflect the uncertainty in beam diameter (±0.5 μm) and in the recovery time (±2.5 msec).

Table 4.2 Diffusion Coefficients

Exp.	Three Point Method ($1x10^{-6}$ cm^2/sec)	Least Squares Fit ($1x10^{-6}$ cm^2/sec)
41	1.11 ± 0.56	0.20 (-0.99)
42	1.30 ± 0.71	0.44 (-0.98)
49	1.13 ± 0.76	0.79 (-0.98)
50	1.15 ± 0.78	0.79 (-0.98)
51	1.13 ± 0.76	0.79 (-0.98)
52	1.36 ± 0.59	0.67 (-0.97)
53	1.36 ± 0.59	0.67 (-0.97)

A technique using a least squares fit of the data predicted lower diffusion coefficients for ($4.6x10^{-6}$ M) rhodamine solutions. The mean diffusion coefficient of the least squares fits was 0.62×10^{-6} cm^2/sec. The mean of the standard deviations of these seven diffusion coefficient values was $\pm 0.22 \times 10^{-6}$ cm^2/sec. The diffusional coefficients calculated using a least squares fit can be seen in Table 4.2. The correlation coefficients for each of the least squares fits are listed in parentheses in Table 4.2.

The five "abnormal" recoveries could not be explained. Yet they did provide substantive information about the laser photobleaching system. These recoveries revealed that a mismatch between the A/D converter and PMT output existed. They showed that shorter experimental run times could be used for rhodamine in water experiments. Finally, these abnormal recoveries showed that the emission intensities were constant for periods much greater than the diffusional characteristic time for the (4.6×10^{-6} M) rhodamine solutions.

5.0 Discussion

The results from the applications of the laser photobleaching system revealed the system is functional within certain limits. These limits were due to problems and situations faced throughout the development and applications of the laser system. The solutions, explanations, or evasions of these problems and situations is presented in this section. Then data reduction methods, and application results are discussed.

The laser photobleaching system hardware posed many problems. The energy generation group, beam manipulation group, and light detection group of the hardware each provided major difficulties.

The energy generation group problems began before the laser and power supply arrived. The laser and power supply initially proposed was an air cooled 300 mW Lexel Argon Laser. Instead a water cooled 4W Spectra Physics Argon Laser was purchased this required the building of a heat exchanger. Constructing the heat exchanger, even though it was simple to design, added extra time to the development of the system.

The arrival of the laser brought further problems. The laser arrived in a damaged shipping crate. It was unknown whether the laser was seriously damaged or not. Also, since this was a demonstration model, no replacement was available. The shipping company refused to cover the loss of the laser until it was shown to be damaged. Many weeks were wasted because there was no service contract or technical support supplied by Spectra Physics for setting up and testing the laser. Ron Hass a technician from M.S.U.'s chemistry department,

finally was able to help show that the laser "worked".

Indeed, the laser "worked" but not to the manufacturer's specifications. The problem apparently stemmed from the mishandling of the laser during shipping. The laser system was brought up to specifications by re-aligning the laser tube within the field magnet and centering the beam in the laser tube. Again, due to the lack of a service contract, Ron Hass provided instruction on centering the tube in the field magnet, centering the beam in the tube, and cleaning the laser optics. Many weeks were spent optimizing the lasers output to meet the manufacturer's specifications. Complete details for these power optimizations methods are presented in Appendix A.

More extensive usage of the laser disclosed one last major problem with the energy generation group. The laser field magnet required a large current to handle its enhancement for ultra violet work. This large current requirement kept blowing fuses in the power supply when run above 30 amps. It was found ultimately that Spectra Physics had mistakenly supplied and installed the fuses for a non-ultra violet enhanced laser. Ron Hass and Spectra Physics spent many more weeks trying to isolate this problem.

After the laser was operating properly, difficulties were discovered in the beam manipulation group. The problems were in the areas of attenuation, direction, filtration, fixation, position, and reduction.

The problem with attenuation of the laser beam was that multiple reflections of the beam seen at the sample were introduced by the neutral density filters. The multiple reflections were caused by the beam reflecting off the surface of a neutral density filter and

striking another filter. The mistaken belief that the filters had to be critically aligned to avoid these reflections wasted time unnecessarily. The multiple reflections were in fact eliminated by arbitrarily positioning the neutral density filters to avoid having the beam reflect on any other filters.

Multiple reflections were also a problem with the filter sets for the laser system. These reflections occurred where the beam reflected off the surfaces of the excitation, barrier and dichroic mirrors of the filter sets. Some of the reflections were removed by eliminating the excitation filters from the beam path. The excitation filters were deemed unnecessary because the laser was being operated in single wavelength mode at 514.5 nm. Other reflections of the beam were eliminated by aligning the barrier filters properly in the epi-illuminator housing of the microscope.

The last multiple reflection was caused by the dichroic mirror. The dichroic mirror had been improperly installed. This caused the beam to be reflected down to the sample by the front (substrate) surface and then the (reflective) coated surface. The proper orientation of the dichroic mirror was obtained by placing the coated surface nearest to the beam's source. This re-orientation of the dichroic mirror eliminated the final multiple reflection.

The next difficulty which arose in the beam manipulation group was the alignment of the optical axis of the laser beam with the optical axis of the microscope. The first step was to affix all the equipment to the optical table. This provided a frame reference for the optical paths of the laser and microscope. The next step was to direct the beam

to the back of the microscope keeping it parallel or orthogonal to the optical table (reference frame) at all times. The final step was to use the multiple degree of freedom microscope mounting plate to align the optical path of the microscope to the beam. Attempts at aligning the beam optical path to the microscope optical path failed. Complete alignment techniques are presented in Appendix A.

The most time consuming problem with the beam manipulation group was the reduction of the beam. Three methods were applied to reduce the beam from 1.25 mm at the source to the 1-2 μm diameter desired at the sample focal plane. Two of these methods proved unsatisfactory. The first unsatisfactory method came directly from optical physics. Parallel light (source) passed through two convex lenses with the correct ratio of focal lengths (magnification) produces parallel light at a reduced diameter an "inverted telescope". The problem with this "inverted telescope" method was that the incoming beam did not fill the entire lens. That caused an interference pattern to also be visible at the sample focal plane. This interference pattern appeared as concentric rings with the desired spot diameter in the center point.

The second unsuccessful beam reduction method was a variation of the "inverted telescope" method. This method tried to eliminate the concentric interference rings with pinhole stops. The pinhole stops seemed to work, but, there was no way to mount and position them accurately. If these stops were positioned improperly other interference patterns were introduced.

Finally, a method suggested by Jack Holland, Ph.D., a professor for the Biochemistry Department at M.S.U. solved the beam reduction problem. He suggested placing a convex lens in the beam path and matching its

focal point to the effective focal length of the microscope focusing objective. This method worked for observing intensities but a diffuse emission pattern could be seen around the 2 μm emission spot of the beam for bleaching intensities greater than level 7. This diffuse emission pattern occurred because the beam did not diverge enough thus exciting any fluorescent probe in the area surrounding the beam. This reduction method was used even though the fluorescent probe surrounding emission spot was being excited during bleaching. It was assumed that the fluorophore in this diffuse region would not receive enough energy to be bleached thus not affecting the recovery. The spot FRAP experiments showed that this assumption was valid.

Two final problems of the beam manipulation group did not become apparent until the system was being used for the FRAP application experiments. The first problem was that the beam was only able to scan 11 μm at the sample focal plane. This limited scanning length was due to the diffraction of the beam as it was moved off the optical center of the reducing lens and the microscope objective. This problem was evaded by limiting the scanning length to 10 μm (±5 μm from a center point).

The other problem found while trying to apply the system to was FRAP experiments was the low reflectivity of the scanning mirror. When at bleaching or observing intensity the scanning mirror permitted an undetermined portion of the beam to be transmitted through its reflective surface. This problem had no effect on the determination of the diffusion coefficient since normalized intensities were used. However, the theoretical fluorescence could not be calculated since attenuated power supplied to the sample was unknown because of this low reflectivity.

Difficulties with the light detection group appeared during the quantization application experiments using the laser photobleaching system. The erratic output of the photometer system during characterization experiments was a major concern. A typical erratic emission response from a 2 μm spot of a fluorescently labeled liposome can be seen in Figure 4.6 in chapter 4. The erratic outputs did not permit the determination of anything but trends from liposomes and microspheres. This erratic output was corrected by eliminating 60 cycle noise and high frequency noise. The addition of a 470 μF capacitor to the reference voltage circuit of the photomultiplier tube eliminated the high frequency noise. The 60 cycle noise was eliminated by converting the PMT measuring amplifier ground from an earth ground to a floating ground .

Another problem with the light detection group which was found during characterization experiments was a low frequency drift of the photometer output. The period of this drift has been measured to be 11-13 minutes. The magnitude of the drift was approximately 15-20% of the total output. This amplitude drift has not been solved. A "period" of the low frequency drift (oscillating output) of the photometer can be seen in Figure 5.1.

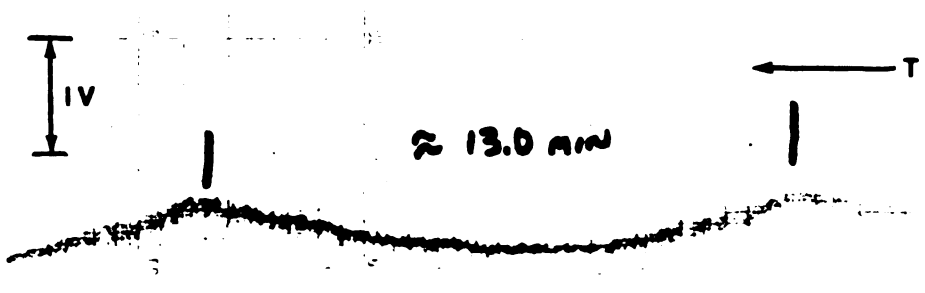

Fig. 5.1 Low frequency drift of the photometer output.

The noise present in Figure 5.1 was due to the expanded scale used (5 V fullscale). This low frequency drift was avoided by using rhodamine in water with an 8 msec characteristic time for diffusion.

The final difficulty with the light detection group was a mismatch of the PMT analog output and the A/D range of the computer. This problem resulted from not understanding that a bipolar 10.24 V A/D board meant ±5.12 V not ±10.24 V. The mismatch went unnoticed until the final stages of this study. This mismatch has not been corrected.

As the system development progressed problems with the software also became apparent. The first software difficulty to arise was the inability to change the output filename interactively using FORTRAN IV. The software was rewritten to allow the changing of the run number after each experiment. The output filename now has the form of FTN--.DAT, where the spaces represent a number between 10 and 99.

Another software problem was the limited speed in which the interrupt service routines (ISRs) could be operated. The ISRs could only be operated at 200 Hz (5 msec) response without causing a computer failure. It is believed this rate may be improved by using machine language programs. Data collected at 5 msec rate supplied 5-6 points to calculate the diffusion coefficient of rhodamine solution using the least squares fit.

Application of the system revealed another fault with the software. The software only acquired one intensity reading for the initial concentration intensity of the sample before bleaching. Taking just one reading did not allow for the possibility of an extraneous intensity reading. Extraneous intensities were compensated by taking ten readings

and then using the mean of these as the initial concentration intensity. Further use of the system showed that a larger sample should be taken, but this change was not incorporated into the software.

Still another difficulty with the laser photobleaching system software was it did not allow the operator to plot results. This graphics capability has not been incorporated into the operating system because of the incompatibility of an ADM-3A terminal with the graphics package PLOT 10 (TEKTRONICS). The ability to see the "recovery" for an experiment would provide the operator with an immediate check for the proper functioning of the system.

The final problem with the software evolved from the conversion from the multi-point-technique to the spot technique. The conversion was made because the recovery of the 2 μm bleached spot was too fast for the 55 m sec cycle of the multi-point technique. The only software change to the multi-point operation was not to move the scanning mirror. In making this change the system was able to collect data at 5 msec intervals, required to be moved from one position to the next in the multi-point technique. The 5 msec interval was fast enough to obtain at least 5-6 points before the bleached spot of the rhodamine solution had recovered. It was from these 5-6 points that the diffusion coefficients were determined. Separation of the two techniques should improve on the 5 msec sampling rate presently obtainable for the spot technique.

The application and data reduction of the FRAP experiments disclosed other difficulties. The 5 msec cycling time of the spot technique did not permit the measurement of the fluorescence intensity immediately after bleaching. The intensity at zero time was required for Axelrod's three point fit method (1) to determine the diffusion coefficient. In

this study, the zero time intensity had to be approximated. The approximation was made assuming $f(t) = 1 - e^{-t/\tau_o}$ for the fluorescence recovery. Using a least squares fit of the recovery data in the following form

$$\ln\left(\frac{(F(\infty) - F(t))v}{10\ v}\right) = \left(-\frac{4D}{w^2}\right) t + \ln\left(\frac{(F(\infty) - F(o)v}{10v}\right) \quad (5.1)$$

provided the slope $(-4D/w^2)$ and the fluorescence at t=0. This approximation was the basis for the least squares method of determining the diffusion coefficient D. The diffusion coefficient was determined from the slope of equation 5.1. A complete derivation of this equation can be seen in Appendix G.

Diffusion coefficients determined using the three point fit method (1) were compared to the values determined from the slope of the least squares fit method. The values can be seen in Table 4.2. The mean values of the diffusion coefficients for each method generally varied by a factor of two. The largest difference predicted by the two methods varied by a factor of five. Determination of which method was predicting the more accurate value for the diffusion coefficient was not made. It should be noted that Axelrod (1) uses only three points to determine the diffusion coefficient. The least squares fit method used all the recovery intensities.

The possible differences in the calculated diffusion coefficients of this study originated from a few sources. One source was the assumption made in developing the least squares fit using $f(t) = 1-e^{-t/\tau_o}$ instead of $f(t) = 1 + Be^{-t/\tau_D}$ or the infinite series solution Equation 2.6. By assuming B = 1.0 it was assumed that complete recovery occurred. This

assumption was supported by the mobile fractions of greater than 95% found by (1) for rhodamine solution. Another source of the differences in the predicted values was that one Axelrod's method used only three points F(0), F(∞), and $F(\tau_{1/2})$ to predict D, while the least squares fit used all the data points.

The results obtained during this study by using the three point fit method (1) for determining the diffusion coefficients were compared to those obtained by Axelrod et al (1). They agreed within stated error ranges. Axelrod predicted the diffusion coefficients to be (1.2 ± .3) x 10^{-6} cm^2/sec for solutions of rhodamine in water (1) in 12 samples. The laser photobleaching system predicted a mean diffusion coefficient to be (1.22 ± .68) x 10^{-6} cm^2/ sec for a sample size of 7.

The larger error for the diffusion coefficient prediction was due to the sample size of only 7, a 25% uncertainty in the effective beam diameter, the uncertainty of the fluorescence immediately after bleaching (F(0)) and the uncertainty of the actual time when the normalized fluorescence recovery was equal to one half ($f_k^m(t) = 0.5$). The effective beam diameter as stated in Table 3.3 was (2.0 ± 0.5) μm for the 20x objective. The time uncertainty of ± 2.5 msec for the normalized fluorescence intensity reflected 5 msec sampling rate.

The quantization application results raised questions of intensity requirements, probe selection, single bilayer standard formation, and normalizing standard selection. Concerns about the intensity requirements appeared during the characterizations of the fluorescently labeled microspheres and the liposomes. The long term exposures shown Figure 4.5 and 4.6 both displayed a definite decrease in intensity over ten minutes using a 16.7 μW observing intensities. It was initially

believed that this observing intensity had too high an energy density. This high density caused "bleaching" of the fluorescent probe in the sample region. However, when the energy densities used by others (18, 19, 40) were compared to the energy densities supplied by the laser photobleaching system the density level was not too high. In Table 5.1 the energy densities used by others for bleaching the diI fluorescent probe are presented.

Table 5.1 - Bleaching Energy Densities

System	energy density $w/\mu m^2$	Length of Exposure
Axelrod (40)	6.5×10^{-3} $w/\mu m^2$	1.0 sec
Schlessinger (18)	9.1×10^{-3} $w/\mu m^2$	0.5 sec
Wolf (19)	9.1×10^{-3} $w/\mu m^2$	0.15 sec

The energy density used in this study (2.6×10^{-6} $w/\mu m^2$) was three orders of magnitude less than the bleaching energy density recommended by (18, 19, 40) for observation. Some decrease of fluorescence was expected for long exposures, but 30-50% decrease in 10 minutes was unacceptable.

The diI probe had been selected because of its widespread use. No characterization of the probe was attempted before its use. Therefore no further explanation of the decrease in emission intensity of the probes in the 10 minute exposure to the 16.7 μW intensity can be made at this time.

The results from the single bilayer formation and transferring attempt showed that the microbiology background and experience in successfully forming a single bilayer standard was absent. A basic understanding of what BLMS were and how they were formed was obtained. However, a physical understanding of why the BLM formation and transportation failed has yet to be ascertained.

The results from the characterization experiments of normalizing standards showed that the microspheres were of no use when using diI as the fluorescent probe in liposomes. The emission intensities of the fluorescently labeled microspheres and liposomes could not be taken with the same PMT gain settings. Further, the emission from the microspheres decreased twice as much as fluorescently labeled liposomes for 10 minute exposures.

6.0 Conclusions

Based upon the results of this study the following conclusions can be made:

1) A laser photobleaching system for use with unique microscopy techniques has been developed.

2) The laser photobleaching system has been shown to be functional although limitations exist. These limitations include: a) low frequency drift in photometer output, b) low reflectivity of the scanning mirror, c) confined beam scanning length on a sample focal plane, and d) mismatched analog to digital input range (±5.12 V) photometer output range (0 - 10 V).

3) The laser photobleaching system using a spot Fluorescence Recovery After Photobleaching technique has predicted lateral diffusion coefficients. The predicted lateral diffusion coefficients of rhodamine (4.6×10^{-6} M) in water were within the same order of magnitude as values published by Axelrod (1).

4) The laser photobleaching system was unable to monitor the recovery of rhodamine in water using a multi-point Fluorescence Recovery After Photobleaching technique. The 55 m sec cycling

time for the multi-point technique was too slow for the calculated 8 m sec characteristic time for diffusion of rhodamine in water.

5) The development of a microscopy technique using the laser photobleaching system to quantitatively determine the number of bilayers present in liposomes produced inconclusive results. Both the fluorescence contrast and comparison methods of quantization of the number of bilayers require further study to be classified as valid quantization techniques.

6) To use the laser photobleaching system to its maximum potential will require more collaboration with researchers who have an extensive background in biochemistry or microbiology.

7.0 Suggestions for future work

The results of this study displayed only some of the capabilities and limitations of the laser photobleaching system. During this study several components of the laser system required corrections. However, some applications of this laser system worthy of future investigations were disclosed.

Many components of the laser photobleaching system hardware need to be replaced, repaired, or redesigned before further applications are conducted. Of the items requiring replacement the scanning mirror with its poor reflectivity should have the top priority. Uncertainty about its reflectivity prevented precise energy density calculations. Measurement of the energy lost due to this mirror should be made. Other system components that should be considered for replacement are the stand for the fifth neutral density filter and the convex lens which reduces the beam diameter. The fifth neutral density filter, which allows additional attenuation of the beam, is held in place with masking tape. The convex reducing lens produces a diffuse emission pattern around the focused beam for bleaching intensities and should be replaced.

Some of the computer system components should also be considered for replacement. The printer should be replaced as soon as possible. It is needed for both printed hardcopy and graphics. Another component which should be replaced is the graphics terminal because its resolution is too low. The last computer component that should be replaced is the parallel line unit (transistor logic input-output board DRV11, D.E.C.). On the DRV11 board presently in use 4 of the 16 channels are not functioning.

The only laser photobleaching system component which required repair was the photometer. The existing low frequency drift of the photometer output will effect the results of FRAP experiments with larger characteristic times for diffusion. The problem has been isolated to the photomultiplier tube or the electronics in the photomultiplier tube housing. The electronics of the measuring amplifier box have been thoroughly examined and eliminated as the source of the low frequency drift of the output of the photometer.

Components that should be considered for redesigning are the beam directing and reducing groups and the beam scanning system. The beam directing and reducing groups presently direct the beam in the rear of the microscope to be reduced. If replacement of the reducing convex lens does not eliminate the emission pattern around the focused beam, an alternate beam path to the sample should be considered. The beam scanning system must be repositioned to allow for a longer scanning range at the sample focal plane. The scanning range is presently only 10 μm at the sample focal plane.

Some components of the laser photobleaching system software need to be modified or added to the operating systems before further applications are conducted. The highest priority for operating system software should be given to separating the spot and multi-point FRAP techniques into different subroutines. Separation of the two techniques should elicit a quicker data collection cycle for the spot FRAP technique than the present rate of 5 msec. Compensation for the A/D board and photometer output in the operating system software should be made until the hardware mismatch can be eliminated. The interrupt services routines also need to be changed. The cycling times of the spot and

multi-point techniques should be reduced by converting the interrupt service routines from FORTRAN IV to MACRO-11. A final software component that needs to be added to the operating system is graphics. Graphics capability would allow immediate recognition of problems with fluorescence recoveries. Before graphics capability can be added the present graphics software needs to be made compatible with the present terminal.

After making the necessary corrections to the laser system components, proposed applications of the system should include further verification of application experiments conducted in this study as well as some new applications. The spot FRAP technique should be the first application to be verified. This verification is needed to understand and explain why both "expected" and "abnormal" recoveries were detected in this study. Next the multi-point FRAP technique should be examined. The present study failed to verify the FRAP multi-point technique capability. Both techniques of quantization using laser photobleaching require further investigation. The fluorescent contrast method of quantization showed some promise as a technique for determining single bilayer liposomes. However no solid conclusions could be drawn from the small sample size of fluorescence contrast information. The comparison method of quantization method is based on a sound hypothesis, but an easily formed single bilayer standard and a stable normalizing standard are needed. Finding two workable standards would allow evaluation of this technique as a method of quantization.

The new applications that should be investigated are the next logical step in the progression of the laser photobleaching system's capabilities. The most interesting application would be the integration of image analysis with the present system. With such an integration

researchers could eliminate the photometer system. This elimination would be possible because the image analysis system has the capability to resolve intensity differences. The image analysis system would also allow for data collection over an increased area. This increased area would be equal to the viewing area of the low light video camera. The image analysis system would not reduce the 5 m sec cycling time of the interrupt service routines (ISRs) for the multi-point system. The image analysis is limited by the 33 m sec per frame state of video tapes. Intensities at multiple points could be monitored simultaneously by the image analysis system.

Even without image analysis integration, there are other new applications which would expand the capabilities of the laser photobleaching system. The first two applications that should be conducted involve using the laser photobleaching system on a membrane system. These studies would be important for further verification of the spot and multi-point FRAP technique capabilities of the system.

Another interesting application for the system would be determining the effect of temperature on the diffusion of molecules in a membrane. This study, if used in conjunction with image analysis, could disclose some of the mechanisms which cause damage during freezing.

The present study has demonstrated that the laser photobleaching system is operational. The system has some limitations, but once these are resolved a very powerful experimental tool will exist. It will then be up to other researchers to expand and refine the laser photobleaching system and its applications.

APPENDICES

APPENDIX A: LASER OPERATION AND CARE

Laser Operation and Care

A.1: Laser Start Up

1. Turn on tap water to heat exchanger (5-6 gal/min)
2. Open valve for flow and pressure bleed off downstream pump.
3. Turn on pump for recirculating cooling water.
4. Adjust water pressure going to laser to be 40 psi (30-50 psi limits for laser) this will provide ≈ 2.5-3.0 gal/min. Replace water filter if under 2.2 gal/min.
5. Turn on the main wall circuit breaker box.
6. Turn power supply key on.
7. Set the control settings to the following:
   + meter - 30 A
   + current - 50% of fullscale
   + mode - current
   + field - maximum
8. Check for water indication light on the power supply
9. Turn on power supply circuit breakers
10. Wait 30 seconds (start light will glow) and press start
11. If fails refer to operating manual.
12. One half hour is required for stabilized operation.

A.2: Laser Shut Down

1. Turn off laser at power supply circuit breakers
2. Turn off key of power supply

3. Turn off main circuit breaker
4. Continue running heat exchanger until water returning from laser is 20°C
5. Open pressure valve, pressure should drop to zero.
6. Turn off recirculating pump, and then close pressure valve.
7. Turn off tap water.

A.3: Mode - Selection

The laser has two operating modes, the current mode and the light mode. The current mode regulates the power output by regulating the current supplied to the laser tube. The light mode regulates the power output by sampling beam and adjusting the current accordingly. The current mode is claimed to have a stability of ± 3% after 30 minute warm up and only 1% rms noise (at 514.5 nm) (49). The light mode is claimed to have a stability of ± 0.5% after 2 hour warm up and 0.2% rms noise (49). The current mode is safer to use since any time the beam is blocked the light mode supplies maximum current to counter the drop in the sampling intensity.

The selection of modes should be done after start-up. The settings used before changing modes should be at start-up values. Then to select mode flip mode selection switch.

A.4: Cleaning and Peaking Output

In lasers losses due to unclean or misaligned optics, which might be negligible in other optical systems, can cause enough loss to disable a laser. Dust, films, and atmospheric contaminants on any of the optical surfaces can cause loss of output power or operational failure. Even with clean optical surfaces the output power may be limited by misalignment of the optics. The required materials and procedures for general cleaning and peaking are listed below. If the laser output still does not meet specifications refer to advanced techniques listed in the operating manual (49).

Materials -

1. Filtered, Nitrogen (25 μm limit on filter mesh)
2. Lens cleaning tissue
3. Cotton swabs
4. Hemostat (non-metallic preferred)
5. Acetone (CH_3COCH_3 spectroscopic grade
6. Methanol (CH_3OH) spectroscopic grade
7. Power meter

Procedure - If specified power output is achieved prior to the completion of all the steps, the remaining steps are optional.

I. Peaking Output - Complete peaking only required once every 2 months or before laser changed to single-line mode. Before any cleaning step the laser should be peaked for maximum output using multi-line lasing. Note: when peaking output of laser work at one end only. In the case of losing lasing capabilities it will be easy to get lasing again. If lasing is lost refer to operation manuals trouble shooting section (49).

1. Before laser start-up remove cover and insert cover interlock defeat plug.

2. Start-up laser. Allow a half hour for warm-up.

3. Always start at rear end plate (anode end, where cooling water is supplied) adjust vertical adjustment thumbwheel until power is peaked. Next adjust horizontal adjustment thumbwheel until power is peaked. If lasing is lost do not touch any other adjustment. Using adjustment thumbwheel used when lasing was lost turn back slowly until lasing re-established. Finish peaking using that thumbwheel. Repeat cycle at the rear end plate until no more output power is gained.

4. After peaking power at rear end peak output power at the front end plate (cathode end, where laser beam is emitted). Do not touch any other adjustments until peaking at the front plate is completed. Again start with vertical adjustment peak then go to horizontal. If lasing lost use that adjustment set screw without touching another to get lasing to return. Repeat cycle at the front end plate until no more output power is gained.

5. Repeat steps 3 & 4 until no more output power is gained.

6. If specified power is not achieved yet start cleaning procedure.

II. Cleaning - Clean only one optical component at a time. If lasing lost after cleaning and replacement refer to operation manual (49).

1. Always start with rear end mirror, then front end, then front Brewster window, and then rear Brewster window.

2. Repeat steps 1-4 for each optical component.

    1. Blow away dust with filtered air.
    2. Use folded (1/2" square) lens tissue held in hemostat when applying methanol or acetone.

3. Start wiping with methanol. Wet tissue rid excess by shaking. Wipe with even pressure only once over surface. Replace lens tissue. Repeat but wipe in a direction 90 degrees to previous wipe. Repeat two more times.
4. Replace optical component.
5. If specified power still is not achieved then repeat above cleaning steps 2-4 but adding wiping with acetone before wiping with methanol.
6. If specified power still is not achieved try peaking power again.
7. If still does not achieve specified power levels go to advanced cleaning steps outlined in operation manual (49).

NOTE: Cleaning prism for single-line operation DO NOT TAKE APART. Use cotton swabs with methanol or acetone to wipe surfaces clean.

A.5: Lasing -- Multi-Line

Multi-line operation is used for complete peaking laser power (required every 2 months or before laser changed to single-line operation). Multi-line operation achieved by just inserting planar rear end mirror into rear end plate. Peak power only at rear end plate after insertion. Other end should not have changed.

A.6: Lasing - Single-Line

Laser must be completely peaked before inserting prism for single-line operation. The following procedure is then required to peak the power for single line operation. According to Spectra-Physics the prism for single-line operation and the planar mirror for multi-line operation can be interchanged without realignment. But for this laser realignment is required to peak power of the single line. If the prism has not been jarred the laser should lase when the prism replaces the planar mirror after complete peaking (APPENDIX A.4).

Peaking single-line power is required if laser output at the desired wavelength is less than 25% of the "ideal" specified power. (49). Always peak prism initially at the 514.5 nm (bright green) wavelength.

Warning: the procedure in the operation manual is the only way to peak the power for single line operation.

The procedure sounds easy in theory but is very difficult in practice. Listed below are a few hints that will help.

1. After each adjustment to the prism replaces the planar mirror (multi-line). Repeak using rear end plate only then replace prism to see if further adjustment is needed.

If lasing is lost use "rocking procedure" discussed in manual (49) to find which direction the prism must be moved.

2. To see where prism is deflecting beam insert piece of paper in path of beam. Center reflection about laser tube using adjustments in step 3 to get it to lase if it was not lasing before. If the laser was lasing the rocking procedure discussed in manual (49) will indicate which adjustments in step three will be needed.

APPENDIX B: LASER PHOTOBLEACHING SYSTEM PROGRAMS

B.1 CONTROL ONLY OPERATING SYSTEM

```
      PROGRAM LOPERA
C-----------------------------------------------------------------
C       THIS IS A PROGRAM THAT CONTROLS THE NEUTRAL DENSITY FILTERS,
C       AND SCANNING MIRROR FOR BASIC LASER OPERATION (LOPERA). THE
C       PROGRAM COMBINES MANY OF THE SAME SUBROUTINES USED IN THE
C       LASCON PROGRAM (F.R.A.P. CONTROL). THE PROGRAM MUST BE LINKED
C       WITH THE FOLLOWING SUBROUTINES MPOSIT,FPLACE,DTACHA,AND CLOCIN
C-----------------------------------------------------------------
C
C-----------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------
      COMMON /DTOA/IVLTS1,IVLTS2,IVLTS3,ICOUNT,ICYCLE,IVB,ISLOPE,
     +IDONE,IDACHA
      COMMON /CLOCK/ ICLBPR,ICLCSR,I2COMP,ICLKST,ICLKGO
      COMMON /SHUT/ IDRV11,ILSHUT,IPSHUT,IBSHUT,ISHUT,ISHUTN
      COMMON /FILTER/ OBSERV,OBSVAL,BLECHV,BLEVAL,TRNSO,TRNSB
      COMMON /FILE/ FNAME
      INTEGER OBSVAL,OBSERV,BLEVAL,BLECHV
      REAL RESP
      REAL*8 FNAME
      DIMENSION IDLIST(21)
      EXTERNAL DTACHA
C
C-----------------------------------------------------------------
C       IDLIST FOR RESETTING REGISTERS TO ORIGINAL STATUS
C-----------------------------------------------------------------
      IDLIST(1)="167770              !DRV11 C.S.R.
      IDLIST(2)=IPEEK("167770)       !CONTENTS DRV11 C.S.R.
      IDLIST(3)="167772              !DRV11 OUTPUT BUFFER
      IDLIST(4)=IPEEK("167772)       !CONTENTS DRV11 O.B.
      IDLIST(5)="170420              !R.T. CLOCK C.S.R.
      IDLIST(6)=IPEEK("170420)       !CONTENTS R.T. CLOCK C.S.R.
      IDLIST(7)="170422              !R.T. CLOCK B.R.
      IDLIST(8)=IPEEK("170422)       !CONTENTS R.T. CLOCK B.R.
      IDLIST(9)="177000              !A/D C.S.R.
      IDLIST(10)=IPEEK("177000)      !CONTENTS A/D C.S.R.
      IDLIST(11)="177002             !A/D B.R.
      IDLIST(12)=IPEEK("177002)      !CONTENTS A/D B.R.
      IDLIST(13)="170440             !D/A C.S.R. A
      IDLIST(14)=IPEEK("170440)      !CONTENTS D/A C.S.R. A
      IDLIST(15)="170442             !D/A C.S.R. B
      IDLIST(16)=IPEEK("170442)      !CONTENTS C.S.R.B
      IDLIST(17)="170444             !D/A C.S.R. C
      IDLIST(18)=IPEEK("170444)      !CONTENTS C.S.R. C
      IDLIST(19)="170446             !D/A C.S.R. D
      IDLIST(20)=IPEEK("170446)      !CONTENTS C.S.R. D
      IDLIST(21)=0                   !END OF LIST FLAG
C-----------------------------------------------------------------
      CALL DEVICE(IDLIST)     !UPON EXIT RESETS REGISTERS IN IDLIST
C-----------------------------------------------------------------
```

```
C       INITIAL VALUES
C-------------------------------------------------------------------
        IDRV11="167772  !DRV11 OUTPUT BUFFER ADDRESS
        ILSHUT="20      !OCTAL VALUE TO SHUT LASER SHUTTER
        IPSHUT="40      !OCTAL VALUE TO SHUT PMT SHUTTER
        IBSHUT="60      !OCTAL VALUE TO SHUT BOTH SHUTTERS
        ISHUTN=3                !SHUTTER FLAG BOTH CLOSED
        ISHUT=IBSHUT            !OCTAL VALUE FOR BOTH CLOSED
        OBSERV=1                !INTENSITY LEVEL 1
        OBSVAL="0               !OCTAL VALUE FOR LEVEL 1
        TRNSO=.000050           !PERCENTAGE TRANSMIT OBS.MODE
        BLECHV=7                !INTENSITY LEVEL 7
        BLEVAL="60000           !OCTAL VALUE FOR LEVEL 7
        TRNSB=.050              !PERCENTAGE TRANSMIT BL. MODE

C-------------------------------------------------------------------
        CALL IPOKE(IDRV11,IBSHUT)       !CLOSES BOTH SHUTTERS
10      CONTINUE
        WRITE(7,1)
        WRITE(7,1)' LASER OPERATION MENU--ENTER E-TO EXIT PROGRAM,'
        WRITE(7,1)' I-TO SELECT BEAM INTENSITY AND SELECT SHUTTER'
        WRITE(7,1)' CONDITIONS,P-TO POSITION BEAM'
        READ(7,20) RESP
20      FORMAT(A1)
        IF(RESP.EQ.'E')GO TO 999
        IF(RESP.EQ.'I')GO TO 40
        IF(RESP.EQ.'P')GO TO 30
        WRITE(7,1)' UNABLE TO DETERMINE RESPONSE'
        GO TO 10
30      CONTINUE
        CALL MPOSIT
        GO TO 10
40      CONTINUE
        CALL FPLACE
        GO TO 10
999     CONTINUE
        CALL IPOKE(IDRV11,"0)           !SHUTS OFF EVERYTHING
        WRITE(7,1)' NORMAL PROGRAM EXIT'
C-------------------------------------------------------------------
        CALL EXIT       !RESETS REGISTERS TO INITIAL VALUES OF IDLIST
C-------------------------------------------------------------------
        END
```

```
      SUBROUTINE DTACHA
C-----------------------------------------------------------------
C       THIS SUBROUTINE IS AN I.S.R. FOR THE SCANNING MIRROR CONTROL
C       PROGRAM OR SUBROUTINE. THIS I.S.R. POKES A DIGITAL EQUIVALENT
C       OF A VOLTAGE TO D/A CHANNEL 1. IT ALSO KEEPS TRACK OF THE
C       NUMBER OF COMPLETED CYCLES. MUST BE LINKED WITH MCNTRL AND
C       MPOSIT SUBROUTINES, WHICH ARE PART OF THE PROGRAMS LASCON
C       AND LOPERA RESPECTIVELY.
C-----------------------------------------------------------------
C
C-----------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------
      COMMON /DTOA/IVLTS1,IVLTS2,IVLTS3,ICOUNT,ICYCLE,IVB,ISLOPE,
     +IDONE,IDACHA
      COMMON /CLOCK/ ICLBPR,ICLCSR,I2COMP,ICLKST,ICLKGO
      COMMON /SHUT/ IDRV11,ILSHUT,IPSHUT,IBSHUT,ISHUT,ISHUTN
      COMMON /FILTER/ OBSERV,OBSVAL,BLECHV,BLEVAL,TRNSO,TRNSB
      COMMON /FILE/ FNAME
      INTEGER OBSVAL,OBSERV,BLEVAL,BLECHV
      REAL RESP
      REAL*8 FNAME
C-----------------------------------------------------------------
      CALL IPOKE(ICLCSR,ICLKGO)       !RESETS CLOCK POKES GO BIT
      IF(ISLOPE) 10,20,20             !CHECKS POS. OR NEG SLOPE
10    CONTINUE
      IVLTS1=IVLTS1+1
      IF(IVLTS1.GT.IVB) GO TO 110
      CALL IPOKE(IDACHA,IVLTS1)       !POKES NEXT VOLTAGE INCREMENT
      GO TO 190
20    CONTINUE
      ISLOPE=0                        !RESETS SLOPE FLAG
      IVLTS1=IVLTS1-1
      IF(IVLTS1.LT.IVLTS2) GO TO 100
      CALL IPOKE(IDACHA,IVLTS1)       !POKES NEXT VOLTAGE INCREMENT
      GO TO 190
100   CONTINUE
      ISLOPE=-1                       !REDIRECTS SLOPE FLAG
      GO TO 190
110   CONTINUE
      ICOUNT=ICOUNT+1
      IVLTS1=IVB                      !RESETS INITIAL VOLTAGE
      ISLOPE=1                        !REDIRECTS SLOPE FLAG
      IF(ICOUNT.LT.ICYCLE) GO TO 190
      CALL IPOKE(ICLCSR,'0)           !POKES CLOCK OFF
      IDONE=1                         !SETS DONE FLAG
190   RETURN
      END
```

```
      SUBROUTINE FPLACE
C
C     THIS SUBROUTINE IS USED TO CONTROL THE NEUTRAL DENSITY FILTER
C     BOX AND TO CONTROL THE SELECTION OF SHUTTER CONDITIONS. THE
C     FILTER BOX CAN ATTENUATE THE LASER BEAM BY PLACING ANY
C     COMBINATION OF THE FOUR NEUTRAL DENSITY FILTERS IN THE
C     LASER'S PATH. THERE ARE FOURTEEN DIFFERENT LEVELS OF INTENSITY
C     POSSIBLE. THIS SUBROUTINE IS PART OF THE PROGRAM LOPERA.
C     THIS FILE ALSO CONTAINS THE SUBROUTINES TRANSV AND SHUTTR
C     WHICH ARE CALLED BY FPLACE
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /DTOA/IVLTS1,IVLTS2,IVLTS3,ICOUNT,ICYCLE,IVB,ISLOPE,
     +IDONE,IDACHA
      COMMON /CLOCK/ ICLBPR,ICLCSR,I2COMP,ICLKST,ICLKGO
      COMMON /SHUT/ IDRV11,ILSHUT,IPSHUT,IBSHUT,ISHUT,ISHUTN
      COMMON /FILTER/ OBSERV,OBSVAL,BLECHV,BLEVAL,TRNSO,TRNSB
      COMMON /FILE/ FNAME
      INTEGER OBSVAL,OBSERV,BLEVAL,BLECHV
      REAL RESP
      REAL*8 FNAME
C
C  FOR LATER USE WITH DATA ACQUISITION PORTION FOR LASER SYSTEM
C
C     WRITE(7,*)' ENTER DATA FILE NAME'
C     READ(7,10) FNAME
C10   FORMAT(A8)
C     WRITE(7,20) FNAME
C20   FORMAT(1X,A8)
C
25    CONTINUE
      WRITE(7,*)
      WRITE(7,*)
      WRITE(7,100) OBSERV,TRNSO
100   FORMAT(' OBS. LEVEL',I3,':',F10.7,' PERCENT OF BEAM')
      WRITE(7,*)
      WRITE(7,110) BLECHV,TRNSB
110   FORMAT(' BLEACH LEVEL',I3,':',F12.7,' PERCENT OF BEAM')
      WRITE(7,*)
      IF(ISHUTN.EQ.1) WRITE(7,*)' LASER-OPEN,PMT-CLOSED'
      IF(ISHUTN.EQ.2) WRITE(7,*)' LASER-CLOSED,PMT-OPEN'
      IF(ISHUTN.EQ.3) WRITE(7,*)' LASER-CLOSED,PMT-CLOSED'
      IF(ISHUTN.EQ.4) WRITE(7,*)' LASER-OPEN,PMT-OPEN'
      WRITE(7,*)
      WRITE(7,*)
      WRITE(7,*)' ENTER A RESPONSE FROM THE FOLLOWING MENU:'
      WRITE(7,*)' CO-TO CHANGE OBSERVATION INTENSITY'
      WRITE(7,*)' CB-TO CHANGE BLEACH INTENSITY'
      WRITE(7,*)' CS-TO CHANGE SHUTTER SETTINGS'
```

```
      WRITE(7,*)' EX-TO EXIT INTENSITY MODULE'
      WRITE(7,*)' OK-IF SATISFIED WITH LEVELS AND SETTINGS'
      READ(7,27) RESP
27    FORMAT(A2)
      IF(RESP.EQ.'CO') GO TO 30
      IF(RESP.EQ.'CB') GO TO 40
      IF(RESP.EQ.'CS') GO TO 45
      IF(RESP.EQ.'EX') GO TO 199
      IF(RESP.EQ.'OK') GO TO 50
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 25
30    CONTINUE
      WRITE(7,*)
      WRITE(7,*)' ENTER OBSERVING INTENSITY,1 LOWEST-6 HIGHEST'
      READ(7,*) OBSERV
      IF(OBSERV.GT.6) GO TO 30
      IF(OBSERV.LT.1) GO TO 30
      CALL OCTREP(OBSERV,OBSVAL)
      CALL TRANSV(OBSERV,TRNSO)
      TRNSO=100*TRNSO
      GO TO 25
40    CONTINUE
      WRITE(7,*)
      WRITE(7,*)' ENTER BLEACHING INTENSITY,7 LOWEST-14 HIGHEST'
      READ(7,*) BLECHV
      IF(BLECHV.LT.7) GOTO 40
      IF(BLECHV.GT.14) GO TO 40
      CALL OCTREP(BLECHV,BLEVAL)
      CALL TRANSV(BLECHV,TRNSB)
      TRNSB=TRNSB*100
      GO TO 25
45    CONTINUE
      CALL SHUTTR
      GO TO 25
50    CONTINUE
      WRITE(7,*)
      WRITE(7,*)' ENTER CHOICE FROM BELOW'
      WRITE(7,*)' C-CHANGE SETTINGS'
      WRITE(7,*)' O-OBSERVATION MODE'
      WRITE(7,*)' B-BLEACH MODE'
      WRITE(7,*)' E-EXIT INTENSITY MODULE'
      READ(7,55)RESP
55    FORMAT(A1)
      IF(RESP.EQ.'C')GO TO 25
      IF(RESP.EQ.'O')GO TO 60
      IF(RESP.EQ.'B')GO TO 70
      IF(RESP.EQ.'E')GO TO 199
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 50
60    CONTINUE
      INTENT=ISHUT.OR.OBSVAL
```

```
      CALL IPOKE(IDRV11,INTENT)
      GO TO 25
70    CONTINUE
C
C   NEED TO INSERT AN INTERRUPT SERVICE ROUTINE TO TIME BLEACH AND DATA
C   ACQUISTION TO BE ADDED LATER
      WRITE(7,1)' BLEACH MODE'
      INTENT=ISHUT.OR.BLEVAL
      CALL IPOKE(IDRV11,INTENT)
C     DO 90 I=1,1000
C     I1=1
C90   CONTINUE
C     WRITE(7,1)' OBSERVATION MODE'
C     INTENT=ISHUT.OR.OBSVAL
C     CALL IPOKE(IDRV11,INTENT)
      GO TO 25
199   CONTINUE
      RETURN
      END



      SUBROUTINE TRANSV(IANS,TVAL)
C
C     THIS SUBROUTINE MATCHES THE AMOUNT OF LASER OUTPUT ALLOWED THROUGH
C     TO MICROSCOPE TO THE INTENSITY VALUE ENTERED.
C
      IF(IANS.EQ.1) TVAL=.00000050
      IF(IANS.EQ.2) TVAL=.0000010
      IF(IANS.EQ.3) TVAL=.0000050
      IF(IANS.EQ.4) TVAL=.000010
      IF(IANS.EQ.5) TVAL=.000050
      IF(IANS.EQ.6) TVAL=.00010
      IF(IANS.EQ.7) TVAL=.00050
      IF(IANS.EQ.8) TVAL=.0010
      IF(IANS.EQ.9) TVAL=.0050
      IF(IANS.EQ.10) TVAL=.010
      IF(IANS.EQ.11) TVAL=.050
      IF(IANS.EQ.12) TVAL=.10
      IF(IANS.EQ.13) TVAL=.50
      IF(IANS.EQ.14) TVAL=1.00
      RETURN
      END


      SUBROUTINE SHUTTR
C
C     SUBROUTINE THAT CONTROLS THE LASER SHUTTER AND P.M.T. SHUTTER.
C     THIS IS PART OF FPLACE SUBROUTINE WHICH IS PART OF THE LOPERA
C     OR LASCOM PROGRAMS
C
```

```
C-----------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------
        COMMON /DTOA/IVLTS1,IVLTS2,IVLTS3,ICOUNT,ICYCLE,IVB,ISLOPE,IDONE,
     +IDACHA
        COMMON /CLOCK/ ICLBPR,ICLCSR,I2COMP,ICLKST,ICLK60
        COMMON /SHUT/ IDRV11,ILSHUT,IPSHUT,IBSHUT,ISHUT,ISHUTN
        COMMON /FILTER/ OBSERV,OBSVAL,BLECHV,BLEVAL,TRNSO,TRNSB
        COMMON /FILE/ FNAME
        INTEGER OBSVAL,OBSERV,BLEVAL,BLECHV
        REAL RESP
C-----------------------------------------------------------------
5       CONTINUE
        WRITE(7,1)' LC-LASER SHUTTER CLOSED / PMT SHUTTER OPEN'
        WRITE(7,1)' PC-PMT SHUTTER CLOSED / LASER SHUTTER OPEN'
        WRITE(7,1)' BC-BOTH SHUTTERS CLOSED'
        WRITE(7,1)' BO-BOTH SHUTTERS OPEN'
        READ(7,10) RESP
10      FORMAT(A2)
        IF(RESP.EQ.'LC')GO TO 20
        IF(RESP.EQ.'PC')GO TO 30
        IF(RESP.EQ.'BC')GO TO 40
        IF(RESP.EQ.'BO')GO TO 50
        WRITE(7,1)' UNABLE TO DETERMINE RESPONSE'
        GO TO 5
20      CONTINUE
        ISHUT=ILSHUT            !OCTAL VALUE TO CLOSE LASER
        ISHUTN=2                !FLAG FOR LASER CLOSED
        GO TO 99
30      CONTINUE
        ISHUT=IPSHUT            !OCTAL VALUE TO CLOSE PMT
        ISHUTN=1                !FLAG FOR PMT CLOSED
        GO TO 99
40      CONTINUE
        ISHUT=IBSHUT            !OCTAL VALUE TO CLOSE BOTH
        ISHUTN=3                !FLAG FOR BOTH CLOSED
        GO TO 99
50      CONTINUE
        ISHUT=0                 !OCTAL VALUE TO OPEN BOTH
        ISHUTN=4                !FLAG FOR BOTH OPEN
99      CONTINUE
        RETURN
        END


        SUBROUTINE OCTREP(INPUT,IOUT)
C
C       SUBROUTINE TAKES AN INTEGER INPUT AND RETURNS OCTAL
C       REPRESENTATION OF DESIRED INTENSITY
```

```
C
      IF(INPUT.EQ.1) IOUT="0
      IF(INPUT.EQ.2) IOUT="100000
      IF(INPUT.EQ.3) IOUT="40000
      IF(INPUT.EQ.4) IOUT="140000
      IF(INPUT.EQ.5) IOUT="20000
      IF(INPUT.EQ.6) IOUT="120000
      IF(INPUT.EQ.7) IOUT="60000
      IF(INPUT.EQ.8) IOUT="160000
      IF(INPUT.EQ.9) IOUT="50000
      IF(INPUT.EQ.10) IOUT="150000
      IF(INPUT.EQ.11) IOUT="30000
      IF(INPUT.EQ.12) IOUT="130000
      IF(INPUT.EQ.13) IOUT="70000
      IF(INPUT.EQ.14) IOUT="170000
      RETURN
      END
```

```
      SUBROUTINE MPOSIT
C
C     THIS SUBROUTINE IS TO BE PART OF THE LASER OPERATION PROGRAM
C     (LOPERA) IT ALLOWS FOR SELECTION OF THE OPERATIONAL MODE,
C     TO SCAN MIRROR OR TO POSITION MIRROR. IT WILL ALSO BE THE
C     BASIS OF THE SUBROUTINE TO BE USED BY LASCON PROGRAM (F.R.A.P.
C     LASER CONTROL PROGRAM). THIS SUBROUTINE MUST ALSO BE LINKED
C     WITH THE SUBROUTINE DTACHA AND CLOCIN
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /DTOA/IVLTS1,IVLTS2,IVLTS3,ICOUNT,ICYCLE,IVB,ISLOPE,
     +IDONE,IDACHA
      COMMON /CLOCK/ ICLBPR,ICLCSR,I2COMP,ICLKST,ICLKGO
      COMMON /SHUT/ IDRV11,ILSHUT,IPSHUT,IBSHUT,ISHUT,ISHUTN
      COMMON /FILTER/ OBSERV,OBSVAL,BLECHV,BLEVAL,TRNSO,TRNSB
      COMMON /FILE/ FNAME
      COMMON /VOLT/ VINT,VFIN,VMAX
      INTEGER OBSVAL,OBSERV,BLEVAL,BLECHV
      REAL RESP
      REAL*8 FNAME
      DIMENSION IDLIST(21)
      EXTERNAL DTACHA
C-----------------------------------------------------------------
C     INITIAL VALUES
C-----------------------------------------------------------------
      IVEC='440       !CLOCK OVERFLOW VECTOR
      ID=1            !ISR ID NUMBER
      IPRTY=7         !PRIORITY NUMBER
      ICOUNT=0        !COUNT OF NUMBER OF CYCLES
      IZERO='7777     !DIGITAL VALUE FOR 0.0 D/A OUTPUT
      ISLOPE=1        !SETS INITIAL SLOPE FOR VOLTAGE INCREMENTS
      ICLBPR='170422  !REAL-TIME CLOCK BPR
      ICLCSR='170420  !REAL-TIME CLOCK CSR
      IDACHA='170440  !D TO A CH. A (NON-D.E.C. STANDARD REG.)
C .
C-----------------------------------------------------------------
C
2400  CONTINUE
      WRITE(7,2402)
2402  FORMAT('0','IN MPOSIT MODULE, WANT TO CONTINUE?(Y,N)')
      READ(7,2404) RESP
2404  FORMAT(A1)
      IF(RESP.EQ.'Y') GO TO 2406
      IF(RESP.EQ.'N') GO TO 2499
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 2400
2406  CONTINUE
C-----------------------------------------------------------------
C     OPERATING MODE CHOICE
```

```
C-------------------------------------------------------------------
      WRITE(7,*)
      WRITE(7,2408)
2408  FORMAT('0','WHICH MODE IS DESIRED? (S-SCAN,P-POSITION,
     +OR E-TO EXIT MODULE)')
      READ(7,2410)RESP
2410  FORMAT(A1)
      IF(RESP.EQ.'S') GO TO 2412
      IF(RESP.EQ.'P') GO TO 2450
      IF(RESP.EQ.'E') GO TO 2499
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 2406
C-------------------------------------------------------------------
C     FOLLOWING ALLOWS THE PROGRAM TO BE REPEATED
C-------------------------------------------------------------------
2412  CONTINUE
      ICOUNT=0              !RESETS COUNTER FOR REPEATED CYCLES
      IDONE=0               !RESETS DONE FLAG
C-------------------------------------------------------------------
C     SETTING D/A CHANNEL ONE TO ZERO
C-------------------------------------------------------------------
      CALL IPOKE(IDACHA,IZERO)        !D/A(REG.NONSTANDARD)TO 0.0 V
C-------------------------------------------------------------------
C     SETTING UP I.S.R.
C-------------------------------------------------------------------
      CALL CLOCIN     !SUBROUTINE ALLOWING MULTIPLE CLOCK SETTINGS
      CALL IPOKE(ICLBPR,I2COMP)       !SETS BPR 2'S COMP INT./CYC.
      CALL IPOKE(ICLCSR,ICLKST)       !SETS CSR TO STANDBY
C
      IERR=INTSET(IVEC,IPRTY,ID,DTACHA)       !SETS ISR PARAMETERS
C-------------------------------------------------------------------
C     DELAY FOR I.S.R. TO BE INSTALLED
C-------------------------------------------------------------------
2414  CONTINUE
      WRITE(7,*)' ARE YOU READY TO CONTINUE? (Y,N)'
      READ(7,2416) RESP
2416  FORMAT(A1)
      IF(RESP.EQ.'Y') GO TO 2418
      IF(RESP.EQ.'N') GO TO 2414
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 2414
C-------------------------------------------------------------------
C     PROGRAM INPUTS # OF CYCLES & VOLTAGE RANGE INPUTS
C-------------------------------------------------------------------
2418  CONTINUE
      WRITE(7,*)' ENTER INITIAL CYCLE VOLTAGE, RANGE 0.0--5.12 V'
      READ(7,*) VINT
      IF(VINT.LT.0.0.OR.VINT.GT.10.24) GO TO 2418
2420  CONTINUE
      WRITE(7,*)' ENTER FINAL CYCLE VOLTAGE, RANGE VINT--5.12V'
      READ(7,*) VFIN
```

```
      IF(VFIN.LT.VINT.OR.VFIN.GT.10.24) GO TO 2420
      VMAX=5.12              !MAXIMUM VOLTAGE ALLOWED
      CALL CONVRT
      WRITE(7,*)
      WRITE(7,*)' STARTING MIRROR POSITION: ',IVLTS1
      WRITE(7,*)' FINISHING MIRROR POSITION: ',IVLTS2
      WRITE(7,*)
      WRITE(7,*)' ENTER THE NUMBER OF CYCLES TO BE RUN.'
      READ(7,*) ICYCLE
C-----------------------------------------------------------------
C     STARTING CLOCK AND END OF PROGRAM CHECK
C-----------------------------------------------------------------
      CALL IPOKE(IDACHA,IVLTS1)       !POKES INIT. VOLTAGE
      CALL IPOKE(ICLCSR,ICLKGO)       !POKES CLOCK GO BIT
      WRITE(7,*)' WAITING FOR PROGRAM TO CYCLE'
      WRITE(7,*)
2422  IF(ISLOPE.EQ.1) WRITE(7,*)' COMPLETED CYCLE #',ICOUNT
      IF(IDONE.EQ.0) GO TO 2422
      WRITE(7,*)' PROGRAM COMPLETED CYCLING'
2424  CONTINUE
      WRITE(7,*)
      WRITE(7,*)' ENTER ONE OF THE FOLLOWING CHOICES-- S-RESET SCAN'
      WRITE(7,*)' PARAMETERS, C-RETURN TO MIRROR MODE CHOICE, OR'
      WRITE(7,*)' E-TO EXIT MPOSIT MODULE'
      READ(7,2426) RESP
2426  FORMAT(A1)
      IF(RESP.EQ.'C') GO TO 2400
      IF(RESP.EQ.'S') GO TO 2412
      IF(RESP.EQ.'E') GO TO 2499
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 2424
2450  CONTINUE
      WRITE(7,*)' POSITIONING MODE'
      VINT=0.0
      VFIN=2.56
      VMAX=5.12
      CALL CONVRT
      CALL IPOKE(IDACHA,IVLTS2)       !POKES CENTERING VOLTAGE
      WRITE(7,*)
      WRITE(7,*)' LOWEST MIRROR POSITION LIMIT: ',IVLTS1
      WRITE(7,*)' HIGHEST MIRROR POSITION LIMIT: ',IVLTS3
      WRITE(7,*)
      WRITE(7,*)'PRESENT MIRROR POSITION: ',IVLTS2
      CALL MOVEM
2452  CONTINUE
      WRITE(7,*)
      WRITE(7,*)' ENTER ONE OF THE FOLLOWING CHOICES-- P-REPOSITION'
      WRITE(7,*)' MIRROR, C-RETURN TO MIRROR MODE CHOICE, OR E-TO'
      WRITE(7,*)' EXIT MPOSIT MODULE'
      READ(7,2454)RESP
2454  FORMAT(A1)
```

```
      IF(RESP.EQ.'C') GO TO 2400
      IF(RESP.EQ.'P') GO TO 2450
      IF(RESP.EQ.'E') GO TO 2499
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 2452
2499  CONTINUE
      RETURN
      END


      SUBROUTINE CONVRT
C
C-----------------------------------------------------------------
C     THIS SUBROUTINE CONVERTS A REAL VOLTAGES INTO INTEGER
C     EQUIVALENTS. THIS SUBROUTINE IS USED BY MPOSIT SUBROUTINE,
C     WHICH IS ALSO PART OF THE PROGRAM LOPERA
C-----------------------------------------------------------------
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /DTOA/IVLTS1,IVLTS2,IVLTS3,ICOUNT,ICYCLE,IVB,ISLOPE,
     +IDONE,IDACHA
      COMMON /VOLT/ VINT,VFIN,VMAX
C
      VINCRM=10.24/(8**4-1)   !NUMBER OF VOLTAGE INCREMENTS
      VOLTS1=(VINT/VINCRM)    !REAL INITIAL VOLTAGE REP.
      VOLTS2=(VFIN/VINCRM)    !REAL FINAL VOLTAGE REP.
      VOLTS3=(VMAX/VINCRM)    !REAL FINAL VOLTAGE REP.
      IVLTS1=VOLTS1           !INTEGER VOLT. REP.
      IVLTS2=VOLTS2           !INTEGER VOLT. REP.
      IVLTS3=VOLTS3           !INTEGER VOLT. REP.
      DEC1=VOLTS1-IVLTS1
      IF(DEC1.GT.0.5) IVLTS1=IVLTS1+1 !CORRECTS FOR REMAINDERS
      IVLTS1="7777-IVLTS1             !CORRECTS FOR INVERTED D/A
      DEC2=VOLTS2-IVLTS2
      IF(DEC2.GT.0.5) IVLTS2=IVLTS2+1 !CORRECTS FOR REMAINDERS
      IVLTS2="7777-IVLTS2             !CORRECTS FOR INVERTED D/A
      DEC3=VOLTS3-IVLTS3
      IF(DEC3.GT.0.5) IVLTS3=IVLTS3+1 !CORRECTS FOR REMAINDERS
      IVLTS3="7777-IVLTS3             !CORRECTS FOR INVERTED D/A
      IVB=IVLTS1                      !INIT VOLTAGE USED IN DTACHA
      RETURN
      END



      SUBROUTINE MOVEM
C
C-----------------------------------------------------------------
C     THIS SUBROUTINE INCREMENTS OR DECREMENTS DTA VOLAGE OUTPUT
C     TO THE MIRROR CONTROL BOX. ROUTINE KEEPS LOOPING UNTIL USER
```

```
C       IS SATISFIED WITH LASER BEAM LOCATION. THIS SUBROUTINE IS
C       USED BY THE MPOSIT SUBROUTINE, WHICH IS PART OF THE PROGRAM
C       LOPERA
C-----------------------------------------------------------------
C
C-----------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------
        COMMON /DTOA/IVLTS1,IVLTS2,IVLTS3,ICOUNT,ICYCLE,IVB,ISLOPE,
     +IDONE,IDACHA
        REAL RESP
2470    CONTINUE
        WRITE(7,*)
        WRITE(7,*)' MIRROR COARSE ADJUSTMENT--ENTER U-TO MOVE'
        WRITE(7,*)' MIRROR UP,D-TO MOVE MIRROR DOWN,F-TO GO TO'
        WRITE(7,*)' FINE ADJUSTMENT,L-TO LEAVE POSITIONING LOOP'
        READ(7,2472) RESP
2472    FORMAT(A1)
        IF(RESP.EQ.'F')GO TO 2460
        IF(RESP.EQ.'D')GO TO 2474
        IF(RESP.EQ.'U')GO TO 2474
        IF(RESP.EQ.'L')GO TO 2469
        WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
        GO TO 2470
2474    CONTINUE
        INCREM=0                        !RESETS VALUE
        WRITE(7,*)'ENTER THE NUMBER INCREMENTS DESIRED'
        READ(7,2476) INCREM
2476    FORMAT(I4)
        IF(RESP.EQ.'D')IVT2=IVLTS2+INCREM
        IF(RESP.EQ.'U')IVT2=IVLTS2-INCREM
        IF(IVT2.GT.IVLTS1.OR.IVT2.LT.IVLTS3)GO TO 2473
        IVLTS2=IVT2
        CALL IPOKE(IDACHA,IVLTS2)
        WRITE(7,*) 'PRESENT LOCATION: ',IVLTS2
        GO TO 2470
2473    CONTINUE
        WRITE(7,*)' THE ENTERED NUMBER WAS OUT OF RANGE'
        WRITE(7,*)'PRESENT LOCATION: ',IVLTS2
        GO TO 2470
2460    CONTINUE
        WRITE(7,*)
        WRITE(7,*)' ENTER U-TO MOVE MIRROR UP, D-TO MOVE MIRROR DOWN,'
        WRITE(7,*)' C-TO GO TO COARSE ADJUSTMENT,OR L-TO LEAVE LOOP'
        READ(7,2462)RESP
2462    FORMAT(A1)
        IF(RESP.EQ.'L')GO TO 2469
        IF(RESP.EQ.'C')GO TO 2470
        IF(RESP.EQ.'U')GO TO 2463
        IF(RESP.EQ.'D')GO TO 2464
        GO TO 2460
```

```
2463    CONTINUE
        IVLTS2=IVLTS2-1
        GO TO 2465
2464    CONTINUE
        IVLTS2=IVLTS2+1
2465    CONTINUE
        IF(IVLTS2.GE.IVLTS1.OR.IVLTS2.LE.IVLTS3) GO TO 2467
        CALL IPOKE(IDACHA,IVLTS2)
        WRITE(7,*)'PRESENT LOCATION: ',IVLTS2
        GO TO 2460
2467    CONTINUE
        WRITE(7,*)' OUT OF RANGE MUST MOVE IN OTHER DIRECTION'
        WRITE(7,*)'PRESENT LOCATION: ',IVLTS2
        GO TO 2460
2469    CONTINUE
        RETURN
        END
```

```
      SUBROUTINE CLOCIN
C-----------------------------------------------------------------------
C       THIS SUBROUTINE IS USED WITH THE SCANNING MIRROR CONTRL(MCNTRL)
C       PROGRAM/SUBROUNTINE. IT ALLOWS FOR THE SELECTION OF REAL-TIME
C       CLOCK FREQUENCIES AND MODE OF OPERATION. THESE SELECTION ARE
C       THEN POKED INTO THE CSR AND BPR, RESPECTIVELY. MUST BE LINKED
C       WITH SUBROUTINES MCNTRL OR MPOSIT, WHICH ARE PART OF THE
C       PROGRAMS LASCON AND LOPERA RESPECTIVELY.
C-----------------------------------------------------------------------
C
C-----------------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------------
      COMMON /DTOA/IVLTS1,IVLTS2,IVLTS3,ICOUNT,ICYCLE,IVB,ISLOPE,
     +IDONE,IDACHA
      COMMON /CLOCK/ ICLBPR,ICLCSR,I2COMP,ICLKST,ICLK60
      COMMON /SHUT/ IDRV11,ILSHUT,IPSHUT,IBSHUT,ISHUT,ISHUTN
      COMMON /FILTER/ OBSERV,OBSVAL,BLECHV,BLEVAL,TRNSO,TRNSB
      COMMON /FILE/ FNAME
      INTEGER OBSVAL,OBSERV,BLEVAL,BLECHV
      REAL RESP
      REAL*8 FNAME
C
C-----------------------------------------------------------------------
C
10    CONTINUE
      WRITE(7,*)' THE POSSIBLE FREQUENCIES OF THE REAL-TIME CLOCK'
      WRITE(7,*)' CAN BE SET TO ANY OF THE FOLLOWING:'
      WRITE(7,*)
      WRITE(7,*)' 100 HZ  - ENTER 2 BELOW'
      WRITE(7,*)' 60 HZ   - ENTER 1 BELOW'
      WRITE(7,*)
      WRITE(7,*)' ENTER THE NUMBER ABOVE CORRESPONDING TO THE'
      WRITE(7,*)' DESIRED REAL-TIME CLOCK FREQUENCY'
      READ(7,20,ERR=10) IFREQ
20    FORMAT(I2)
      IF(IFREQ.LT.1.OR.IFREQ.GT.2) GO TO 10
      IF(IFREQ.EQ.2) GO TO 70
       IF (IFREQ.EQ.1) GO TO 80
      GO TO 10
70    CONTINUE
      ICLKST="152             !SETS CSR TO STANDBY 100 HZ
      ICLK60="153             !SETS CSR GO BIT
      CYC=-1.0E2              !NEG. OF NUMBER OF CYCLES/SEC
      GO TO 90
80    CONTINUE
      ICLKST="172             !SETS CSR TO STANDBY 60 HZ
      ICLK60="173             !SETS CSR GO BIT
      CYC=-6.0E1              !NEG. OF NUMBER OF CYCLES/SEC
90    CONTINUE
      WRITE(7,*)' THE DEFAULT VALUE FOR 2'S COMPLIMENT FOR THE'
```

```
      WRITE(7,*)' NUMBER OF INTERRUPTS PER CYCLE IS SET TO ONE'
      WRITE(7,*)' DO YOU WANT THE DEFAULT VALUE? (Y,N)'
      READ(7,95) RESP
95    FORMAT(A1)
      IF(RESP.EQ.'N') GO TO 100
      IF(RESP.EQ.'Y') GO TO 120
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 90
100   CONTINUE
      WRITE(7,*)' ENTER THE NUMBER OF COUNTS BEFORE AN INTERRUPT'
      WRITE(7,*)' IS TO OCCUR. THE NUMBER OF COUNTS CANNOT BE'
      WRITE(7,*)' GREATER THAN THE NUMBER OF CYCLES ',CYC,'OR'
      WRITE(7,*)' LESS THAN ONE.'
      READ(7,*) COUNTS
      R2COMP=(CYC/COUNTS)     !CYC IS NEG. SO GIVES 2'S COMP.
      IF(R2COMP.LT.-1.OR.R2COMP.GT.CYC) GO TO 125
      WRITE(7,*)' 2"S COMPLIMENT IS OUT OF RANGE'
      GO TO 100
125   CONTINUE
      WRITE(7,*)' IF 2"S COMPLIMENT IS GREATER THAN 32,000 RESET TO'
      WRITE(7,*)' A SMALLER CLOCK FREQUENCY'
      IF(R2COMP.GT.32000) GO TO 10
      I2COMP=INT(R2COMP)
      GO TO 130
120   CONTINUE
      I2COMP=(-1)
130   CONTINUE
      WRITE(7,*)' THE NEW VALUES ARE:'
      WRITE(7,*)
      WRITE(7,*)'CLOCK FREQUENCY',CYC,'CYCLES/SEC'
      WRITE(7,*)'2"S COMPLIMENT IS',I2COMP
      WRITE(7,*)
      WRITE(7,*)' DO YOU WANT TO CHANGE EITHER OF THESE VALUES?(Y/N)'
      READ(7,140) RESP
140   FORMAT(A1)
      IF(RESP.EQ.'Y') GO TO 10
      IF(RESP.EQ.'N') GO TO 199
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 130
199   CONTINUE
      RETURN
      END
```

B.2 DATA ACQUISITION AND CONTROL OPERATING SYSTEM

```
      PROGRAM LASCON
C
C     THIS PROGRAM CONTROLS ALL MAJOR FUNCTIONS OF FLUORESCENCE RECOVERY
C     AFTER PHOTOBLEACHING SYSTEM FOR THE BTP LABORATORY. IT FOLLOWS A
C     PROGRAM GIVEN TO US BY JOHN HOLLAND,PH.D., M.S.U.  WITH PERMISSION
C     OF MERIDIAN INSTRUMENTS, OKEMOS, MICHIGAN.
C
C-----------------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------------
      COMMON /ATOD/ IADBUF,IADCSR,INITD,IREADY
      COMMON /DRV11/ IDRV11,INTENB,INTENO
      COMMON /DTOA/ IDACHA,IPCNT,IPOST
      COMMON /FLAGS/ LPRINT,LTAKE
      COMMON /FILE/ FNAME,IUNIT
      COMMON /FILTER/ BLECHV,BLEVAL,OBSERV,OBSVAL,TRNSO,TRNSB
      COMMON /ISRS/ ID,IPRTY,IVEC,LD,LPRTY,LVEC
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLKGO,ICLKST,I2COMP
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      COMMON /VALUE/ IELEVN,IONE,IVOUT,IZERO
      COMMON /ZTICK/ ICOL,ITICK,TICK
      DIMENSION IVOUT(11,280)
      DIMENSION IDLIST(7)
      DIMENSION IPOST(11)
      EXTERNAL ATDC13,LTIME
      INTEGER BLECHV,BLEVAL,OBSERV,OBSVAL
      REAL RESP
      REAL*8 FNAME
C
C-----------------------------------------------------------------------
C     SETTING UP IDLIST FOR RESETTING REGISTERS TO ORIGINAL STATUS
C-----------------------------------------------------------------------
C
      IDLIST(1)="167772              !DRV11 OUTPUT BUFFER
      IDLIST(2)=IPEEK("167772)       !CONTENTS DRV11 O.B.
      IDLIST(3)="170420              !R.T. CLOCK C.S.R.
      IDLIST(4)=IPEEK("170420)       !CONTENTS R.T. CLOCK C.S.R.
      IDLIST(5)="177546              !LTC C.S.R.
      IDLIST(6)=IPEEK("177546)       !CONTENTS LTC C.S.R.
      IDLIST(7)="0                   !END OF LIST FLAG
C-----------------------------------------------------------------------
C     CALL DEVICE(IDLIST)     !UPON EXIT RESETS REGISTERS IN IDLIST
C-----------------------------------------------------------------------
C
C-----------------------------------------------------------------------
C     SUBROUTINE THAT SETS INITIAL VALUES FOR PROGRAM AND SUBROUTINES
C-----------------------------------------------------------------------
      CALL VALUES
C-----------------------------------------------------------------------
30    CONTINUE
```

```
      WRITE(7,*) ' PLEASE TYPE IN THE TWO LETTER CODE FROM LIST BELOW'
      WRITE(7,*) ' FOR THE OPTION YOU WISH TO COMPLETE.'
      WRITE(7,*)
      WRITE(7,*) ' CP - CHANGE PARAMETERS'
      WRITE(7,*) ' EX - EXIT PROGRAM'
      WRITE(7,*) ' FN - CHANGE FILE NAME'
      WRITE(7,*) ' HE - HELP'
      WRITE(7,*) ' LP - LIST PARAMETERS'
      WRITE(7,*) ' RB - RUN BLEACH EXPERIMENT'
      WRITE(7,*) ' RL - RUN LASER SET UP'
      WRITE(7,*) ' SB - STAND BY'
      WRITE(7,*)
      READ(7,20) ANS
20    FORMAT(A2)
C
C-----------------------------------------------------------------------
C     OPTION DECISION BLOCK
C-----------------------------------------------------------------------
C
      IF(ANS.EQ.'CP') GO TO 40
      IF(ANS.EQ.'EX') GO TO 60
      IF(ANS.EQ.'FN') GO TO 80
      IF(ANS.EQ.'HE') GO TO 100
      IF(ANS.EQ.'LP') GO TO 120
      IF(ANS.EQ.'RB') GO TO 160
      IF(ANS.EQ.'RL') GO TO 180
      IF(ANS.EQ.'SB') GO TO 200
      WRITE(7,*) ' UNABLE TO DETERMINE RESPONSE'
      GO TO 30
C
C-----------------------------------------------------------------------
C     SUBROUTINE CALLS
C-----------------------------------------------------------------------
C
40    CONTINUE
      CALL CPARAM
      GO TO 30
C
80    CONTINUE
      CALL NAMEF
      GO TO 30
C
100   CONTINUE
      CALL HELPME
      GO TO 30
C
120   CONTINUE
      CALL LPARAM
      GO TO 30
C
160   CONTINUE
```

```
        CALL EXPRUN
        GO TO 30
C
180     CONTINUE
        CALL LASET
        GO TO 30
C
200     CONTINUE
        CALL STNDBY
        GO TO 30
C
60      CONTINUE
        WRITE(7,*)' NORMAL END OF LASCON PROGRAM'
        CALL IPOKE(LTCCSR,LDISAB)       !TURNS OFF LTC
        CALL IPOKE(ICLCSR,LDISAB)       !TURNS OFF RTC
        CALL IPOKE(IDRV11,IZERO)        !CLEARS DRV11 OUTPUT BUFFER
C
C-------------------------------------------------------------------
C
C       CALL EXIT       !ACTIVATES DEVICE SUBROUTINE, RESETS REGISTERS
C
C-------------------------------------------------------------------
C
        END

        SUBROUTINE VALUES
C-------------------------------------------------------------------
C       THIS SUBROUTINE SETS DEFAULT AND INITIAL VALUES USED BY LASCON
C       PROGRAM AND ITS SUBROUTINES.
C-------------------------------------------------------------------
C-------------------------------------------------------------------
C       DECLARATIONS
C-------------------------------------------------------------------
        COMMON /ATOD/ IADBUF,IADCSR,INITD,IREADY
        COMMON /DRV11/ IDRV11,INTENB,INTENO
        COMMON /DTOA/ IDACHA,IPCNT,IPOST
        COMMON /FLAGS/ LPRINT,LTAKE
        COMMON /FILE/ FNAME,IUNIT
        COMMON /FILTER/ BLECHV,BLEVAL,OBSERV,OBSVAL,TRNSO,TRNSB
        COMMON /ISRS/ ID,IPRTY,IVEC,LD,LPRTY,LVEC
        COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
        COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLKGO,ICLKST,ICCOMP
        COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
        COMMON /VALUE/ IELEVN,IONE,IVOUT,IZERO
        COMMON /ZTICK/ ICOL,ITICK,TICK
        DIMENSION IVOUT(11,280)
        DIMENSION IPOST(11)
        EXTERNAL ATOC13,LTIME
        INTEGER BLECHV,BLEVAL,OBSERV,OBSVAL
        REAL RESP
        REAL*8 FNAME
```

```
C-------------------------------------------------------------------
C       INITIAL VALUES
C-------------------------------------------------------------------
        BLECHV=7                !BLEACH INTNESITY CHOICES (7-14)
        BLEVAL="60000           !VALUE FOR BLEACH LEVEL 7
        BTIME=0.5*60.           !INTEGER REP. OF BLEACH TIME 0.5SEC
        DTIME=1.00              !TIME BETWEEN RTC ISR INTERRUPTS
        FNAME='FRAP.DAT'        !DEFAULT NAME PUT INTO FILE
        IADBUF="177002          !ATD BPR ADDRESS
        IADCSR="177000          !ATD CSR ADDRESS
        IBOPEN="0               !VALUE TO OPEN LASER AND PMT SHUTTERS
        IBSHUT="60              !VALUE TO CLOSE LASER AND PMT SHUTTERS
        ICLBPR="170422          !RTC BPR ADDRESS
        ICLCSR="170420          !RTC CSR ADDRESS
        ICLKGO="143             !SETS RTC CSR MODE1,1 KHZ,CLK GO
        ICLKST="142             !SETS RTC CSR MODE1,1 KHZ,CLK OFF
        ICOL=1                  !INITIAL VOUT COLUMN POINTER
        ID=2                    !RTC ISR ID NUMBER
        IDACHA="170440          !DTA CHANNEL A CSR
        IDRV11="167772          !DRV11 OUTPUT BUFFER
        IELEVN=11               !UPPER POSITION LIMIT FOR MIRROR
        ILSHUT="20              !VALUE TO CLOSE LASER SHUTTER
        INITD="6401             !VALUE INITIATES DATA CONVERSION CH13
        INTENO=OBSVAL.OR.IBOPEN !OBS. INT 1 AND SHUT COND BO
        INTENB=BLEVAL.OR.IPSHUT !BLEACH INT 7 AND SHUT COND PC
        IONE=1                  !LOWER POSITION LIMIT FOR MIRROR
        IPCNT=6                 !POINTER MIRROR POSITION ARRAY
C       IPOST(1)=3046           !INT REP. MIRROR POSITION ONE
C       IPOST(2)=3051           !EACH POSITION IS APPROXIMATELY
C       IPOST(3)=3056           !2 MICROMETERS DISTANT FROM
C       IPOST(4)=3061           !POSITION ABOVE OR BELOW CURRENT
C       IPOST(5)=3066           !POSITION
C       IPOST(6)=3071           !INT. REP. MIRROR CENTER PT.
C       IPOST(7)=3076           !ALLOWS FOR PLUS/MINUS 5 MICRONS
C       IPOST(8)=3081           !FROM CENTER POSITION
C       IPOST(9)=3086
C       IPOST(10)=3091
C       IPOST(11)=3096          !INT. REP. MIRROR POSITION 11
        DO 333 I=1,11
        IPOST(I)=3071
333     CONTINUE
        IPRTY=6                 !RTC ISR PRIORITY NUMBER
        IPSHUT="40              !VALUE TO CLOSE PMT SHUTTER
        IREADY="6600            !VALUE DATA CONVERSION DONE CH13
        ISHUT="60               !VALUE SET FROM I*SHUT VALUES
        ISHUTN=4                !FLAG FOR SHUTTER COND. BOTH OPEN
        ITICK=0                 !INTERRUPT COUNTER
        IUNIT=10                !DEFAULT OUTPUT LOGICAL UNIT
        IVEC="440               !RTC INTERRUPT OVERFLOW VECTOR
        IZERO=0                 !VALUE OF ZERO
        I2COMP=(-1)*100         !2'COMP 100 COUNTS/INTERRUPT RTC BPR
```

```
LD=1                    !LTC ISR ID NUMBER
LDISAB="0               !VALUE TO DISABLE LTC
LENABL="100             !VALUE TO ENABLE INTERRUPT BIT 6
LPRTY=7                 !LTC ISR PRIORITY NUMBER
LTCCSR="177546          !LTC CSR ADDRESS
LVEC="100               !LTC INTERRUPT OVERFLOW VECTOR
OBSERV=1                !OBS. INTENSITY CHOICES (1-6)
OBSVAL="0               !VALUE FOR OBS. INT LEVEL 1
RTIME=5.*60.            !INTEGER REP. OF RUNNING TIME 5 SEC
TICK=0.0                !TIME KEEPER
TIME=0.                 !COUNTER FOR TIME FROM RTC
TRNSB=0.05              !PERCENTAGE OF BEAM TRANSMITTED BLEACH
TRNSO=0.00005           !PERCENTAGE OF BEAM TRANSMITTED OBS.
RETURN
END
```

```
      SUBROUTINE CPARAM
C
C     THIS SUBROUTINE IS PART OF THE LASER CONTROL PROGRAM (LASCON).
C     ITS FUNCTION IS TO ALLOW THE USER TO CHANGE THE PROGRAMS
C     PARAMETERS OR USE DEFAULT PARAMETERS.
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /ATOD/ IADBUF,IADCSR,INITD,IREADY
      COMMON /DRV11/ IDRV11,INTENB,INTENO
      COMMON /DTOA/ IDACHA,IPCNT,IPOST
      COMMON /FLAGS/ LPRINT,LTAKE
      COMMON /FILE/ FNAME,IUNIT
      COMMON /FILTER/ BLECHV,BLEVAL,OBSERV,OBSVAL,TRNSO,TRNSB
      COMMON /ISRS/ ID,IPRTY,IVEC,LD,LPRTY,LVEC
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLKGO,ICLKST,I2COMP
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      COMMON /VALUE/ IELEVN,IONE,IVOUT,IZERO
      COMMON /ZTICK/ ICOL,ITICK,TICK
      DIMENSION IVOUT(11,280)
      DIMENSION IPOST(7)
      INTEGER BLECHV,BLEVAL,OBSERV,OBSVAL
      REAL RESP
      REAL*8 FNAME
C
C-----------------------------------------------------------------
C
      WRITE(7,*)
      WRITE(7,*)' OUTPUT FILE NAME IS IN FORM FTN__.DAT, THE NUMBER'
      WRITE(7,*)' IN FTN__.DAT IS ICREMENTED BY ONE FOR EACH RUN'
      WRITE(7,*)' DEFAULT NAME WRITTEN INTO FILE FTN__.DAT IS'
      WRITE(7,*)' FRAP.DAT, USER MAY CHANGE IF DESIRED.'
      WRITE(7,*)
      WRITE(7,*)' HIT RETURN TO CONTINUE'
      READ(7,403) RESP
403   FORMAT(A1)
406   CONTINUE
      CALL LPARAM
C
C-----------------------------------------------------------------
C
408   CONTINUE
      WRITE(7,*)
      WRITE(7,*)' ENTER CODE FOR THE PARAMETER YOU WISH CHANGE'
      WRITE(7,*)
      WRITE(7,*)' FN-FILENAME FOR DATA OUTPUT'
      WRITE(7,*)' IS-INTENSITY LEVELS AND OBSRV.SHUTTER CONDITIONS'
      WRITE(7,*)' ST-SET TIME: BLEACH, DATA, RUN'
      WRITE(7,*)' LP-LIST PARAMETERS'
```

```
      WRITE(7,1)' OK-SATISFIED WITH PARAMETERS'
      READ(7,410) RESP
410   FORMAT(A2)
      IF(RESP.EQ.'IS')GO TO 412
      IF(RESP.EQ.'ST')GO TO 416
      IF(RESP.EQ.'LP')GO TO 406
      IF(RESP.EQ.'FN')GO TO 424
      IF(RESP.EQ.'OK')GO TO 499
      WRITE(7,1)' UNABLE TO DETERMINE RESPONSE'
      GO TO 408
C
C-----------------------------------------------------------------
C
412   CONTINUE
      CALL INSHUT
      GO TO 406
C
C-----------------------------------------------------------------
C
416   CONTINUE
      CALL SETIME
      GO TO 406
C
C-----------------------------------------------------------------
C
424   CONTINUE
      CALL NAMEF
      GO TO 406
C
C-----------------------------------------------------------------
C
499   CONTINUE
      WRITE(7,1)'RETURNING TO LASCON MENU'
      RETURN
      END
```

```
      SUBROUTINE INSHUT
C
C     THIS SUBROUTINE IS USED TO CONTROL THE NEUTRAL DENSITY FILTER
C     BOX AND TO CONTROL THE SELECTION OF SHUTTER CONDITIONS. THE
C     FILTER BOX CAN ATTENUATE THE LASER BEAM BY PLACING ANY
C     COMBINATION OF THE FOUR NEUTRAL DENSITY FILTERS IN THE
C     LASER'S PATH. THERE ARE FOURTEEN DIFFERENT LEVELS OF INTENSITY
C     POSSIBLE. THIS SUBROUTINE IS PART OF THE PROGRAM LOPERA.
C     THIS FILE ALSO CONTAINS THE SUBROUTINES TRANSV AND SHUTTR
C     WHICH ARE CALLED BY INSHUT
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /DRV11/ IDRV11,INTENB,INTENO
      COMMON /FILTER/ BLECHV,BLEVAL,OBSERV,OBSVAL,TRNSO,TRNSB
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      INTEGER BLECHV,BLEVAL,OBSERV,OBSVAL
      REAL RESP
C
25    CONTINUE
      WRITE(7,*)
      WRITE(7,*)
      WRITE(7,100) OBSERV,TRNSO
100   FORMAT(' OBS. LEVEL',I3,':',F10.7,' PERCENT OF BEAM')
      WRITE(7,*)
      WRITE(7,110) BLECHV,TRNSB
110   FORMAT(' BLEACH LEVEL',I3,':',F12.7,' PERCENT OF BEAM')
      WRITE(7,*)
      WRITE(7,*)'-----OBSERVATION SHUTTER CONDITIONS-----'
      IF(ISHUTN.EQ.1) WRITE(7,*)' LASER-OPEN,PMT-CLOSED'
      IF(ISHUTN.EQ.2) WRITE(7,*)' LASER-CLOSED,PMT-OPEN'
      IF(ISHUTN.EQ.3) WRITE(7,*)' LASER-CLOSED,PMT-CLOSED'
      IF(ISHUTN.EQ.4) WRITE(7,*)' LASER-OPEN,PMT-OPEN'
      WRITE(7,*)
      WRITE(7,*)
      WRITE(7,*)' ENTER A RESPONSE FROM THE FOLLOWING MENU:'
      WRITE(7,*)' CO-TO CHANGE OBSERVATION INTENSITY'
      WRITE(7,*)' CB-TO CHANGE BLEACH INTENSITY'
      WRITE(7,*)' CS-TO CHANGE SHUTTER SETTINGS'
      WRITE(7,*)' EX-TO EXIT INTENSITY MODULE'
      WRITE(7,*)' OK-IF SATISFIED WITH LEVELS AND SETTINGS'
      READ(7,27) RESP
27    FORMAT(A2)
      IF(RESP.EQ.'CO') GO TO 30
      IF(RESP.EQ.'CB') GO TO 40
      IF(RESP.EQ.'CS') GO TO 45
      IF(RESP.EQ.'EX') GO TO 50
      IF(RESP.EQ.'OK') GO TO 50
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 25
```

```
30      CONTINUE
        WRITE(7,*)
        WRITE(7,*)' ENTER OBSERVING INTENSITY,1 LOWEST-6 HIGHEST'
        READ(7,*) OBSERV
        IF(OBSERV.GT.6) GO TO 30
        IF(OBSERV.LT.1) GO TO 30
        CALL OCTREP(OBSERV,OBSVAL)
        CALL TRANSV(OBSERV,TRNSO)
        TRNSO=100*TRNSO
        GO TO 25
40      CONTINUE
        WRITE(7,*)
        WRITE(7,*)' ENTER BLEACHING INTENSITY,7 LOWEST-14 HIGHEST'
        READ(7,*) BLECHV
        IF(BLECHV.LT.7) GOTO 40
        IF(BLECHV.GT.14) GO TO 40
        CALL OCTREP(BLECHV,BLEVAL)
        CALL TRANSV(BLECHV,TRNSB)
        TRNSB=TRNSB*100
        GO TO 25
45      CONTINUE
        CALL SHUTTR
        GO TO 25
50      CONTINUE
C       SETS VALUES TO BE POKED INTO DRV11 CSR
        INTENO=ISHUT.OR.OBSVAL          !VALUE FOR OBS. AND SHUT COND.
        INTENB=IPSHUT.OR.BLEVAL         !VALUE FOR BLEACH AND PMT SHUT
        RETURN
        END



        SUBROUTINE TRANSV(IANS,TVAL)
C
C       THIS SUBROUTINE MATCHES THE AMOUNT OF LASER OUTPUT ALLOWED THROUGH
C       TO MICROSCOPE TO THE INTENSITY VALUE ENTERED.
C
        IF(IANS.EQ.1) TVAL=.00000050
        IF(IANS.EQ.2) TVAL=.0000010
        IF(IANS.EQ.3) TVAL=.0000050
        IF(IANS.EQ.4) TVAL=.000010
        IF(IANS.EQ.5) TVAL=.000050
        IF(IANS.EQ.6) TVAL=.00010
        IF(IANS.EQ.7) TVAL=.00050
        IF(IANS.EQ.8) TVAL=.0010
        IF(IANS.EQ.9) TVAL=.0050
        IF(IANS.EQ.10) TVAL=.010
        IF(IANS.EQ.11) TVAL=.050
        IF(IANS.EQ.12) TVAL=.10
        IF(IANS.EQ.13) TVAL=.50
        IF(IANS.EQ.14) TVAL=1.00
```

```
      RETURN
      END


      SUBROUTINE SHUTTR
C
C     SUBROUTINE THAT CONTROLS THE LASER SHUTTER AND P.M.T. SHUTTER.
C     THIS IS PART OF FPLACE SUBROUTINE WHICH IS PART OF THE LOPERA
C     OR LASCON PROGRAMS
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      REAL RESP
C-----------------------------------------------------------------
5     CONTINUE
      WRITE(7,1)' LC-LASER SHUTTER CLOSED / PMT SHUTTER OPEN'
      WRITE(7,1)' PC-PMT SHUTTER CLOSED / LASER SHUTTER OPEN'
      WRITE(7,1)' BC-BOTH SHUTTERS CLOSED'
      WRITE(7,1)' BO-BOTH SHUTTERS OPEN'
      READ(7,10) RESP
10    FORMAT(A2)
      IF(RESP.EQ.'LC')GO TO 20
      IF(RESP.EQ.'PC')GO TO 30
      IF(RESP.EQ.'BC')GO TO 40
      IF(RESP.EQ.'BO')GO TO 50
      WRITE(7,1)' UNABLE TO DETERMINE RESPONSE'
      GO TO 5
20    CONTINUE
      ISHUT=ILSHUT            !OCTAL VALUE TO CLOSE LASER
      ISHUTN=2                !FLAG FOR LASER CLOSED
      GO TO 99
30    CONTINUE
      ISHUT=IPSHUT            !OCTAL VALUE TO CLOSE PMT
      ISHUTN=1                !FLAG FOR PMT CLOSED
      GO TO 99
40    CONTINUE
      ISHUT=IBSHUT            !OCTAL VALUE TO CLOSE BOTH
      ISHUTN=3                !FLAG FOR BOTH CLOSED
      GO TO 99
50    CONTINUE
      ISHUT=IBOPEN            !OCTAL VALUE TO OPEN BOTH
      ISHUTN=4                !FLAG FOR BOTH OPEN
99    CONTINUE
      RETURN
      END


      SUBROUTINE OCTREP(INPUT,IOUT)
C
```

```
C       SUBROUTINE TAKES AN INTEGER INPUT AND RETURNS OCTAL
C       REPRESENTATION OF DESIRED INTENSITY
C
        IF(INPUT.EQ.1) IOUT="0
        IF(INPUT.EQ.2) IOUT="100000
        IF(INPUT.EQ.3) IOUT="40000
        IF(INPUT.EQ.4) IOUT="140000
        IF(INPUT.EQ.5) IOUT="20000
        IF(INPUT.EQ.6) IOUT="120000
        IF(INPUT.EQ.7) IOUT="60000
        IF(INPUT.EQ.8) IOUT="160000
        IF(INPUT.EQ.9) IOUT="50000
        IF(INPUT.EQ.10) IOUT="150000
        IF(INPUT.EQ.11) IOUT="30000
        IF(INPUT.EQ.12) IOUT="130000
        IF(INPUT.EQ.13) IOUT="70000
        IF(INPUT.EQ.14) IOUT="170000
        RETURN
        END
```

```
      SUBROUTINE SETIME
C-----------------------------------------------------------------
C       THIS SUBROUTINE IS PART OF THE LASCON PROGRAM. IT ALLOWS
C       USER TO SELECT LENGTH OF BLEACHING, LENGTH OF RUN (DATA
C       COLLECTION, AND TIME INTERVAL BETWEEN PMT READINGS.
C-----------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLKGO,ICLKST,I2COMP
      REAL RESP
2500  CONTINUE
      TIMEB=BTIME/60.0
      TIMER=RTIME/60.0
      WRITE(7,2502)TIMEB
2502  FORMAT('0','LENGTH OF BLEACH   ',F5.3,' SECONDS')
      WRITE(7,2504)TIMER
2504  FORMAT('0','LENGTH OF RUN      ',F5.1,' SECONDS')
      WRITE(7,2506)DTIME
2506  FORMAT('0','INTERRUPT INTERVAL ',F5.3,' SECONDS')
      WRITE(7,*)
      WRITE(7,*)' PLEASE ENTER CHOICE FROM LIST BELOW'
      WRITE(7,*)' BT-TO SET BLEACHING TIME'
      WRITE(7,*)' RT-TO SET RUNNING TIME / INTERRUPT INTERVAL'
      WRITE(7,*)' OK-IF SATISFIED WITH VALUES'
      READ(7,2510) RESP
2510  FORMAT(A2)
      IF(RESP.EQ.'BT')GO TO 2520
      IF(RESP.EQ.'RT')GO TO 2530
      IF(RESP.EQ.'OK')GO TO 2599
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 2500
2520  CONTINUE
      WRITE(7,*)' ENTER LETTER CORRESPONDING TO BLEACH TIME DESIRED'
      WRITE(7,*)' A = 0.25 SECONDS'
      WRITE(7,*)' B = 0.50 SECONDS'
      WRITE(7,*)' C = 1.00 SECONDS'
      WRITE(7,*)' D = 2.00 SECONDS'
      WRITE(7,*)' ANY OTHER RESPONSE VALUE UNCHANGED'
      READ(7,2522) RESP
2522  FORMAT(A1)
      IF(RESP.EQ.'A') BTIME=0.25*60
      IF(RESP.EQ.'B') BTIME=0.50*60
      IF(RESP.EQ.'C') BTIME=1.00*60
      IF(RESP.EQ.'D') BTIME=2.00*60
      GO TO 2500
2530  CONTINUE
      WRITE(7,*)' ENTER LETTER CORRESPONDING TO DESIRED INTERVAL'
      WRITE(7,*)' A = DATA TAKEN EVERY 0.005   SECOND'
      WRITE(7,*)' B = DATA TAKEN EVERY 0.008   SECOND'
      WRITE(7,*)' C = DATA TAKEN EVERY 0.01    SECOND'
```

```
      WRITE(7,*)' D = DATA TAKEN EVERY 0.1    SECOND'
      WRITE(7,*)' E = DATA TAKEN EVERY 1.0000 SECOND'
      WRITE(7,*)' ANY OTHER RESPONSE VALUE UNCHANGED'
      READ(7,2532)RESP
2532  FORMAT(A1)
      IF(RESP.EQ.'A') DTIME=0.005
      IF(RESP.EQ.'B') DTIME=0.008
      IF(RESP.EQ.'C') DTIME=0.01
      IF(RESP.EQ.'D') DTIME=0.1
      IF(RESP.EQ.'E') DTIME=1.0
2533  CONTINUE
      WRITE(7,*)
      WRITE(7,*)' ENTER LETTER CORRESPONDING TO DESIRED RUN TIME'
      WRITE(7,*)' A =   5 SECONDS'
      WRITE(7,*)' B =  15 SECONDS'
      WRITE(7,*)' C =  30 SECONDS'
      WRITE(7,*)' D =  45 SECONDS'
      WRITE(7,*)' E =  60 SECONDS'
      WRITE(7,*)' F =  90 SECONDS'
      WRITE(7,*)' G = 120 SECONDS'
      WRITE(7,*)' H = 150 SECONDS'
      WRITE(7,*)' I = 180 SECONDS'
      WRITE(7,*)' J = 240 SECONDS'
      WRITE(7,*)' K = 300 SECONDS'
      READ(7,2534) RESP
2534  FORMAT(A1)
      IF(RESP.EQ.'A') GO TO 2536
      IF(RESP.EQ.'B') GO TO 2536
      IF(RESP.EQ.'C') GO TO 2536
      IF(RESP.EQ.'D') GO TO 2536
      IF(RESP.EQ.'E') GO TO 2536
      IF(RESP.EQ.'F') GO TO 2536
      IF(RESP.EQ.'G') GO TO 2536
      IF(RESP.EQ.'H') GO TO 2536
      IF(RESP.EQ.'I') GO TO 2536
      IF(RESP.EQ.'J') GO TO 2536
      IF(RESP.EQ.'K') GO TO 2536
      WRITE(7,*)' UNABLE TO DETERIMNE RESPONSE'
      GO TO 2533
2536  CONTINUE
      IF(RESP.EQ.'A') RUN=5.0/DTIME
      IF(RESP.EQ.'B') RUN=15.0/DTIME
      IF(RESP.EQ.'C') RUN=30.0/DTIME
      IF(RESP.EQ.'D') RUN=45.0/DTIME
      IF(RESP.EQ.'E') RUN=60.0/DTIME
      IF(RESP.EQ.'F') RUN=90.0/DTIME
      IF(RESP.EQ.'G') RUN=120.0/DTIME
      IF(RESP.EQ.'H') RUN=150.0/DTIME
      IF(RESP.EQ.'I') RUN=180.0/DTIME
      IF(RESP.EQ.'J') RUN=240.0/DTIME
      IF(RESP.EQ.'K') RUN=300.0/DTIME
```

```
      IF(RUN.LE.3000) GO TO 2540
      WRITE(7,*)
      WRITE(7,*)' RUN TIME EXCEEDS MAXIMUM ALLOWABLE TIME FOR'
      WRITE(7,*)' INTERRUPT INTERVAL OF ',DTIME,' SECONDS/INTERRUPT'
      GO TO 2533
2540  CONTINUE
      I2COMP=(-1)*DTIME*1000          !#COUNTS / INTERRUPT
      IF(RESP.EQ.'A') RTIME=5.0*60.
      IF(RESP.EQ.'B') RTIME=15.0*60.
      IF(RESP.EQ.'C') RTIME=30.0*60.
      IF(RESP.EQ.'D') RTIME=45.0*60.
      IF(RESP.EQ.'E') RTIME=60.0*60.
      IF(RESP.EQ.'F') RTIME=90.0*60.
      IF(RESP.EQ.'G') RTIME=120.0*60.
      IF(RESP.EQ.'H') RTIME=150.0*60.
      IF(RESP.EQ.'I') RTIME=180.0*60.
      IF(RESP.EQ.'J') RTIME=240.0*60.
      IF(RESP.EQ.'K') RTIME=300.0*60.
      GO TO 2500
2599  CONTINUE
      RETURN
      END
```

```
      SUBROUTINE NAMEF
C
C     THIS SUBROUTINE IS PART OF THE LASER CONTROL PROGRAM (LASCON).
C     ITS FUNCTION IS TO ALLOW THE USER TO CHANGE THE OUTPUT FILE
C     NAMES WHILE RUNNING THE PROGRAM. IT CAN ALSO BE CALLED BY
C     CPARAM SUBROUTINE
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /FILE/ FNAME,IUNIT
      REAL RESP
      REAL*8 FNAME
C
C-----------------------------------------------------------------
C
800   CONTINUE
      WRITE(7,802)
802   FORMAT('0','ENTER YOUR CHOICE FROM BELOW')
      WRITE(7,*)' D-TO USE DEFAULT NAME PUT INTO FILE'
      WRITE(7,*)' C-TO CHOOSE NAME PUT INTO FILE'
      WRITE(7,*)' U-TO CHOOSE OUTPUT FILE UNIT NUMBER'
      WRITE(7,*)' E-TO EXIT NAMEF MODULE'
      READ(7,804) RESP
804   FORMAT(A1)
      IF(RESP.EQ.'D') GO TO 806
      IF(RESP.EQ.'C') GO TO 810
      IF(RESP.EQ.'U') GO TO 830
      IF(RESP.EQ.'E') GO TO 899
      WRITE(7,*)'UNABLE TO DETERMINE RESPONSE'
      GO TO 800
C
C-----------------------------------------------------------------
C
806   CONTINUE
      FNAME='FRAP.DAT'
      WRITE(7,808) IUNIT,FNAME
808   FORMAT('0','OUTPUT FILE=FTN',I2,'.DAT:NAME PUT INTO FILE=',A8)
      GO TO 800
C
C-----------------------------------------------------------------
C
810   CONTINUE
      WRITE(7,820)
820   FORMAT('0','PLEASE ENTER NAME TO BE PUT INTO THE OUTPUT FILE'
C     WRITE(7,*)' IT MUST BE IN THE FORM "____.DAT"'
      READ(7,822) FNAME
822   FORMAT(A8)
      WRITE(7,826) FNAME
826   FORMAT('0','NAME ENTERED WAS ',A8,' , CORRECT? (Y,N)')
      READ(7,828) RESP
```

```
828     FORMAT(A1)
        IF(RESP.EQ.'Y') GO TO 800
        IF(RESP.EQ.'N') GO TO 810
        WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
        GO TO 810
830     CONTINUE
        WRITE(7,832)IUNIT
832     FORMAT('0','PRESENT OUTPUT FILE=FTN',I2,'.DAT')
        WRITE(7,*)
        WRITE(7,*)' PROGRAM INCREMENTS UNIT BY ONE AFTER EXPERIMENTAL'
        WRITE(7,*)' RUN SO PROGRAM MAY BE RUN REPEATEDLY, THE USER'
        WRITE(7,*)' MAY CHANGE UNIT BY ENTERING A NEW UNIT NUMBER'
        WRITE(7,*)' BETWEEN 10 AND 99 BUT IF UNIT NUMBER HAS BEEN USED'
        WRITE(7,*)' BEFORE THE EXISTING FILE WILL BE OVERWRITTEN'
        READ(7,*) IUNIT
        IF(IUNIT.LT.10) GO TO 830
        IF(IUNIT.GT.99) GO TO 830
        WRITE(7,836)IUNIT
836     FORMAT('0','NEW OUTPUT FILE=FTN',I2,'.DAT')
        WRITE(7,*)
        WRITE(7,*)' ENTER Y-CORRECT,N-INCORRECT'
        READ(7,838) RESP
838     FORMAT(A1)
        IF(RESP.EQ.'Y') GO TO 800
        IF(RESP.EQ.'N') GO TO 830
        WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
        GO TO 830
C
C-----------------------------------------------------------------
C
899     CONTINUE
        RETURN
        END
```

```
      SUBROUTINE HELPME
C
C     THIS SUBROUTINE IS PART OF THE LASER CONTROL PROGRAM. ITS
C     FUNCTION IS TO FURTHER EXPLAIN OPTIONS THAT ARE POSSIBLE.
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      REAL RESP
C
C-----------------------------------------------------------------
C
1000  CONTINUE
      WRITE(7,1040)
1040  FORMAT('0','INFORMATION ON EACH OPTION WILL BE GIVEN')
      WRITE(7,*)' ONLY A FEW OPTIONS WILL BE LISTED AT ONE TIME'
      WRITE(7,1080)
1080  FORMAT('0','HIT RETURN TO CONTINUE')
      READ(7,1082) RESP
1082  FORMAT(A1)
      WRITE(7,1042)
1042  FORMAT('1','CP - CHANGE PARAMETERS')
      WRITE(7,*)' THIS OPTION ALLOWS USER TO CHANGE PARAMETERS. IF'
      WRITE(7,*)' NO CHANGES MADE WILL REMAIN AS DEFAULT VALUES OR'
      WRITE(7,*)' VALUES PREVIOUSLY SET.'
      WRITE(7,1044)
1044  FORMAT('0','EX - EXIT PROGRAM')
      WRITE(7,1046)
1046  FORMAT('0','FN - ENTER FILE NAME')
      WRITE(7,*)' THIS OPTION ALLOWS USER TO CHANGE NAME PUT INTO'
      WRITE(7,*)' OUTPUT FILE FTN__.DAT, FTN__.DAT IS INCREMENTED'
      WRITE(7,*)' BY ONE EACH TIME EXPERIMENTAL RUN IS COMPLETED'
      WRITE(7,1048)
1048  FORMAT('0','HE - HELP')
      WRITE(7,1050)
1050  FORMAT('0','LP - LIST PARAMETERS')
      WRITE(7,*)' THIS OPTION LISTS THE PARAMETERS AS THEY EXIST'
      WRITE(7,*)' EITHER DEFAULT OR SET VALUES.'
      WRITE(7,1062)
1062  FORMAT('0','HIT RETURN TO CONTINUE.')
      READ(7,1064) RESP
1064  FORMAT(A1)
      WRITE(7,1054)
1054  FORMAT('1','RB - RUN BLEACH EXPERIMENT')
      WRITE(7,*)' THIS OPTION ALLOWS USER TO INITIATE A BLEACH'
      WRITE(7,*)' SEQUENCE. SUBROUTINES DRIVE FILTERS,SHUTTERS'
      WRITE(7,*)' AND DATA ACQUISTION.'
      WRITE(7,1056)
1056  FORMAT('0','RL - RUN LASER SET UP')
      WRITE(7,*)' THIS OPTION ALLOWS USER TO RUN SYSTEM WITHOUT'
      WRITE(7,*)' BLEACH SEQUENCE. USED FOR ADJUSTING PMT, ETC.'
```

```
      WRITE(7,1058)
1058  FORMAT('0','SB - STAND BY')
      WRITE(7,*)' THIS OPTION ALLOWS THE SYSTEM TO BE PUT IN A'
      WRITE(7,*)' SAFE MODE WHILE SYSTEM IS JUST SITTING IDLE.'
      WRITE(7,1060)
1060  FORMAT('0','HIT RETURN TO CONTINUE.')
      READ(7,1068) RESP
1068  FORMAT(A1)
1074  CONTINUE
      WRITE(7,1070)
1070  FORMAT('0','DO NEED TO SEE OPTIONS AGAIN? (Y,N)')
      READ(7,1072) RESP
1072  FORMAT(A1)
      IF(RESP.EQ.'Y') GO TO 1000
      IF(RESP.EQ.'N') GO TO 1099
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 1074
1099  CONTINUE
      WRITE(7,*)' LEAVING HELP MODULE'
      RETURN
      END
```

```
      SUBROUTINE LPARAM
C-----------------------------------------------------------------------
C     SUBROUTINE LISTS LASCON PROGRAM PARAMETERS ON REQUEST. IT
C     IS ALSO CALLED BY CPARAM SUBROUTINE
C-----------------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------------
      COMMON /FILE/ FNAME,IUNIT
      COMMON /FILTER/ BLECHV,BLEVAL,OBSERV,OBSVAL,TRNSO,TRNSB
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLK60,ICLKST,I2COMP
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      INTEGER BLECHV,BLEVAL,OBSERV,OBSVAL
      REAL RESP
      REAL*8 FNAME
C-----------------------------------------------------------------------
10    CONTINUE
      WRITE(7,20)
20    FORMAT('0','THE PRESENT PARAMETER VALUES ARE:')
      WRITE(7,*)
      WRITE(7,*)' ------INTENSITY LEVELS-----'
      WRITE(7,*)' OBSERVATION=',OBSERV,'  BLEACH=',BLECHV
      WRITE(7,*)' -----OBSERVATION SHUTTER CONDITIONS-----'
      IF(ISHUTN.EQ.1) WRITE(7,*)' LASER-OPEN,PMT-CLOSED'
      IF(ISHUTN.EQ.2) WRITE(7,*)' LASER-CLOSED,PMT-OPEN'
      IF(ISHUTN.EQ.3) WRITE(7,*)' LASER-CLOSED,PMT-CLOSED'
      IF(ISHUTN.EQ.4) WRITE(7,*)' LASER-OPEN,PMT-OPEN'
      WRITE(7,*)' ------OUTPUT FILE PARAMETERS-------'
      WRITE(7,30) IUNIT,FNAME
30    FORMAT(' OUTPUT FILE= FTN',I2,'.DAT; NAME PUT INTO FILE=',A8)
      TIMEB=BTIME/60.0
      TIMER=RTIME/60.0
      WRITE(7,32)TIMEB
32    FORMAT('0','LENGTH OF BLEACH ',F5.3,' SECONDS')
      WRITE(7,34)TIMER
34    FORMAT('0','LENGTH OF RUN    ',F5.1,' SECONDS')
      WRITE(7,36)DTIME
36    FORMAT('0','DATA COLLECTED AT ',F5.3,' SECOND INTERVALS')
      WRITE(7,*)
      WRITE(7,*)' HIT RETURN TO CONTINUE'
      READ(7,40) RESP
40    FORMAT(A1)
      RETURN
      END
```

```
      SUBROUTINE EXPRUN
C-----------------------------------------------------------------------
C       THIS SUBROUTINE IS PART OF THE LASER CONTROL PROGRAM FOR THE
C       F.R.A.P. EXPERIMENTS. (LASCON.FOR). THIS SUBROUTINE WHEN
C       CALLED WILL EXCUTE AN EXPERIMENTAL RUN. IT CONTROLS ALL
C       EQUIPMENT REQUIRED FOR AN EXPERIMENTAL RUN AND ALSO TAKES
C       CHARGE OF THE DATA AQUISITION.
C-----------------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------------
      COMMON /ATOD/ IADBUF,IADCSR,INITD,IREADY
      COMMON /DRV11/ IDRV11,INTENB,INTENO
      COMMON /DTOA/ IDACHA,IPCNT,IPOST
      COMMON /FLAGS/ LPRINT,LTAKE
      COMMON /FILE/ FNAME,IUNIT
      COMMON /FILTER/ BLECHV,BLEVAL,OBSERV,OBSVAL,TRNSO,TRNSB
      COMMON /ISRS/ ID,IPRTY,IVEC,LD,LPRTY,LVEC
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLKGO,ICLKST,I2COMP
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      COMMON /VALUE/ IELEVN,IONE,IVOUT,IZERO
      COMMON /ZTICK/ ICOL,ITICK,TICK
      DIMENSION IVOUT(11,280)
      DIMENSION IPOST(11)
      EXTERNAL ATDC13,LTIME
      INTEGER BLECHV,BLEVAL,OBSERV,OBSVAL
      REAL RESP
      REAL*8 FNAME
1600  CONTINUE
      WRITE(7,*)
      WRITE(7,*)' YOU ARE IN THE EXPERIMENTAL RUN MODE. PLEASE ENTER'
      WRITE(7,*)' YOUR CHOICE FROM THE FOLLOWING LIST:'
      WRITE(7,*)
      WRITE(7,*)' GO - PROCEED WITH F.R.A.P. EXPERIMENT'
      WRITE(7,*)' RE - RETURN TO LASCON MENU'
      READ(7,1610) RESP
1610  FORMAT(A2)
      IF(RESP.EQ.'GO') GO TO 1620
      IF(RESP.EQ.'RE') GO TO 1690
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 1600
1620  CONTINUE
C-----------------------------------------------------------------------
C       SETTING INITIAL VALUES,RUN PARAMETERS,AND I.S.R.'S
C-----------------------------------------------------------------------
      CALL IPOKE(IDRV11,INTENO)       !DRV11 TO OBS. & SHUT COND.
      COUNT=0.          !COUNTER OF CYCLES TO CALCULATE TIME IN SEC.
      ICOL=1            !COLUMN POINTER
      ITICK=1           !INTERRUPT COUNTER
      LPRINT=0          !FLAG TO EXIT INIT. DATA COLLECTION
      LTAKE=0           !FLAG FOR END OF BLEACH TIME
```

```
      TIME=0.          !COUNTER FOR RUNNING TIME
      TICK=0.          !COUNTER FOR INTERVAL TIME
      IPCNT=6          !RESETS MIRROR POSITION POINTER
      DO 1300 IR=1,11
       DO 1302 IC=1,280
      IVOUT(IR,IC)=0          !INITIALIZES VOLTAGE OUT ARRAY
1302  CONTINUE
1300  CONTINUE
      OPEN(UNIT=IUNIT,ERR=1629,TYPE='NEW',INITIALSIZE=300)
      CALL IPOKE(LTCCSR,LDISAB)       !CLEARS LTC CSR FOR I.S.R.
      CALL IPOKE(DTACHA,IPOST(IPCNT)) !SETS MIRROR TO 1ST LOCATION
      CALL IPOKE(ICLBPR,I2COMP)       !SETS NUMBER COUNTS/INTERRUPT
      LERR=INTSET(LVEC,LPRTY,LD,LTIME)        !SETS ISR FOR LTC
      WRITE(7,8)' DELAY TO SET UP LTC ISR, HIT RETURN TO CONTINUE'
      READ(7,1621) RESP
1621  FORMAT(A1)
      IF(LERR.NE.0) GO TO 1662        !CHECKS FOR ISR ERROR
      CALL IPOKE(ICLCSR,LDISAB)       !CLEARS RTC CSR FOR I.S.R.
      IERR=INTSET(IVEC,IPRTY,ID,ATDC13)       !SETS ISR FOR RTC
      WRITE(7,8)' DELAY TO SET UP RTC ISR, HIT RETURN TO CONTINUE'
      READ(7,1623) RESP
1623  FORMAT(A1)
      IF(IERR.NE.0) GO TO 1672        !CHECKS FOR ISR ERROR
C-----------------------------------------------------------------------
C     WRITING INITIAL VALUES TO OUTPUT FILE
C-----------------------------------------------------------------------
      WRITE(IUNIT,1624) FNAME
      WRITE(7,1624) FNAME
1624  FORMAT(A8)
      TIMEB=BTIME/60.
      TIMER=RTIME/60.
      WRITE(IUNIT,1625)TIMEB,TIMER,DTIME
1625  FORMAT(F10.2,' =BTIME ',F12.1,' =RTIME ',F5.3,' =DTIME')
      WRITE(IUNIT,1626)OBSERV,TRNSO,BLECHV,TRNSB
1626  FORMAT(I3,' =OBS L;TRAN=',F12.8,3X,I3,' =BLE L;TRAN=',F12.8)
C     TAKES PREBLEACH,POSITION ZERO READING FOR RUN
      WRITE(7,8)
      WRITE(7,8)' TAKING INITIAL READINGS'
C     CALL IPOKE(IADCSR,INITD)        !INITIATES DATA CONVERSION
C     CHECKS IF INITIAL READING DATA CONVERSION DONE
C1628 IF((IPEEK(IADCSR).AND.IREADY).EQ.IZERO) GO TO 1628
C     IVLOUT=IPEEK(IADBUF)
C     WRITE(IUNIT,8)
C     WRITE(IUNIT,1630) IPCNT,TICK,IVLOUT
C     WRITE(7,1630)IPCNT,TICK,IVLOUT
C1630 FORMAT(I3,2X,F12.3,3X,I12)
      CALL INTDAT             !COLLECTS INITIAL DATA
      IF(LPRINT.EQ.0) GO TO 7000
      TIME=BTIME                      !SETS LTC ISR BLEACH CHECK
      WRITE(7,8)
      WRITE(7,8)' BLEACHING'
```

```
      CALL IPOKE(LTCCSR,"100) !ENABLES INTERRUPT BIT 6 LTC
      CALL IPOKE(IDRV11,INTENB)       !POKES B-INTENT AND SHUT COND.
1644  IF(LTAKE.EQ.0) GO TO 1644       !CHECKS BLEACH DONE FLAG CHANGE
      CALL IPOKE(ICLCSR,ICLKGO)       !POKES GO BIT FOR RTC ISR
      WRITE(7,*)
      WRITE(7,*)' COLLECTING'
C     CHECKS IF RUN IS DONE,IF DONE TURNS OFF RTC ISR
1656  IF(LTAKE.LE.1)GO TO 1656
      CALL IPOKE(ICLCSR,LDISAB)       !TURNS RTC ISR OFF
      CALL IPOKE(LTCCSR,LDISAB)       !TURNS LTC ISR OFF
      WRITE(7,*)
      WRITE(7,*)' WRITING DATA INTO OUTPUT FILE'
      WRITE(IUNIT,1303)IPCNT,TICK,IVOUT(IPCNT,ICOL)   !LAST DATA
1303  FORMAT(I3,2X,F12.3,3X,I12)
      WRITE(IUNIT,*)ICOL,ITICK,TICK
      WRITE(IUNIT,*)
C     WRITING INTEGER VOLTAGE REPRESENTATION INTO OUTPUT FILE
      TIMTMP=DTIME
      DO 1304 IR=6,11
       WRITE(IUNIT,1305)IR,TIMTMP,IVOUT(IR,IONE)
1305   FORMAT(I3,2X,F12.3,3X,I12)
       TIMTMP=TIMTMP+DTIME
1304  CONTINUE
      DO 1306 IC=2,ICOL
       DO 1308 IR=1,11
       WRITE(IUNIT,1307)IR,TIMTMP,IVOUT(IR,IC)
1307   FORMAT(I3,2X,F12.3,3X,I12)
       TIMTMP=TIMTMP+DTIME
1308   CONTINUE
1306  CONTINUE
      WRITE(7,*)
      WRITE(7,*)' NORMAL END OF EXPERIMENTAL RUN'
7000  CONTINUE
      CLOSE (UNIT=IUNIT)
      IUNIT=IUNIT+1           !INCREMENTS UNIT NUMBER
      IF(IUNIT.GT.99) IUNIT=0 !DEFINES IMPOSSIBLE UNIT
      IF(IUNIT.LT.10) IUNIT=0 !DEFINES IMPOSSIBLE UNIT
      GO TO 1600
1629  CONTINUE
      CALL IPOKE(LTCCSR,LDISAB)       !TURNS LTC ISR OFF
      CALL IPOKE(ICLCSR,LDISAB)       !TURNS RTC ISR OFF
      WRITE(7,*)' ERROR OPENING FILE, RUN ABORTED'
      GO TO 1600
1662  CONTINUE
      CALL IPOKE(LTCCSR,LDISAB)       !TURNS LTC ISR OFF
      CALL IPOKE(ICLCSR,LDISAB)       !TURNS RTC ISR OFF
      CLOSE(UNIT=IUNIT)
      WRITE(7,*)' ERROR SETTING UP LTC ISR, RUN ABORTED'
      GO TO 1600
1672  CONTINUE
      CALL IPOKE(LTCCSR,LDISAB)       !TURNS LTC ISR OFF
```

```
      CALL IPOKE(ICLCSR,LDISAB)       !TURNS RTC ISR OFF
      CLOSE(UNIT=IUNIT)
      WRITE(7,*)' ERROR SETTING UP RTC ISR, RUN ABORTED'
      GO TO 1600
1690  CONTINUE
      CALL IPOKE(LTCCSR,LDISAB)       !TURNS LTC ISR OFF
      CALL IPOKE(ICLCSR,LDISAB)       !TURNS RTC ISR OFF
      RETURN
      END
```

```
      SUBROUTINE ATDC13
C-----------------------------------------------------------------
C     THIS SUBROUTINE IS AN INTERRUPT SERVICE ROUTINE FOR A/D DATA
C     SAMPLING AND MIRROR POSITIONING. IT IS PART OF THE EXPRUN
C     SUBROUTINE WHICH IN TURN PART OF THE LASCON PROGRAM.
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /ATOD/ IADBUF,IADCSR,INITD,IREADY
      COMMON /DTOA/ IDACHA,IPCNT,IPOST
      COMMON /FLAGS/ LPRINT,LTAKE
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLK60,ICLKST,I2COMP
      COMMON /VALUE/ IELEVN,IONE,IVOUT,IZERO
      COMMON /ITICK/ ICOL,ITICK,TICK
      DIMENSION IVOUT(11,280)
      DIMENSION IPOST(11)
C-----------------------------------------------------------------
      CALL IPOKE(ICLCSR,ICLK60)       !RESETS CLOCK 60 BIT
      CALL IPOKE(IADCSR,INITD)        !INITIATES DATA CONVERSION
C     CHECKS IF DATA CONVERSION IS DONE
200   IF((IPEEK(IADCSR).AND.IREADY).EQ.IZERO) GO TO 200
      IVOUT(IPCNT,ICOL)=IPEEK(IADBUF) !RETRIEVES DATA FROM BUFFER
      IPCNT=IPCNT+IONE        !INCREMENTS MIRROR POSITION POINTER
      TICK=TICK+DTIME         !KEEPS TIME
      ITICK=ITICK+IONE        !COUNTS INTERRUPTS
C     CHECKS IF INCREMENT RESET IS NEEDED
      IF(IPCNT.LE.IELEVN) GO TO 210
      IPCNT=IONE
      ICOL=ICOL+IONE
210   CONTINUE
      CALL IPOKE(IDACHA,IPOST(IPCNT))   !MIRROR TO NEXT POSITION
      RETURN
      END
```

```
      SUBROUTINE LTIME
C-----------------------------------------------------------------
C       THIS SUBROUTINE IS AN INTERRUPT SERVICE ROUTINE - THAT
C       COUNTS THE NUMBER OF TICKS OF THE LINE TIME CLOCK (LTC)
C       TO CONTROL THE LENGTH OF BLEACHING AND THE LENGTH OF DATA
C       COLLECTING FOR AN EXPERIMENTAL RUN OF F.R.A.P. SYSTEM.
C       THE SUBROUTINE IS PART OF THE LASCON PROGRAM, CALLED BY
C       EXPRUN SUBROUTINE.
C-----------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------
      COMMON /DRV11/ IDRV11,INTENB,INTENO
      COMMON /FLAGS/ LPRINT,LTAKE
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /VALUE/ IELEVN,IONE,IVOUT,IZERO
      DIMENSION IVOUT(11,280)
C
      CALL IPOKE(LTCCSR,LENABL)       !RESETS ENABLE BIT 6 OF LTC
      COUNT=COUNT+IONE
      IF(COUNT.LT.TIME) GO TO 99
      CALL IPOKE(IDRV11,INTENO)       !DRV11 TO OBS. & SHUT COND.
      LTAKE=LTAKE+IONE
      TIME=RTIME
      COUNT=0.0
99    RETURN
      END
```

```
      SUBROUTINE INTDAT
C
C     THIS SUBROUTINE IS PART OF LASCON PROGRAM. IT IS CALLED
C     BY THE SUBROUTINE EXPRUN. IT'S FUNCTION IS TO TAKE THE
C     INITIAL READINGS FOR GIVEN MIRROR POSITIONS AND WRITE
C     THEM INTO THE OUTPUT FILE AND WRITE THE AVERAGE TO THE
C     TERMINAL SCREEN. THIS SUBROUTINE ALSO ALLOWS THE USER TO
C     TO CALIBRATE THE P.M.T. SO THAT THE MAXIMUM INTENSITY IS
C     WITHIN THE 0.0 TO 10.0 VOLT LIMIT OF THE P.M.T.
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /ATOD/ IADBUF,IADCSR,INITD,IREADY
      COMMON /DRV11/ IDRV11,INTENB,INTENO
      COMMON /DTOA/ IDACHA,IPCNT,IPOST
      COMMON /FLAGS/ LPRINT,LTAKE
      COMMON /FILE/ FNAME,IUNIT
      COMMON /FILTER/ BLECHV,BLEVAL,OBSERV,OBSVAL,TRNSO,TRNSB
      COMMON /ISRS/ ID,IPRTY,IVEC,LD,LPRTY,LVEC
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLKGO,ICLKST,I2COMP
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      COMMON /VALUE/ IELEVN,IONE,IVOUT,IZERO
      DIMENSION IDLIST(11)
      DIMENSION IPOST(11)
      DIMENSION AVE(11)
      EXTERNAL ATDC13,LTIME
      INTEGER BLECHV,BLEVAL,OBSERV,OBSVAL
      REAL RESP
      REAL*8 FNAME
6000  CONTINUE
      WRITE(7,*)
      WRITE(7,*)' ENTER YOUR OPTION CHOICE FROM LIST BELOW:'
      WRITE(7,*)' CL-TO CALIBRATE P.M.T.'
      WRITE(7,*)' RE-TO RETURN TO MAIN MENU'
      WRITE(7,*)' TD-TO TAKE DATA FOR OUTPUT FILE'
      READ(7,6010)RESP
6010  FORMAT(A2)
      IF(RESP.EQ.'CL') GO TO 6100
      IF(RESP.EQ.'TD') GO TO 6200
      IF(RESP.EQ.'RE') GO TO 6300
      WRITE(7,*)' UNABLE TO DETERMINE RESPONSE'
      GO TO 6000
6100  CONTINUE
      WRITE(7,*)
      DO 6110 I=1,11
      IVLOUT=0
       DO 6120 J=1,10
        DO 6130 L=1,1000
6130     CONTINUE
```

```
        CALL IPOKE(IADCSR,INITD)
6122    IF((IPEEK(IADCSR).AND.IREADY).EQ.IZERO) GO TO 6122
        IVLOUT=IVLOUT+IPEEK(IADBUF)
        AVE(I)=IVLOUT/J
6120    CONTINUE
       WRITE(7,6112)I,AVE(I)
6112   FORMAT(' POSITION :',I3,' AVERAGE:',F8.1)
6110   CONTINUE
       GO TO 6000
6200   CONTINUE
       LPRINT=1                !FLAG FOR CONTINUING
       WRITE(7,*)
       DO 6210 I=1,11
        IVLOUT=0
        DO 6220 J=1,10
         DO 6230 L=1,1000
6230     CONTINUE
        CALL IPOKE(IADCSR,INITD)       !INITIATES DATA COLLECTION
6222    IF((IPEEK(IADCSR).AND.IREADY).EQ.IZERO) GO TO 6222
        IVTOUT=IPEEK(IADBUF)
        IVLOUT=IVLOUT+IPEEK(IADBUF)
        AVE(I)=IVLOUT/J
        WRITE(IUNIT,6224)I,TICK,IVTOUT
6224    FORMAT(I3,2X,F12.1,3X,I12)
6220   CONTINUE
       WRITE(IUNIT,6226)I,AVE(I)
6226   FORMAT(' POSITION:',I3,' AVERAGE: ',F8.1)
       WRITE(7,6228)I,AVE(I)
6228   FORMAT(' POSITION:',I3,' AVERAGE: ',F8.1)
6210   CONTINUE
       GO TO 6999
6300   CONTINUE
       LPRINT=0
6999   CONTINUE
       RETURN
       END
```

```
      SUBROUTINE LASET
C-----------------------------------------------------------------------
C       THIS SUBROUTINE ALLOWS FOR SET UP AND INITIALIZATION OF THE
C       EQUIPMENT USED IN DATA COLLECTION AND CONTROL OF THE LASER
C       CONTROL PROGRAM (LASCON). IT USES MANY OF THE SAME SUBROUTINES
C       AS THE EXPRUN SUBROUTINE.
C-----------------------------------------------------------------------
C       DECLARATIONS
C-----------------------------------------------------------------------
      COMMON /ATOD/ IADBUF,IADCSR,INITD,IREADY
      COMMON /DRV11/ IDRV11,INTENB,INTENO
      COMMON /DTOA/ IDACHA,IPCNT,IPOST
      COMMON /FLAGS/ LPRINT,LTAKE
      COMMON /FILE/ FNAME,IUNIT
      COMMON /FILTER/ BLECHV,BLEVAL,OBSERV,OBSVAL,TRNSO,TRNSB
      COMMON /ISRS/ ID,IPRTY,IVEC,LD,LPRTY,LVEC
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLKGO,ICLKST,I2COMP
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      COMMON /VALUE/ IELEVN,IONE,IVOUT,IZERO
      COMMON /ZTICK/ ICOL,ITICK,TICK
      DIMENSION IVOUT(11,280)
      DIMENSION IPOST(11)
      INTEGER BLECHV,BLEVAL,OBSERV,OBSVAL
      REAL RESP
      IP=IPCNT                !MIRROR POSITION ELEMENT
3000  CONTINUE
      WRITE(7,3010)
3010  FORMAT('0','IN LASER SET UP MODULE--PLEASE ENTER CHOICE FROM')
      WRITE(7,1)' THE FOLLOWING:'
      WRITE(7,1)' PO-BEAM POSITION SET UP'
      WRITE(7,1)' IN-BEAM INTENSITY SET UP'
      WRITE(7,1)' CP-CHANGE PARAMETERS'
      WRITE(7,1)' LP-LIST PARAMETERS'
      WRITE(7,1)' RE-RETURN TO LASCON MENU'
      READ(7,3020)RESP
3020  FORMAT(A2)
      IF(RESP.EQ.'CP') GO TO 3060
      IF(RESP.EQ.'IN') GO TO 3030
      IF(RESP.EQ.'LP') GO TO 3050
      IF(RESP.EQ.'PO') GO TO 3040
      IF(RESP.EQ.'RE') GO TO 3060
      WRITE(7,1)' UNABLE TO DETERMINE RESPONSE'
      GO TO 3000
3030  CONTINUE
      WRITE(7,3032)
3032  FORMAT('0','BEAM INTENSITY SET UP')
      CALL IPOKE(IDRV11,INTENO)       !POKES INT AND SHUT COND
      GO TO 3000
3040  CONTINUE
      WRITE(7,3042)
```

```
3042    FORMAT('0','BEAM POSITION SET UP')
        WRITE(7,1)
        CALL IPOKE(IDACHA,IPOST(IP))
        WRITE(7,1)' PRESENT MIRROR POSITION IS = ',IP
        WRITE(7,1)
        WRITE(7,1)' ENTER MIRROR POSITION DESIRED, 1-11;6 CENTER:'
        WRITE(7,1)' MIRROR RESET BY EXPRUN SO NO NEED CHANGE BACK'
        WRITE(7,1)' TO ORIGINAL SETTING.'
        READ(7,3044) IP
3044    FORMAT(I2)
        IF(IP.LT.1) GO TO 3040
        IF(IP.GT.11) GO TO 3040
        CALL IPOKE(IDACHA,IPOST(IP))    !POKES IN MIRROR POSITION
        GO TO 3000
3050    CONTINUE
        CALL LPARAM
        GO TO 3000
3060    CONTINUE
        RETURN
        END
```

```
      SUBROUTINE STNDBY
C
C     THIS SUBROUTINE IS PART OF THE LASER CONTROL PROGRAM (LASCON).
C     IT'S FUNCTION IS TO SET ALL NEURTAL DENSITY IN PLACE AND CLOSE
C     ALL SHUTTERS ALLOWING FOR THE USER TO LEAVE SYSTEM ON BUT IN
C     A SAFE MODE.
C
C
C-----------------------------------------------------------------
C     DECLARATIONS
C-----------------------------------------------------------------
      COMMON /DRV11/ IDRV11,INTENB,INTENO
      COMMON /LCLOCK/ BTIME,COUNT,LDISAB,LENABL,LTCCSR,RTIME,TIME
      COMMON /RCLOCK/ DTIME,ICLBPR,ICLCSR,ICLKGO,ICLKST,I2COMP
      COMMON /SHUT/ IBOPEN,IBSHUT,ILSHUT,IPSHUT,ISHUT,ISHUTN
      REAL RESP
C
C-----------------------------------------------------------------
C
2040  CONTINUE
      WRITE(7,1)' YOU WISH TO GO TO THE STANDBY MODE? (Y OR N)'
      READ(7,2000) RESP
2000  FORMAT(A1)
      IF(RESP.EQ.'Y') GO TO 2020
      IF(RESP.EQ.'N') GO TO 2099
      WRITE(7,1)'UNABLE TO DETERMINE RESPONSE.'
      GO TO 2040
2020  CONTINUE
C-----------------------------------------------------------------
C     SET PARAMETERS TO CLOSE BOTH SHUTTERS AND PUT ALL FILTERS IN
C     BEAM PATH.
C-----------------------------------------------------------------
      CALL IPOKE(IDRV11,IBSHUT)       !CLOSES BOTH SHUTTERS
      CALL IPOKE(ICLCSR,ICLKST)       !TURNS OFF RTC ISR
      CALL IPOKE(LTCCSR,LDISAB)       !TUNRS OFF LTC ISR
2060  CONTINUE
      WRITE(7,2050)
2050  FORMAT('0','SYSTEM IN STANDBY MODE. ENTER LE TO LEAVE MODE')
      READ(7,2070) RESP
2070  FORMAT(A2)
      IF(RESP.EQ.'LE') GO TO 2099
      GO TO 2060
2099  CONTINUE
      WRITE(7,2080)
2080  FORMAT('0','LEAVING STANDBY MODE')
      RETURN
      END
```

APPENDIX C: BEAM ALIGNMENT OPERATIONS

Beam Alignment

Beam alignment must be done only after laser has been peaked for power output. Peaking the power output of the laser will assure the beam is along the same optical axis. The procedure that was developed is as follows:

1. Laser should be set to the lowest observation intensity that is visible using the control only program (LOPERA.SAV).

2. Remove first planar mirror from path. Adjust positioning set screws of the mirror until the 2 plats of mirror are equidistant at each corner. Removing the mirror also allows for a longer path for the beam for more precise alignment of the beam.

3. Adjust laser mounting plates until the beam optical axis is parallel to tapped holes of the optical table (reference frame). Aligning optical axis of beam is accomplished by affixing two of the extra mirror mount plates to the optical table parallel (or perpendicular) to a line of tapped holes on the optical table. The mounting plates are to be placed as near to and far from the laser as possible. These 2 mounting plates must be equidistant from all rows of tapped holes, assuring that the edges of the plates are parallel or perpendicular to all the top holes of the optical table reference frame. The location of the beam is then marked on a clear drafting angle (held normal to the optical table by a second

drafting angle) as close to the laser as possible. The angle is then moved to the second mounting plate if it matches a location of the beam marked the laser is aligned to optical table. If locations do not match adjust laser mounts. Repeat until both locations match. (Equidistant from table surface and two mounting plates.)

4. Replace first mirror such that the front plate of positioning mount is at a 45 degree angle to the beam without using the positioning screws.

5. Remove 2nd mirror, adjust so front and back plate of positioning mount are equidistant at each corner.

6. Again repeat positioning the two extra mirror mounting plates as near to and as far from the 1st mirrors surface. Using the two angles make the corrections to the beam location using the positioning screws of the first mirror.

7. Replace third mirror such that the front plate of positioning mount is at a 45 degree angle to the beam without using the positioning screws.

8. Remove scanning mirror positioning mounts but leaving 1" diameter post in place.

9. Adjust position of beam using clear drafting angles. One angle is used to assure second angle is perpendicular to the post (parallel to optical table). The second angle is placed such that the open center of the angle is around the post holding the third mirror. The edge of the second angle can then be placed against the post of the scanning mirror. The location of the beam is marked as near to the surface of the third mirror. The location is again checked at the end of the posts and the beam is corrected using the positioning screws of the mirror mount.

10. The scanning mirror mount is then replaced.

11. Position scanning mirror at center point of scan locations set by software.

12. Affix the two extra mirror mount plates so they will be perpendicular to the intended beam path.

13. Remove microscope and three point mounting plates just leaving the four corner mounting plates affixed to the optical table. (Warning: the beam will be at eye level when sitting!)

14. Adjust the two corner mounting plates so they are equidistant from all rows and columns of tapped holes.

15. Location of the beam is obtained by using a large pane of glass and an angle to assure that the pane is perpendicular to the table.

16. The mount attaching the scanning mirror to the post is adjusted so that the beam lines along a line parallel to the post.

17. The scanning mirror is then raised or lowered or moved to center the beam on the scanning mirror.

18. The scanning mirror is then rotated on the shaft to match locations of beam near and far from scanning mirror. Note rotating mirror may cause beam to deviate from center so check after rotating scanning mirror.

19. Replace microscope and three-point mounting plates. Level mounting plates with level of optical table.

20. Remove epi-illuminator housing and reducing lens from sliding housing.

21. Replace sliding housing center beam on fully closed iris of sliding housing by raising, lowering, or rotating mounting plates. Using glass plane location marked for scanning mirror positioning.

22. When centered replace reducing lens, so it is centered in opening of the sliding housing. Adjust positioning screws of sliding housing to center beam on location marked for scanning mirror positioning.

23. Replace epi-illuminator housing and place fluorescence sample on microscope.

24. Focus the beam on the sample by moving sliding housing toward or away from the epi-illuminator.

25. If emission spot is not symmetrical open iris of sliding housing and repeat 22-24 after changing rotation of microscope about the normal axis of the microscope.

APPENDIX D - SOLUTION TO DIFFUSION PROBLEM

The lateral transport equation with no bulk flow (V=0) gave the diffusion equation (Eq. 25)

$$\frac{\partial C}{\partial t} = D \nabla^2 C \qquad (D.1)$$

with the boundary condition

$$C(\infty,t) = C_0 \qquad (D.2)$$

and the initial condition

$$C(r,0) = C_0 \exp[-\alpha TI(r)] \qquad (D.3)$$

Equation (D.1) can be written using spherical and assuming no concentration dependence on the radius ρ or ϕ leaving only those terms dependent on θ.

$$\frac{\partial C}{\partial t} = \frac{D}{\rho^2} \frac{1}{\sin\theta} \frac{\partial}{\partial \theta} \left(\sin\theta \frac{\partial C}{\partial \theta} \right) \qquad (D.3)$$

The angle θ defines a circular bleached region, an angle of 0 radians denoting a point and an angle of π radians denoting the entire sample region. Equation (D.3) represents the diffusion of the concentration of fluorescently labeled molecules only after bleaching. Therefore if θ_0 describes the circular region of interest immediately after bleaching it is subject to the following

$$C = 0, \qquad 0 \leq \theta \leq \theta_0 \qquad (D.4)$$

$$C = C_0, \qquad \theta_0 \leq \theta \leq \pi \qquad (D.5)$$

Applying separation of variables $C(\theta,t) = T(t)R(\theta)$ where $T(t)$ represents time and $R(\theta)$ represents position.

Leaves

$$\frac{\rho^2}{D}\frac{\partial T}{\partial t} = \frac{1}{\sin\theta}\frac{\partial}{\partial\theta}\left(\sin\theta\,\frac{\partial R}{\partial\theta}\right) = -\lambda \qquad (D.6)$$

where $-\lambda$ is constant.

Solving for the time portion of the Equation (D.6) leaves

$$\frac{\partial T}{\partial t} = -D/\rho^2\lambda \qquad (D.7)$$

The solution to this differential equation is

$$T(t) = e^{-\lambda D/\rho^2 t} \qquad (D.8)$$

Exchanging the radius term with the effective beam diameter w

diameter at 86% intensity of beam at the sample)

$$T(t) = e^{-\lambda D/4w^2 t} \qquad (D.9)$$

Solving for the position portion of the Equation (D.6) leaves

$$-\lambda = \frac{1}{\sin\theta}\,\frac{\partial}{\partial\theta}\left(\sin\theta\,\frac{\partial R}{\partial\theta}\right) \qquad (D.10)$$

Using the substituion $u=\cos\theta$, and the chain rule for $\partial f/\partial\theta = (\partial u/\partial\theta)(\partial f/\partial u)$

$= -\sin\theta\ \partial f/\partial u$ leaves

$$-\lambda = \frac{\partial}{\partial u}\left[\sin^2\theta\,\frac{\partial R}{\partial u}\right] \qquad (D.11)$$

Using the trigonometry identity $\sin^2\theta=1-\cos^2\theta$ and differentiating leaves

$$-2u\,\frac{\partial R}{\partial u} + (1 - u^2)\,\frac{\partial R}{\partial u^2} + \lambda\ R = 0 \qquad (D.12)$$

which is Legendre equation (16,17). The Legendre equation has a general solution in the form

$$R(\theta) = \sum_{n=0}^{\infty} A_n\ P_n(u) \qquad (D.13)$$

The coefficients A_n depend on neither u or t but instead are only determined by the boundary condition and initial condition C(u,0). The Legendre polynomials P_n expressions can be found in many references (16,17). Combining equation (D.9) and (D.13) leave the solution of the diffusion equation

$$C(u,t) = \sum_{n=0}^{\infty} A_n \, P_n(u) \, e^{-\lambda(D/4w^2)} \qquad (D.14)$$

Applying the initial and boundary conditions gives the final solution to the diffusion equation with $V_0 = 0$ as:

$$C/C_0 = 1/2 \, (1+\cos\theta_0) + \sum_{n=1}^{\infty} \frac{(2n+1)}{2(n+1)} \, [\cos\theta_0 \, P_n(\cos\theta_0) - P_{n-1}(\cos\theta_0)] \, P_n(u) \, e^{-n(n+1)(D/4w^2)t} \qquad (D.15)$$

where $(D/4w^2)$ is the characteristic time for diffusion and $u = \cos\theta$ defines detection region.

APPENDIX E - LIPOSOME PREPARATION

Materials - Solvent - 10:2 Choloroform - Methonal

Solutes - Egg Phosphatidylcholine*** (EPC)
- Oxidized Cholesterol**
- diI* (1,1' - dioctadeyl - 3,3,3',3'
- tetramethlindocarbocyanine)

* - Fluorescent Probe - Molecular Probes, Inc.

** - Stabilizing Agent to Match BLMs - supplied by Tien's Laboratory

*** - EPC 99.9% pure Leon Labs

Methods - Combine solutes in the solvent to form a stock solution. The stock solution should contain have molar ratios identical to BLM preparation solution. For example 1 mole should contain 0.8582 moles of EPC, 0.1409 moles of oxidized cholesterol, 0.0009 moles of diI.

- Stock Solution
  + 25 ml 10:1 chloroform - methanol
  + 0.1371 grams of EPC
  + 0.0225 grams of oxidized cholesterol
  + 0.0002 grams of diI

- Procedure
  + 2-3 ml of stock solution evaporated in rotary evaporator minimum of 20 minutes at room temperature

+ Solutes were then hydrated in distilled water at 60°C in a heat bath
+ H_2O level 1-2 mm above solutes on side of rotary flask
+ Sample left in heated water bath until both cooled to room temperature
+ A cloud of liposomes and lipid should be visible in 3 to 4 hours. (Can use liposomes after this 3 to 4 hour period)
+ Liposomes can be used up to 3 to 4 days after formation

APPENDIX F - BLM PREPARATION SOLUTION

The preparation solution used follows prescribed chemical compositions presented in Bilayer Lipid Membrane (BLM): Theory and Practice by H.T. Tien (31).

Materials - Solvents - Octane and Hexane

Solutes - Egg Phosphatidylcholine*** (EPC)

- Oxidized Cholesterol*

- diI (1,1' - dioctadeyl - 3,3,3',3' - tetramethlindocarbocyanine)**.

* - Stabilizing agent for BLMs supplied by Tien's Laboratory.

** - Fluorescent probe from Molecular Probes Inc.

*** - EPC from Leon Labs 99.9% pure

Methods - Combine the solutes according to the following:

6.7% W/W of EPC

1.1% W/W of Oxidized Cholesterol

0.0067% W.W of diI

where W/W = weight of solute/weight of solvent.

Hexane: ≈ 0.682 g/ml

Octane: ≈ 0.695 g/ml

Detailed formation technique used is described in Weiss (39).

APPENDIX G DETERMINATION OF THE DIFFUSION COEFFICIENT USING A LEASE SQUARES FIT

The determination of the diffusion coefficient using a least squares fit was based on the normalized fluorescence recovery. When plotting the normalized fluorescence intensity versus time that the fluorescence intensity was measured, the curve obtained can be represented by the approximation

$$f_k^m(t) = \frac{F(t) - F(o)}{F(*) - F(o)} = 1 - Ce^{-\left(\frac{4D}{w^2}\right)t} \qquad (G.1)$$

Normalizing both the numerator and denominator which have units of volts by dividing through by 10 volts gives

$$\frac{[F(t) - F(o)]V/10.0V}{[F(*) - F(o)]V/10.0V} = 1 - Ce^{\left(-\frac{4D}{w^2}\right)t} \qquad (G.2)$$

Using $\tau_D = w^2/4D$ and $C = 1$ and isolating the exponential term gives

$$1 - \frac{[F(t) - F(o)]V/10.0V}{[F(*) - F(o)]V/10.0V} = e^{-t/\tau_D} \qquad (G.3)$$

Taking the natural logarithm of both sides and simplifying leaves

$$\ln\left[\frac{[F(*) - F(t)]/10}{[F(*) - F(o)]/10}\right] = -\frac{1}{\tau_D}t \qquad (G.4)$$

Rearranging the equation and substituting in for τ_D reduces to an equation of a line ($y = ax + b$):

$$\ln\left([F(*) - F(t)]/10\right) = \left(\frac{4D}{w^2}\right)t + \ln\left(\frac{[F(*) - F(o)]}{10}\right) \qquad (G.5)$$

Therefore determining the slope of the least squares fit line through the measured fluorescence recovery intensities versus time give the diffusion coefficient for a known effective beam diameter w.

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