

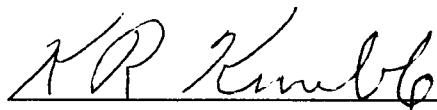
To the Graduate Council:

I am submitting herewith a thesis written by Dennis Howard Bunfield entitled "Simulation of a Single Molecule Detection Experiment." I have examined the final copy of this thesis for form and content and recommend it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Physics.



Dr. Lloyd M. Davis, Major Professor

We have read this thesis
and recommend its acceptance.





Accepted for the Council:



Associate Vice Chancellor
and Dean of The Graduate School

Simulation of a Single Molecule Detection Experiment

**A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville**

**Dennis Howard Bunfield
May, 1997**

DEDICATION

This thesis is dedicated to
my grandmother, Ruth Magee (1908–1986),
and my parents, Dennis & Linda Bunfield
and Kathy & Rick Campbell,
who have influenced and encouraged
me greatly throughout my life.

ACKNOWLEDGMENTS

I owe much thanks to many people. Most of all to my editor-in-chief, Dr. Lloyd Davis, for the direction and the many hours he invested during the writing of this thesis. None of this would have been possible without his guidance. Also, to Dr. George Shi for providing me with a research assistantship and for his patience over the last several months. Thank you George. To Dr. Chris Parigger for providing the computer facilities needed to run the simulation in reasonable periods of time. To Shane Stein for the stimulating political discussions during the many late study nights. To Tom and Christa Markusic for being loyal friends by providing me with moral support and much advice, whether desired or not. Finally, to my loving sister, Amanda, who put up with my teasing and tormenting for many years.

ABSTRACT

A Monte Carlo simulation has been developed to quantitatively understand and explore different physical aspects of a single molecule detection experiment in which individual dye molecules in an aqueous solution pass through a laser beam and emit fluorescence photon bursts. Detected photons are processed by a simple or weighted sliding sum digital filter, and bursts are distinguished from background by a thresholding procedure. The detailed timing of each photon within a burst contains information which is used to produce a spectrum of arrival times in order to estimate the fluorescence lifetime of a molecule. The simulation was applied in this work under three sets of experimental conditions (1) to compare results with those from a past experiment, (2) to determine the feasibility of an experiment for rapid single molecule detection, and (3) to identify each detected molecule according to its fluorescence lifetime. In the last case, the identification is accomplished by three independent algorithms—a weighted averaging technique, a maximum likelihood estimator of the lifetime, and a direct comparison of the fluorescence decay profile data. The simulation of (1) produced comparable results with the experiment, the simulation of (2) determined rapid detection should be possible, and the simulation of (3) correctly identified molecules with $> 85\%$ accuracy when two different types of molecules were to be detected.

Contents

1	Introduction	1
2	The Physics of the Single Molecule Detection Experiment	3
2.1	Overview	3
2.2	Summary of the Experiment	4
2.3	Molecule Transport	7
2.4	Molecule Diffusion	9
2.5	Laser Intensity Profile	10
2.5.1	The M-Factor	12
2.5.2	Elliptical Beams	13
2.6	Dependence of Molecular Excitation on Intensity	14
2.7	Molecule De-excitation Processes	17
2.7.1	Fluorescence	17
2.7.2	Internal Conversion	18
2.7.3	Triplet Crossing and Phosphoresence	18
2.7.4	Photodegradation	19
2.8	The Collection Efficiency Function (CEF)	20
2.9	Sources of Background Noise	23
2.9.1	Dark Noise	23
2.9.2	Rayleigh Scatter	24

2.9.3	Raman Scatter	25
2.9.4	Single Photon Avalanche Diode Afterpulses	28
2.9.5	Induced Fluorescence of Optical Components	28
2.9.6	Impurity Molecules in Solution	28
3	The SMD Simulation	30
3.1	Overview	30
3.2	Random Number Generators	31
3.3	Initializations and Declarations	32
3.4	Overall Flow Diagram	38
3.5	Injecting a Molecule into the System	40
3.6	Advancing Molecules Through the Sheath Flow	42
3.7	Laser-Molecule Interactions	42
3.7.1	Overview	42
3.7.2	Calculation of Laser Intensity at a Molecule's Position	44
3.7.3	Calculating the Excitation Probability for the <i>i</i> -th Molecule	45
3.7.4	Accounting for Intersystem Crossing and Photodegradation	46
3.7.5	Calculating the Probability of Fluorescence Detection	47
3.8	Background Noise, Photon Detection, and Inactive Times	49
3.8.1	Adding Background to the Total Brightness	49
3.8.2	Detection of a Photon and Dead Times	50
3.9	Data Processing Schemes	52
3.9.1	Simple Sliding Sum	52
3.9.2	Weighted Sliding Sum	53
3.9.3	Autocorrelation Function	56

4	Molecule Identification	60
4.1	Overview	60
4.2	Generation of Simulated Fluorescence Decay Data	61
4.3	Selection of the Number of Bins and Their Widths	64
4.4	An Intuitive Approach to Molecule Lifetime Estimation	66
4.5	Maximum Likelihood Estimation	68
4.6	Maximum Likelihood Estimation of the Lifetime of a Molecule	69
4.7	Direct Distinction Between Molecule Types	71
5	Results and Discussion	73
5.1	Overview	73
5.2	Simulation of a Previous Experiment (Condition I)	74
5.3	Simulation of Rapid Detection (Condition II)	76
5.4	Simulation of Molecule Identification (Condition III)	79
6	Summary	88
	Bibliography	89
	Appendix	92
	A Derivation of Fick's Second Law	93
	Vita	95

List of Figures

2.1	Experimental setup.	4
2.2	Photon burst and background photons.	5
2.3	Detection apparatus.	5
2.4	Sheath flow velocity distribution.	8
2.5	Laser intensity profile for a beam propagating in the x-direction with a beam waist ω_0	11
2.6	Fitted Gaussian profile of a real laser beam.	12
2.7	Energy level diagram for a two-level atom.	16
2.8	Simplified energy level diagram for a dye molecule.	16
2.9	Potential well representation of the energy levels for a dye molecule. .	19
2.10	Out-of-focus image of molecule projected on to the spatial filter. . . .	21
2.11	Overlap of two circles representing the collection efficiency function. .	22
2.12	CEF calculated regions.	24
2.13	Time spectrum of Raman scatter (adapted from Reference [2]).	26
3.1	Flow diagram for the SMD simulation.	38
3.2	Flow diagram to generate a molecule.	40
3.3	Flow diagram for the molecular interactions with the laser.	43
3.4	Flow diagram to illustrate the model of the photon detection process.	51
3.5	The interval over which the sliding sum is calculated.	53

3.6	Gaussian weights superimposed upon the signal.	55
3.7	The weighted sliding sum with the phase shift $s(t + \tau)$	57
3.8	The autocorrelation function.	58
3.9	An anomalous experimental result of the autocorrelation function (taken from Reference [9]).	58
4.1	Exponential decay profile for histogram produced for binned data. . .	62
4.2	Representation of bin widths which produce the most distinct distributions for each molecule type.	66
5.1	Experimental photon burst signals from sulforhodamine 101 under Condition I (taken from Reference [9]).	75
5.2	Simulated photon burst signals from sulforhodamine 101 under Condition I.	76
5.3	Simulated photon burst signals under Condition II.	77
5.4	Efficiency analysis for the parameters of Condition II.	78
5.5	Autocorrelation function generated by Condition II.	79
5.6	Simulated photon burst signals under Condition III.	80
5.7	Peak height frequency under Condition III.	81
5.8	Lifetime estimations by the weighted averaging method using equal bin widths.	84
5.9	Lifetime estimations by the weighted averaging method using optimal bin widths.	84
5.10	Lifetime estimations by the maximum likelihood estimator using equal bin widths.	85
5.11	Lifetime estimations by the maximum likelihood estimator using optimal bin widths.	85

5.12 Multinomial distribution predictions using equal bin widths. A confidence level < 0.5 indicates a prediction of molecule type 1.	87
5.13 Multinomial distribution predictions using optimal bin widths.	87
A.1 Concentration change due to diffusion from a plane source.	93

List of Tables

3.1	Random number generators used by simulation	31
3.2	Laser parameters.	32
3.3	Parameters for light collection efficiency and experimental geometry. .	32
3.4	Dead time parameters.	33
3.5	Background noise parameters.	33
3.6	Molecular parameters for sulforhodamine 101 (molecule 0) and rho- damine 640 (molecule 1).	33
3.7	Other molecule and flow parameters.	34
3.8	Structure containing information for molecules in simulation.	35
3.9	Structure containing information on photons detected on the current and previous time steps.	35
3.10	Other temporary storage space used by the simulation.	35
3.11	Variables that are evaluated by the initialization routine.	37
5.1	Experimental conditions for the simulation.	73
5.2	Statistics for comparison of analysis methods of binned fluorescence decay profiles for molecule identification.	82

Chapter 1

Introduction

Single Molecule Detection (SMD) in aqueous solution was first demonstrated in 1990 [1]. At The University of Tennessee Space Institute, Li performed an experiment in which single molecules of sulforhodamine 101 dye were injected into a flow stream and efficiently detected, as reported in his Ph.D. dissertation [2]. The purpose of this thesis is first, to explain the development of a Monte Carlo simulation of that experiment, and second, to extend the simulation to consider the possibility for identification of different types of molecules by the spectral information obtained as they are individually detected. The development of the simulation has utilized an earlier simulation that has been used to visualize how changing an experimental parameter would affect the experiment and to easily enable new data analysis algorithms to be tested and incorporated into the experiment's data analysis program [3]. However, substantial improvements and modifications have been made to more accurately account for the physical processes that occur in the experiment.

These major improvements include properly accounting for (1) inactive times (or dead times) of the photon detector and of the electronics, which occurs after each photon detection event, (2) times when the molecule will be inactive, which occur after excitation and crossing to a metastable state (intersystem crossing), (3) the mechanism by which the photons released by an excited molecule are spatially fil-

tered and collected, (4) the dependence of background noise on the experimental geometry, (5) the random molecular orientation with respect to the laser excitation polarization, and (6) the imperfect laser beam mode quality. Also the simulation was adapted to operate under the X Window System on a UNIX platform; such adaptations required re-writing the graphics. In addition to these improvements, the simulation has been extended to generate data corresponding to the nanosecond time-scale delay between excitation and emission of each fluorescence photon and to analyze this data to identify different types of molecules.

The physical processes involved in the SMD experiment are explained in Chapter 2. Chapter 3 elaborates how these processes are implemented in the code. Details of data analysis methods, such as the simple sliding sum and weighted sliding sum (WSS) are deferred to the end of this chapter. Chapter 4 describes the extension of the simulation to address the identification of the molecules according to their fluorescence lifetimes. It is discussed how each detected photon is allocated to a number of discrete bins according to its random nanosecond emission time. Three different algorithms based upon likelihood estimation have been developed for molecule identification using the nanosecond time information. Chapter 5 presents the results of the simulation, comparisons between experimental results, and evaluation and comparison of the three molecule identification schemes. Finally, Chapter 6 is a summary of the thesis and offers suggestions for future work.

Chapter 2

The Physics of the Single Molecule Detection Experiment

2.1 Overview

The overall process of single molecule detection (SMD) in an ultra-dilute solution consisting of water and dye molecules is explained in Section 2.2. Several stages are involved in the detection process. A detailed understanding of the physical process involved in each stage is necessary for modeling the overall experiment. Sections 2.3 to 2.9 derive or explain the equations used to model each stage, in the order in which each stage occurs in the experiment.

First, the molecule emerges from the injection capillary and is transported through the laser beam by the bulk flow of the sheath, as discussed in Section 2.3, and by diffusion, as discussed in Section 2.4. Second, as the molecule passes through the laser, it experiences a laser intensity dependent on its position, as discussed in Section 2.5, causing it to become excited with a certain probability, as discussed in Section 2.6. The molecule excitation may lead to spontaneous emission, radiationless relaxation, or photodegradation, as discussed in Section 2.7. If a photon is emitted by the molecule, the probability that it will be collected depends on the position of the molecule with respect to the spatial filter, as discussed in Section 2.8. Background noise contributes

to the number of photons detected by the single photon avalanche diode (SPAD). The sources of background are described in Section 2.9.

2.2 Summary of the Experiment

In a Single Molecule Detection experiment, individual dye molecules are excited in an ultra-dilute solution sample and subsequently detected with high efficiency. Figure 2.1 shows the setup in which molecules pass one-by-one from an injection capillary tip into a sheath flow, which carries them through a focused laser beam. The molecule may

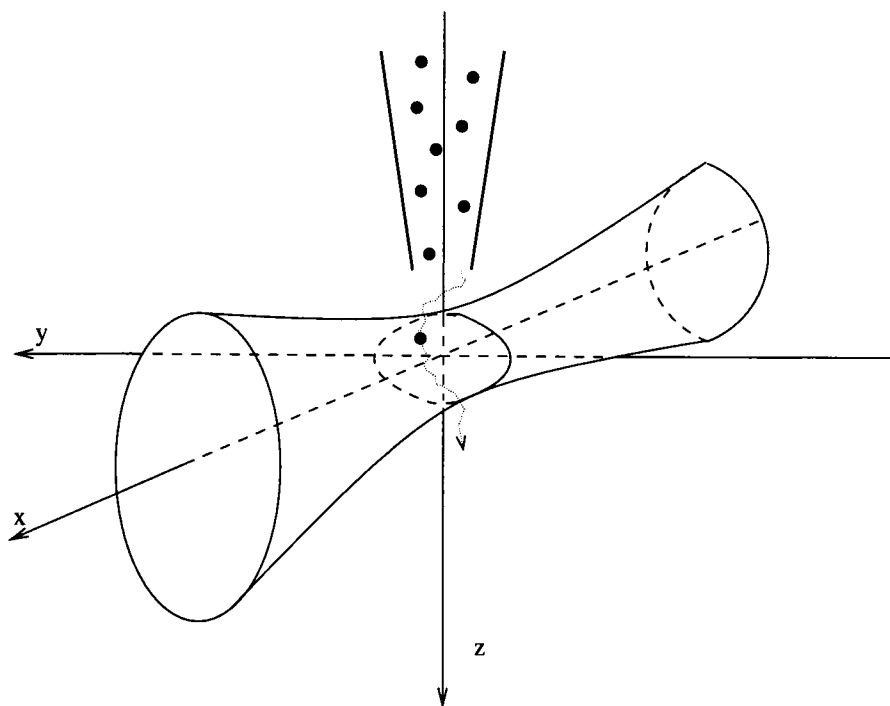


Figure 2.1: Experimental setup.

be repeatedly excited, thereby releasing a burst of photons, as shown in Figure 2.2. Many of the photons pass through the detection apparatus, which is perpendicular to both the propagation of the laser beam and the direction of the molecule solution flow. Figure 2.3 shows the components of the detection apparatus.

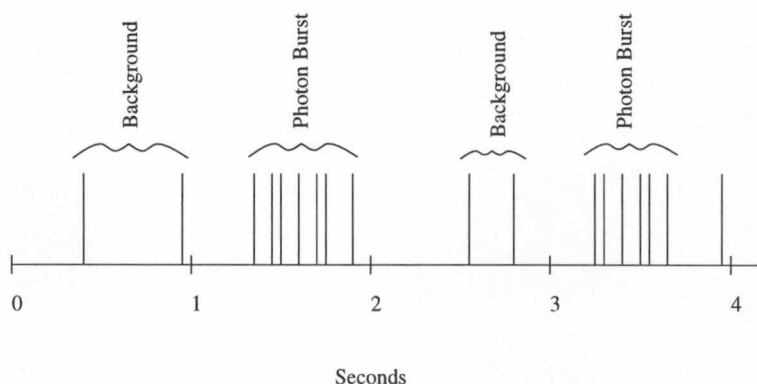


Figure 2.2: Photon burst and background photons.

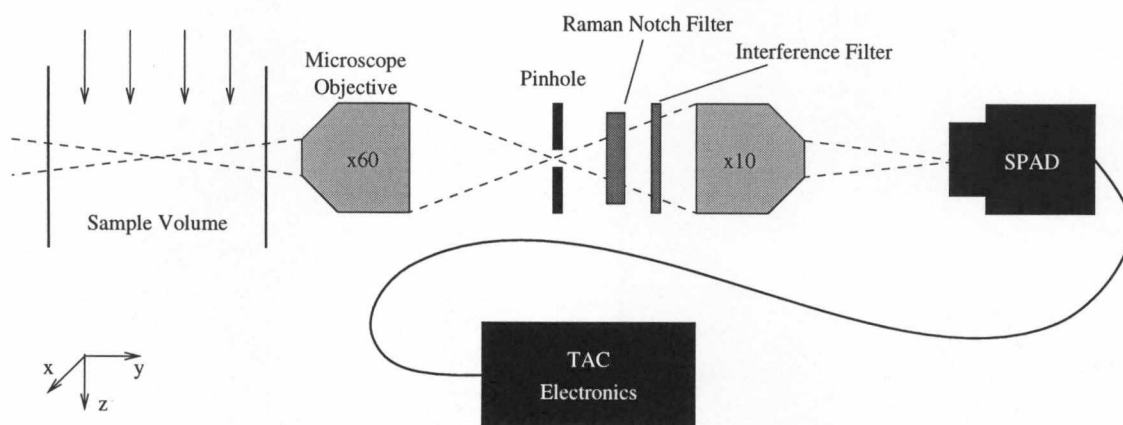


Figure 2.3: Detection apparatus.

The first component is a microscope objective, which collects light from the sample. Following the microscope objective, are a pinhole, spectral filters, another microscope objective, and the SPAD detector. The overall collection efficiency is determined by the solid angle over which the first objective collects light, and the net transmission of this objective and the subsequent optics at the fluorescence wavelengths.

The purpose of the pinhole is to filter out any stray photons which happen to be collected by the first objective but which do not originate from the sample volume. Thus the pinhole serves as a spatial filter. Its size and the magnification of the

collection objective determine the region from which light is efficiently gathered.

After the spatial filter there are a series of high quality, low fluorescence spectral filters. Without these, the noise from other scattered light, such as Rayleigh scatter from the solvent, would overwhelm the signal from the molecule. Because Rayleigh scatter is effectively filtered out in the experiment it is not included by the simulation. Unfortunately, the filter will not block other types of background noise. There will be some photons from Raman scatter from the solvent. To discriminate against noise from Raman scatter, a method known as time gating is used.

Time gating is accomplished by the use of a rapidly-pulsed laser and by electronically blocking instantaneously scattered light whereas fluorescence photons from a dye molecule with an exponential decay profile may be counted. A typical time-resolved data record consists of a large peak at the origin due to scattered light, followed by an exponential decay curve, with a lifetime equal to the dye molecule's fluorescence lifetime. The instantaneous nature of the scatter of the laser pulse gives rise to a Raman peak at the origin, named the experimental prompt. Time gating is achieved by electronically blocking photon signals that occur within that peak. If the laser were continuous, the scatter would occur at all times, and therefore it would not be distinguishable from the fluorescence. The theory of Raman scatter and time gating are further explained in Section 2.9.3.

The last component in the detection apparatus is the single photon detector or SPAD. The SPAD has a high quantum efficiency ($> 50\%$) and sub-nanosecond timing performance. Following the SPAD is the time gate which consists of a custom subnanosecond electronic anticoincidence circuit [2], which passes only those photon signals that are delayed with respect to the experimental prompt. From here, the photon signals may pass either directly to a multichannel scalar for detection of the photon bursts, or through a time to amplitude converter (TAC) for measurement of

the nanosecond arrival time of each photon.

Following each photon detection event, there is an associated time during which the SPAD detector will not be able to detect photons. This is called the SPAD dead time. Also after each real signal, there is a small chance that the SPAD will produce an artificial signal called an afterpulse. These are unavoidable and must be taken into account by the simulation. Finally, if a time to amplitude converter (TAC) is used in the detection electronics, whenever the time gating circuit yields a pulse, the TAC will experience a certain dead time. During this time, any fluorescence photons detected will not be registered by the acquisition hardware.

2.3 Molecule Transport

The molecule is introduced into the system through the capillary tip shown in Figure 2.1. The molecule is then carried downstream by the surrounding sheath flow. This flow is laminar because the Reynolds number, R_e , is much less than the critical value, which is approximately 2300. The Reynolds number is determined by

$$R_e = \frac{\bar{v}_{max}\rho d}{\eta}, \quad (2.1)$$

where $\bar{v}_{max}$ is the maximum mean flow velocity, ρ is the fluid density, d is the tube diameter, and η is the dynamic viscosity. Currently the tube used is square with inside dimensions of 0.8 mm $\times$ 0.8 mm, but to calculate an upper limit for R_e , an approximation of a cylindrical tube with $d \approx 0.8$ mm is made. For water at 20°C, $\eta = 0.01$ g/cm/sec, $\rho = 1.0$ g/cm³, and $\bar{v}_{max} = 2$ cm/s yield a Reynolds number of 16.

The velocity profile of the flow is qualitatively as shown in Figure 2.4. An exact solution may be derived by solving the Navier-Stokes equation for steady flow

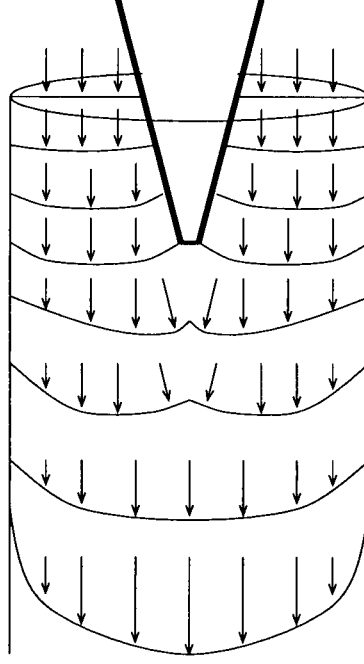


Figure 2.4: Sheath flow velocity distribution.

$$(\partial \vec{v} / \partial t = 0),$$

$$(\vec{v} \vec{\nabla}) \vec{v} = \frac{1}{\rho} \vec{\nabla} p^* + \frac{\eta}{\rho} \vec{\nabla}^2 \vec{v}, \quad (2.2)$$

where p^* is the excess mean pressure, and the equation of continuity for an effectively incompressible fluid is

$$\frac{\partial v_x}{\partial x} + \frac{\partial v_y}{\partial y} + \frac{\partial v_z}{\partial z} = 0. \quad (2.3)$$

For a hydrostatically driven flow, the velocity is zero at the boundaries. Given the boundary conditions for a simple system, such as a cylindrical tube without the capillary tip, the flow velocity at a radial distance, $\mathbf{r}$, from the $\mathbf{z}$ -axis has the well-known parabolic Poiseuille profile given by,

$$v(\mathbf{r}) = 2\bar{v} \left(1 - \frac{r^2}{R^2} \right), \quad (2.4)$$

where R is the radius of the tube and $\bar{v}$ is the mean flow velocity. Here,

$$\bar{v} = \frac{Q}{F}, \quad (2.5)$$

with Q being the volumetric flow rate and F being the tube's cross sectional area.

The boundary conditions for the system used in the experiment, as Figure 2.4 depicts, are such that an analytic solution is not possible. Existing solutions for the Navier-Stokes equations of simpler but similar setups, such as two concentric cylindrical tubes, can yield a qualitative understanding of the flow characteristics in the experiment. Due to the boundary conditions, the velocity of the sheath flow at the walls of the sample injection capillary is zero, and some distance downstream from the capillary tip, the sheath flow attains a steady velocity profile similar to the Poiseuille flow within a cylindrical tube, as shown in Figure 2.4. Because the diameter of the injection capillary tip is less than 1 micron, existing solutions of similar setups indicate that the sheath flow will reach its steady state velocity a very short distance downstream from the tip. Thus in the simulation, the molecule is assumed to be transported by the sheath flow at a uniform velocity. However, in future work, it would be desirable to numerically calculate the actual flow profile, to check this approximation. Also, the actual flow profile could be used by the simulation to better account for the sheath flow transport of the molecule.

2.4 Molecule Diffusion

In addition to being carried by the sheath flow, the molecule undergoes Brownian diffusion as it passes from the injection capillary and through the laser beam.

The diffusion of the molecules in the experiment is evaluated using Fick's second law of diffusion,

$$\frac{\partial c}{\partial t} = D \nabla^2 c, \quad (2.6)$$

where c is the concentration of the molecules in the solvent and D is the diffusion coefficient of the molecules. Equation 2.6 is derived in Appendix A.

Given the initial condition,

$$c(t = 0) = c_0 \delta(\mathbf{x}, \mathbf{y}, \mathbf{z}), \quad (2.7)$$

which represents a concentration in which the molecule(s) may only be found at the origin, as time evolves, the concentration will be the solution of Equation 2.6, which is

$$c(\mathbf{x}, \mathbf{y}, \mathbf{z}, t) = \frac{c_0}{(12Dt\pi)^{3/2}} \exp \left[-\frac{\mathbf{x}^2 + \mathbf{y}^2 + \mathbf{z}^2}{12Dt} \right]. \quad (2.8)$$

In the experiment the concentration is such that only one molecule is present. As a result, Equation 2.8 represents the probability density for finding the molecule at the location $\mathbf{x}, \mathbf{y}, \mathbf{z}$ at time t . The molecule's position is therefore determined by calculating a three-dimensional Gaussian deviate about the initial position.

2.5 Laser Intensity Profile

Figure 2.5 is a graphical representation of the intensity profile. For an ideal diffraction-limited focused Gaussian laser beam propagating in the $\mathbf{x}$ direction, the intensity profile is given by

$$I(\mathbf{x}, \mathbf{y}, \mathbf{z}) = \frac{2P}{\pi\omega^2(\mathbf{x})} \exp \left[\frac{-2(\mathbf{y}^2 + \mathbf{z}^2)}{\omega^2(\mathbf{x})} \right], \quad (2.9)$$

where P is the power of the laser. The square of the spot size at a position $\mathbf{x}$ is

$$\omega^2(\mathbf{x}) = \omega_0^2 \left[1 + \mathbf{x}^2/\mathbf{x}_0^2 \right], \quad (2.10)$$

with ω_0 being the beam waist and $\mathbf{x}_0$ being the Rayleigh range, which is defined by

$$\mathbf{x}_0 = \frac{\pi\omega_0^2}{\lambda}. \quad (2.11)$$

Here,

$$\lambda = \frac{\lambda_0}{n}, \quad (2.12)$$

where λ_0 is the wavelength of the laser in the air, and n is the refractive index of water. When $x = x_0$, Equation 2.10 becomes,

$$\omega(x_0) = \omega_0 \sqrt{2}. \quad (2.13)$$

Thus the Rayleigh range represents the region for which the beam size has twice the area of the focus waist, before it asymptotically diverges. For $x \gg x_0$ the beam will diverge at the angle of

$$\theta = \frac{\omega_0}{x_0} = \frac{\lambda}{\pi \omega_0}. \quad (2.14)$$

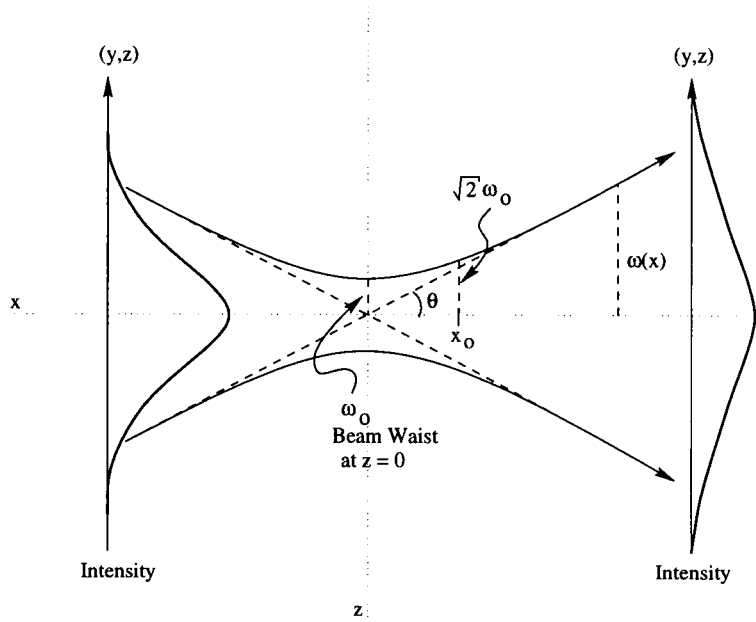


Figure 2.5: Laser intensity profile for a beam propagating in the x -direction with a beam waist ω_0 .

2.5.1 The M-Factor

Real laser beams do not have only a first order transverse electromagnetic (TEM) mode, TEM₀₀, representing a Gaussian intensity profiles but have small contributions from higher order TEM modes. Such contributions may exist in the beam as it emerges from the laser or they may be introduced by aberrations in the focusing optics. For example, the real focused beam may be comprised of

$$85\% \text{ TEM}_{00} + 10\% \text{ TEM}_{10} + 4\% \text{ TEM}_{20} + \dots \quad (2.15)$$

If the contribution from higher modes is small, a Gaussian curve may be fit to the real profile, as shown in Figure 2.6. The width of the fitted Gaussian, $\bar{\omega}_0$, is larger

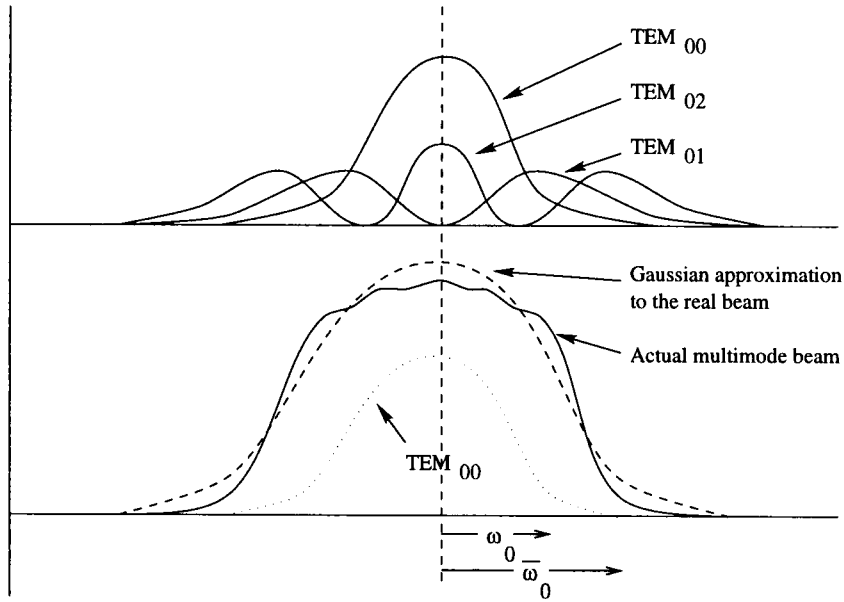


Figure 2.6: Fitted Gaussian profile of a real laser beam.

than that of an ideal TEM₀₀ mode, ω_0 . This difference is typically expressed by the use of the so-called M-Factor,

$$M = \frac{\bar{\omega}_0}{\omega_0}. \quad (2.16)$$

Because the area of the focused spot will be

$$A = M^2 \pi \omega_0^2, \quad (2.17)$$

it is the value of M^2 rather than M that is usually quoted when specifying the quality of a Gaussian beam.

Equations 2.9 through 2.14 may now be modified to more accurately represent the real laser beam by the use of the M^2 parameter. The beam size at any point along the x direction becomes

$$\bar{\omega}^2(x) = \bar{\omega}_0^2 \left[1 + x^2/x_0^2 \right], \quad (2.18)$$

whereas the Rayleigh range remains unchanged from Equation 2.11,

$$\bar{x}_0 = \frac{\pi \bar{\omega}_0^2}{M^2 \lambda} = x_0. \quad (2.19)$$

The beam divergence is now

$$\bar{\theta} = \frac{\bar{\omega}_0}{x_0} = \frac{\lambda}{\pi \bar{\omega}_0}. \quad (2.20)$$

Thus the intensity profile, Equation 2.9, becomes

$$\bar{I}(x, y, z) = \frac{2P}{\pi \bar{\omega}^2(x)} \exp \left[-\frac{2(y^2 + z^2)}{\bar{\omega}^2(x)} \right]. \quad (2.21)$$

Because the M-factor is an empirical factor, it may be used to account for any number of other effects, at least in an approximate manner. For example, diffraction from the aperture of the lens could be accounted for by increasing M.

2.5.2 Elliptical Beams

Cylindrical lenses may be used to transform a beam with a round cross-sectional profile into an elliptical beam with different waists in the y and z dimensions. In this case, the intensity profile is

$$I(x, y, z) = \frac{2P}{\pi \bar{\omega}_y(x) \bar{\omega}_z(x)} \exp \left[-\frac{2y^2}{\bar{\omega}_y^2(x)} \right] \exp \left[-\frac{2z^2}{\bar{\omega}_z^2(x)} \right], \quad (2.22)$$

where

$$\bar{\omega}_i^2(\mathbf{x}) = \bar{\omega}_{i0}^2 \left[1 + \left(\frac{\mathbf{x} - \mathbf{x}_i}{x_{i0}} \right)^2 \right], \quad (2.23)$$

$$\bar{x}_{i0} = \frac{\pi \bar{\omega}_{i0}^2}{M^2 \lambda} = x_{i0}, \quad (2.24)$$

and

$$\bar{\omega}_{i0} = M_i \omega_{i0}, \quad (2.25)$$

in which i refers to y or z , and $\mathbf{x}_i$ refers to the location of the y or z waist along the $\mathbf{x}$ -axis.

2.6 Dependence of Molecular Excitation on Intensity

As the molecule passes through the focused laser beam the probability that it will become excited depends upon the intensity, I , at its present location, $\mathbf{x}, \mathbf{y}, \mathbf{z}$.

If the intensity is relatively weak, no saturation occurs, and the probability of excitation per laser pulse is linearly dependent upon the photon flux, I/E_γ , where E_γ is the energy of each photon. In the case of a pulsed laser with R laser pulses per second, the photon flux per laser pulse, F , at a given location is,

$$F(\mathbf{x}, \mathbf{y}, \mathbf{z}) = \frac{I(\mathbf{x}, \mathbf{y}, \mathbf{z})}{R E_\gamma}. \quad (2.26)$$

For a wavelength of $\lambda_0 = 585$ nm, $E_\gamma = 3.4 \times 10^{-19}$ J and for the simulations conducted in this thesis, R is taken as $7.6 \times 10^7 \text{s}^{-1}$. The excitation probability is also linearly dependent upon the absorption cross section, σ_a . For sulforhodamine 101 dye molecules, $\sigma_a \sim 2.3 \times 10^{-16} \text{cm}^2$.

If the laser were linearly polarized and the molecule transition dipole moment were aligned at θ, ϕ with respect to the polarization direction, the probability for

excitation per laser pulse would be

$$P_E = \sigma_a F \cos^2 \theta \sin^2 \phi. \quad (2.27)$$

Because the molecule tumbles due to rotational diffusion with a time constant $\tau_R \sim 100$ ps, which is much less than the time between laser excitation pulses, for each excitation event it is randomly oriented in space. By averaging over all orientations using

$$\frac{1}{2\pi} \int_{-\pi}^{\pi} \int_0^{\pi} \sin^2 \phi \cos^2 \theta \sin \theta d\theta d\phi = \frac{1}{3}, \quad (2.28)$$

the mean probability of excitation per laser pulse is obtained as

$$\bar{P}_E = \frac{1}{3} \sigma_a F = \frac{\sigma_a I}{3RE_\gamma}. \quad (2.29)$$

Note however, that if $I > 3RE_\gamma/\sigma_a$, $\bar{P}_E > 1$, whereas according to probability theory, $\bar{P}_E \leq 1$. Therefore, this is not a complete analysis of the problem, and for high intensities, molecule saturation must be considered.

In the case of saturation for a two-level atom, if the laser intensity is high, the molecule may be excited and then may be returned to its ground state by stimulated emission, as illustrated in Figure 2.7. In this case, if the transition dipole moment is aligned with the excitation polarization direction, as $I \gg 3RE_\gamma/\sigma_a$, $P_E \rightarrow \frac{1}{2}$. However, dye molecules in solution are not well represented by a two-level energy system—they have a band structure, as illustrated in Figure 2.8. This band structure must be taken into account when considering saturation effects.

Soon after the dye molecule becomes excited it will rapidly relax within the upper vibrational manifold due to collisions with surrounding solvent molecules. For dye molecules in aqueous solution, this relaxation typically occurs on a time scale of ~ 0.5 – 1.0 ps. Provided that the laser pulses are longer than this relaxation time, stimulated emission is negligible, and therefore, as $I \gg 3RE_\gamma/\sigma_a$, $P_E \rightarrow 1$ in an

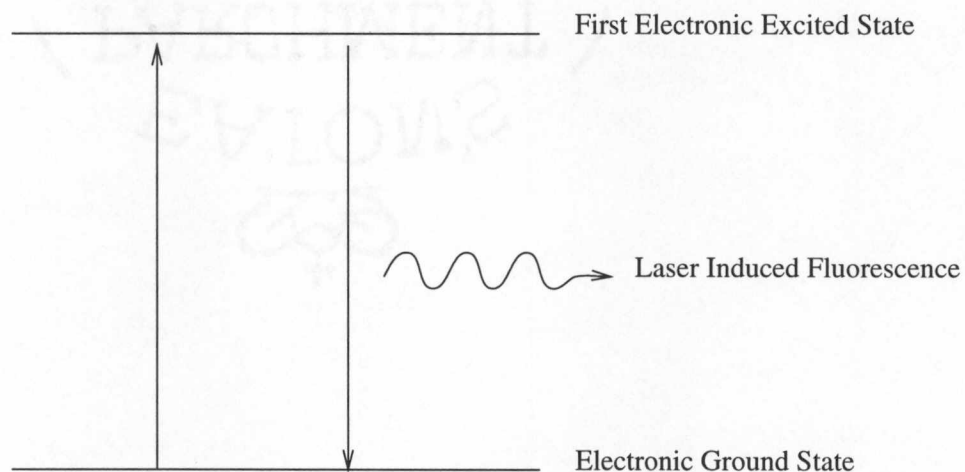


Figure 2.7: Energy level diagram for a two-level atom.

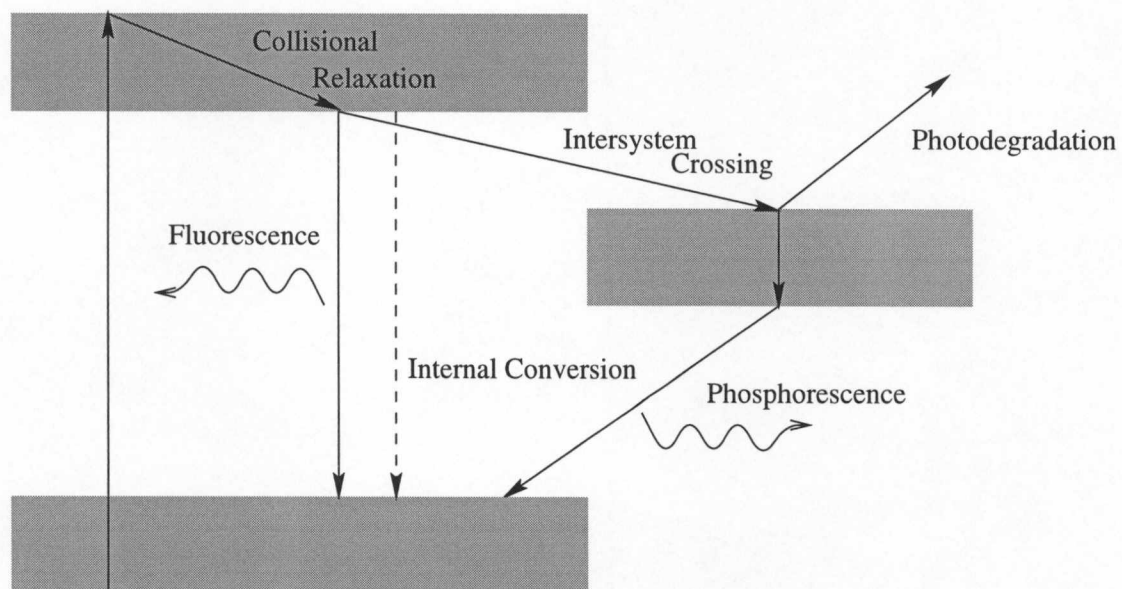


Figure 2.8: Simplified energy level diagram for a dye molecule.

exponential manner. This is approximately the situation when using pulses from the synchronously-pumped dye laser, which gives pulses $\sim 5\text{--}10$ ps. Thus vibrational relaxation within the electronically-excited band causes the probability of excitation per laser pulse to be

$$P_E = 1 - \exp \left[-\frac{\sigma_a I}{R E_\gamma} \cos^2 \theta \sin^2 \phi \right], \quad (2.30)$$

where the intensity, I , is calculated from Equation 2.22.

2.7 Molecule De-excitation Processes

2.7.1 Fluorescence

Once a molecule is excited, it will become vibrationally deactivated within the first electronically excited state, as discussed above. From here, the molecule may return to the electronic ground state via several different pathways. There is a large probability that the molecule will return to the electronic ground state by an electric dipole radiative transition, that is, the release of a fluorescence photon.

The probability for a photon to be emitted per laser excitation pulse is

$$P_F = P_E \Phi_F, \quad (2.31)$$

where Φ_F is the fluorescence quantum efficiency. Φ_F is given by

$$\Phi_F = \frac{k_F}{k_F + k_{IC} + k_{ISC}}, \quad (2.32)$$

where k_F is the rate for fluorescence emission, k_{IC} is the rate for radiationless de-excitation by internal conversion, and k_{ISC} is the rate for intersystem crossing to the triplet manifold. If a molecule is excited and a fluorescence photon is emitted, the time between excitation and emission is random with an exponential distribution with a fluorescence lifetime given by

$$\tau_F = \frac{1}{k_F + k_{IC} + k_{ISC}} \approx 1 - 10 \text{ ns}. \quad (2.33)$$

The fluorescence emission will be isotropic in space because τ_F is much longer than the rotation time constant τ_R .

2.7.2 Internal Conversion

The molecule may become deactivated by transferring its energy into the form of vibrational energy. The energy goes into the internal degrees of motion of the molecule. This is called internal conversion and it causes the molecule to return to the electronic ground state without the release of a photon. Once internal conversion has occurred, the internal motion of the molecule is damped due to collisions with solvent molecules on a time scale of ~ 0.1 – 1 ps.

In Figure 2.9, the energy levels are plotted versus the mean inter-atomic spacing, and the form of the vibrational potential energy wells of the molecule is shown for the ground and first electronically excited states. After the molecule is excited to the first electronic state it will vibrationally deactivate to a lower level within this manifold. Then, if internal conversion occurs, the molecule crosses from this point to a vibrationally excited level in the ground electronic state that is close in energy. Because internal conversion competes with the radiative emission of a photon, it acts to quench fluorescence.

2.7.3 Triplet Crossing and Phosphorescence

Another mechanism that competes with fluorescence is intersystem crossing into the triplet manifold. Crossing into the triplet manifold occurs if the electron of an electronically excited orbital has its spin flipped due to some interaction, such as a collision with another molecule in which angular momentum is transferred. The probability for this to happen is quite small, on the order of 2×10^{-3} [4]. During the time that the molecule is in the triplet state, it is unavailable for subsequent excitation and

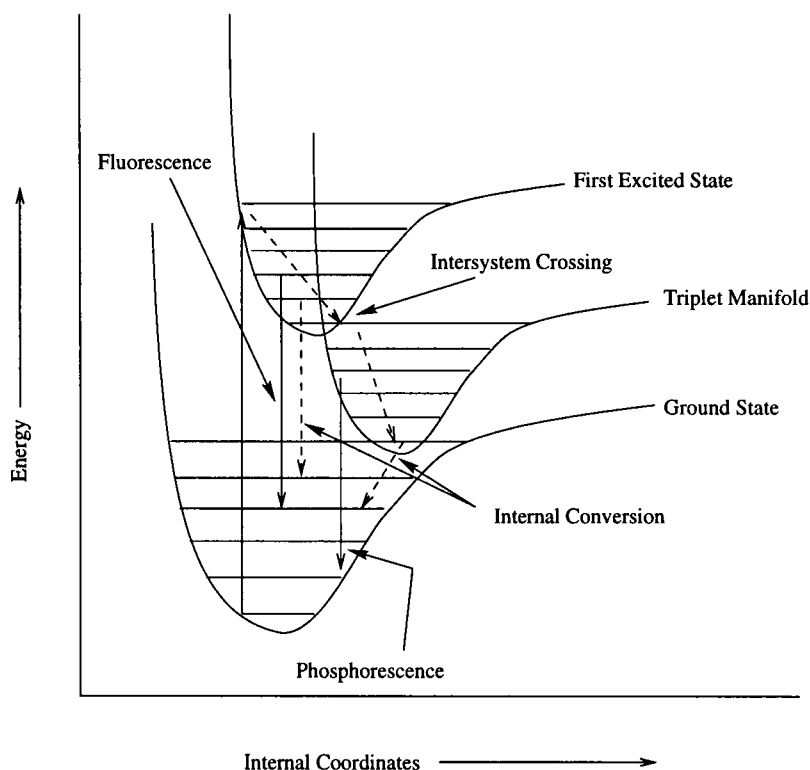


Figure 2.9: Potential well representation of the energy levels for a dye molecule.

fluorescence emission events. Thus the burst of fluorescence from the molecule will be interrupted. From the triplet state, the radiative transition back to the ground state, which is called phosphorescence, is dipole-forbidden because the electron spin must flip again. Hence the return back to the ground state usually occurs via a quenching mechanism, such as vibrational deactivation and collisional transfer of energy to solvent molecules. The time between excitation with triplet crossing and subsequent return to the ground state is random with an exponential distribution with a typical lifetime of $\sim 4 \times 10^{-6}$ s [4].

2.7.4 Photodegradation

While the molecule is in the triplet state, it may be photodegraded (i.e., photo-bleached). The exact mechanisms for photodegradation are not well known but are

generally thought to involve a collision with another agent, such as dissolved oxygen, which causes a chemical reaction in which the bonds of the molecule are broken resulting in a change in physical form.

On average, photodegradation occurs after the molecule undergoes about 10^2 to 10^3 electronic state transitions. After photodegradation the molecule may no longer be excited by the laser. As a result it will no longer be able to produce fluorescence photons.

2.8 The Collection Efficiency Function (CEF)

When a molecule releases a fluorescence photon, because the emission is isotropic in space, the maximum efficiency with which each photon is collected is determined by the fraction of solid angle subtended by the numerical aperture (N.A.) of the microscope collection objective. This has a value of

$$\text{CEF}_{\text{max}} = (1 - \cos \alpha)/2 \quad (2.34)$$

where

$$\alpha = \sin^{-1}(\text{N.A.}/n), \quad (2.35)$$

and n is the refractive index of the solution. The maximum collection efficiency is obtained if the molecule is at the origin, i.e., at the center of the object space virtual image of the pinhole used as the spatial filter. If the molecule is located elsewhere when it emits the photon, the collection efficiency may be reduced. The efficiency with which the optics collects the fluorescence relative to the maximum collection efficiency is called the collection efficiency function (CEF)[5]. Geometrically, the CEF is evaluated by calculating the intersection of the spatial filter and the disk that is the out-of-focus image of the emission point, as Figure 2.10 shows. Thus, the

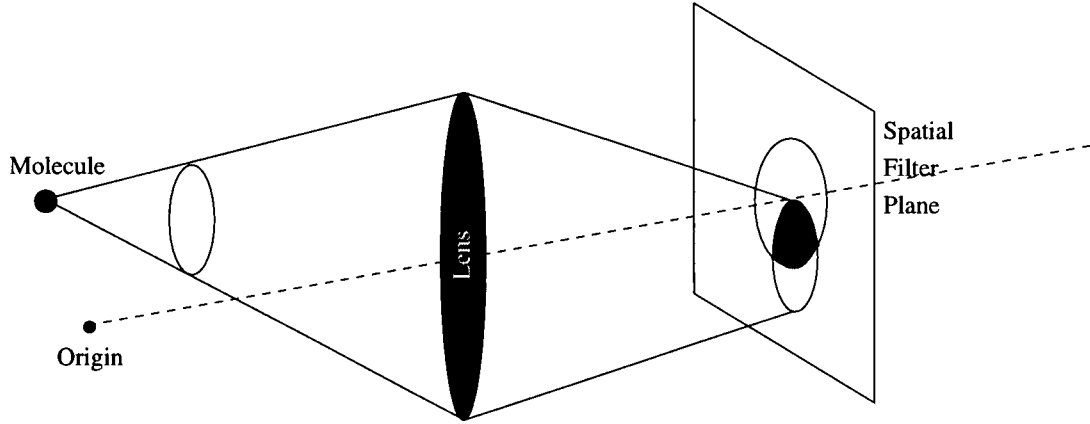


Figure 2.10: Out-of-focus image of molecule projected on to the spatial filter.

geometrical CEF is obtained in terms of a two-dimensional convolution integral of two circle functions,

$$\text{CEF}(\mathbf{R}, \mathbf{Z}) = \begin{cases} \frac{1}{\pi Z^2} \int \text{circ}(\mathbf{R}_1) \text{circ}\left(\frac{\mathbf{R}_1 - \mathbf{R}}{Z}\right) d\mathbf{R}_1, & 1 - R < Z < 1 + R, \\ \frac{1 - Z/\sqrt{\tan^2 \alpha + Z^2}}{\pi(1 - \cos \alpha)} \int \text{circ}(\mathbf{R}_1) \text{circ}\left(\frac{\mathbf{R}_1 - \mathbf{R}}{Z}\right) d\mathbf{R}_1, & 1 + R < Z < R - 1, \end{cases} \quad (2.36)$$

where

$$\text{circ}(\mathbf{r}) = \begin{cases} 1, & \mathbf{r} < 1 \\ 0, & \mathbf{r} \geq 1 \end{cases} \quad (2.37)$$

and $\mathbf{Z}$ is the normalized coordinate in the y-direction, defined by

$$\mathbf{Z} = \frac{\mathbf{y} \tan \alpha}{s_0}, \quad (2.38)$$

and $\mathbf{R}$ is the normalized radial coordinate of the spatial filter, defined by

$$\mathbf{R} = \frac{\sqrt{\mathbf{x}^2 + \mathbf{z}^2}}{s_0}, \quad (2.39)$$

where s_0 is the radius of the virtual image of the spatial filter as determined by the pinhole radius divided by the magnification of the lens.

The convolution of two circle functions may be analytically calculated using basic geometry. Consider one circle of radius $a = 1$ and another circle of radius $b = Z$. The distances between the centers of the two circles is $c = R$. This is illustrated in Figure 2.11. From this figure, circle A represents the pinhole and circle B represents

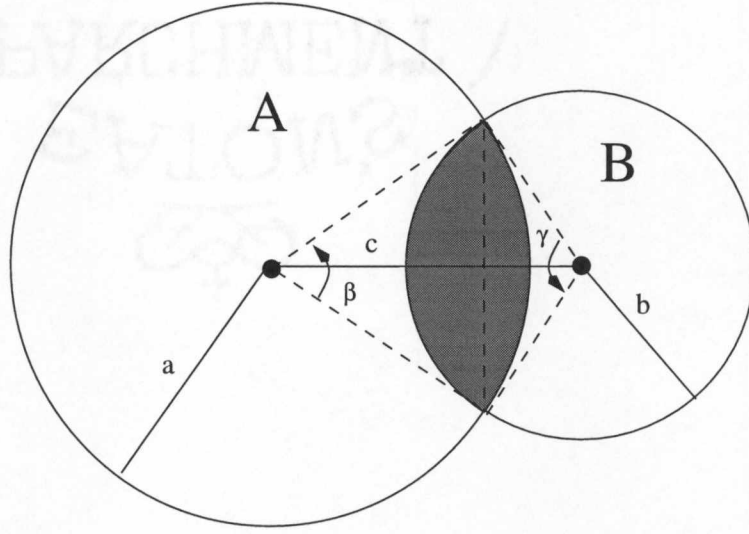


Figure 2.11: Overlap of two circles representing the collection efficiency function.

the disk that is the out-of-focus image, which is the intersection of the solid angle of light with the spatial filter plane. The area of overlap between the two circles for any a , b , and c is

$$A(a, b, c) = s_1 + s_2 - \Delta_1 - \Delta_2, \quad (2.40)$$

where the area of the sectors s_1 and s_2 are given by $s_1 = \beta a^2$ and $s_2 = \gamma b^2$, the areas of the triangles Δ_1 and Δ_2 are given by $\Delta_1 = a \sin \beta \cos \beta$ and $\Delta_2 = b \sin \gamma \cos \gamma$, and the sector angles β and γ are $\beta = \cos^{-1}[(a^2 + c^2 - b^2)/2ac]$ and $\gamma = \cos^{-1}[(b^2 + c^2 - a^2)/2bc]$. For two special cases when $c < a + b$ and $c > a + b$, the area of overlap will be 1 and

0 respectively. This leads to a normalized analytic form of Equation 2.36,

$$\text{CEF}(\mathbf{R}, \mathbf{Z}) = \begin{cases} 1, & \mathbf{Z} < 1 - \mathbf{R} \\ \mathbf{A}(1, \mathbf{Z}, \mathbf{R}) \times \frac{1}{\pi \mathbf{Z}^2}, & 1 - \mathbf{R} < \mathbf{Z} < 1 + \mathbf{R} \\ \left[1 - \frac{\mathbf{Z}}{\sqrt{\tan^2 \alpha + \mathbf{Z}^2}}\right] / (2\pi \text{CEF}_{\max}), & 1 + \mathbf{R} < \mathbf{Z} < \mathbf{R} - 1 \\ 0, & \mathbf{R} - 1 < \mathbf{Z} \end{cases}, \quad (2.41)$$

where

$$\mathbf{A}(1, \mathbf{Z}, \mathbf{R}) = \beta + \gamma \mathbf{Z}^2 - (\sin 2\beta + \mathbf{Z} \sin 2\gamma)/2, \quad (2.42)$$

$$\beta = \cos^{-1}[(1 + \mathbf{R}^2 - \mathbf{Z}^2)/2\mathbf{R}], \quad (2.43)$$

and

$$\gamma = \cos^{-1}[(\mathbf{Z} + \mathbf{R}^2 - 1^2)/2\mathbf{R}\mathbf{Z}]. \quad (2.44)$$

The regions where a molecule may be located within the sample volume in which Equation 2.41 is calculated is illustrated by Figure 2.12.

Equation 2.41 is an approximation of the CEF using geometrical ray tracing. To account for diffraction and aberrations of the collection optics, an approximation closer to the actual CEF could be determined by convolving the geometric CEF with the 3-dimensional point spread function of the collection optics if this were known. This results in a smoothing between the geometric CEF and the actual CEF. However, the difference is reportedly small if a high quality, moderate numerical aperture microscope objective is used [5].

2.9 Sources of Background Noise

2.9.1 Dark Noise

The dark noise is due largely to thermal generation of avalanche pulses at the SPAD detector. It is experimentally determined by accumulating counts with the laser beam blocked. The number of counts that pass through the time gate divided by the

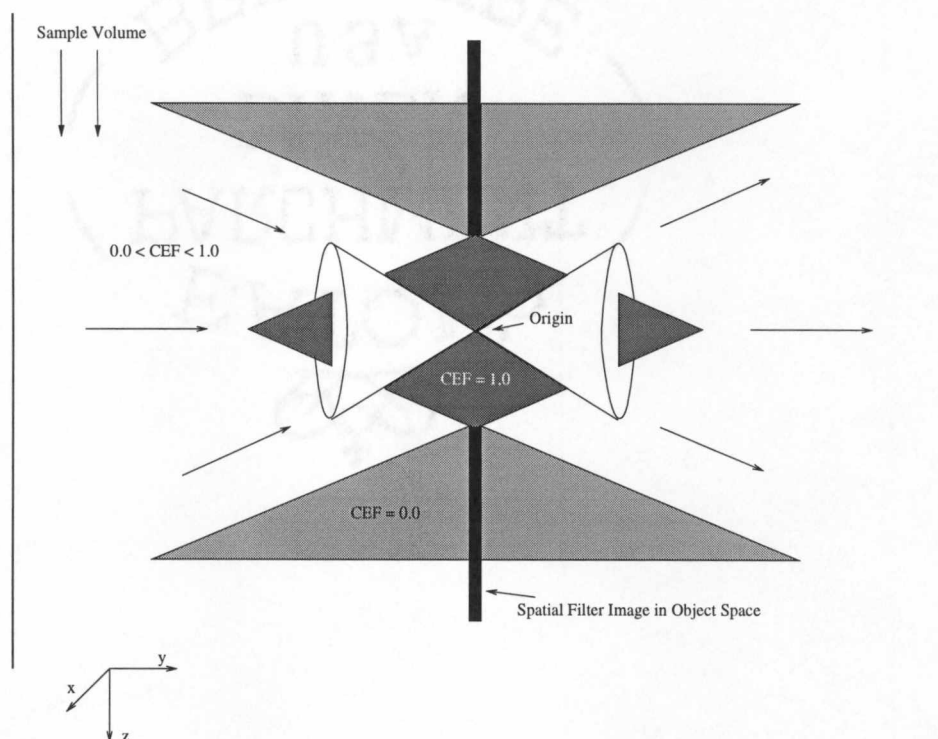


Figure 2.12: CEF calculated regions.

number of seconds of accumulation time yields the dark counts per second, B_{Dark} . The probability of a dark count per laser pulse is B_{Dark}/R .

2.9.2 Rayleigh Scatter

Radiation that is elastically scattered from solvent molecules without a change in its wavelength is called Rayleigh scatter. In the experiment, the Rayleigh scatter may be effectively filtered out using one or more spectral interference filters. The wavelengths of the fluorescence photons generated by the molecules that are to be detected are different than those of the Rayleigh scattered photons. Therefore a Raman notch filter with an optical density of 7 at the laser wavelength can be used to block Rayleigh scattered light so that it becomes negligible. However, if a small

amount of the Rayleigh scatter were to make it through the filters to the detector, it could be treated in the same manner as Raman scatter.

2.9.3 Raman Scatter

The Raman scatter originates from the solvent molecules. For an experimental sample volume of 3.72×10^{-7} l, there are about 10^{15} water molecules and just one dye molecule, so Raman scatter can overwhelm the fluorescence signal. More specifically, the water molecules are vibrationally excited by laser excitation photons, causing the OH-stretching and OH-bending vibrational modes to become excited and resulting in inelastic scatter of the excitation photons. The scattered light is shifted to longer wavelengths that overlap the spectral range of the fluorescence. Thus, spectral interference filters cannot completely discriminate Raman scatter which originates from the solvent molecules, from fluorescence signals which originate from the molecule to be detected.

However, for pulsed laser excitation, Raman scattered photons occur essentially instantaneously or promptly whereas the fluorescence photons are delayed with respect to the excitation pulses. When the effect of the timing jitter of the SPAD detector is included, Raman scatter appears prominent for about the first nanosecond after the laser pulse. This is illustrated in Figure 2.13.

As shown in the figure, the Raman scatter, also known as the experimental prompt, can be significantly reduced by the method of time gating. Only the photons that arrive within the time gate are passed to the subsequent detection electronics.

Although the Raman scatter is mostly rejected by the time gate, the tail of the experimental prompt falls within the time gate. This unrejected scattered light gives rise to a major source of noise in the experiment.

The scattered light from a small volume element $d\mathbf{x} d\mathbf{y} d\mathbf{z}$ located at $\mathbf{x}, \mathbf{y}, \mathbf{z}$

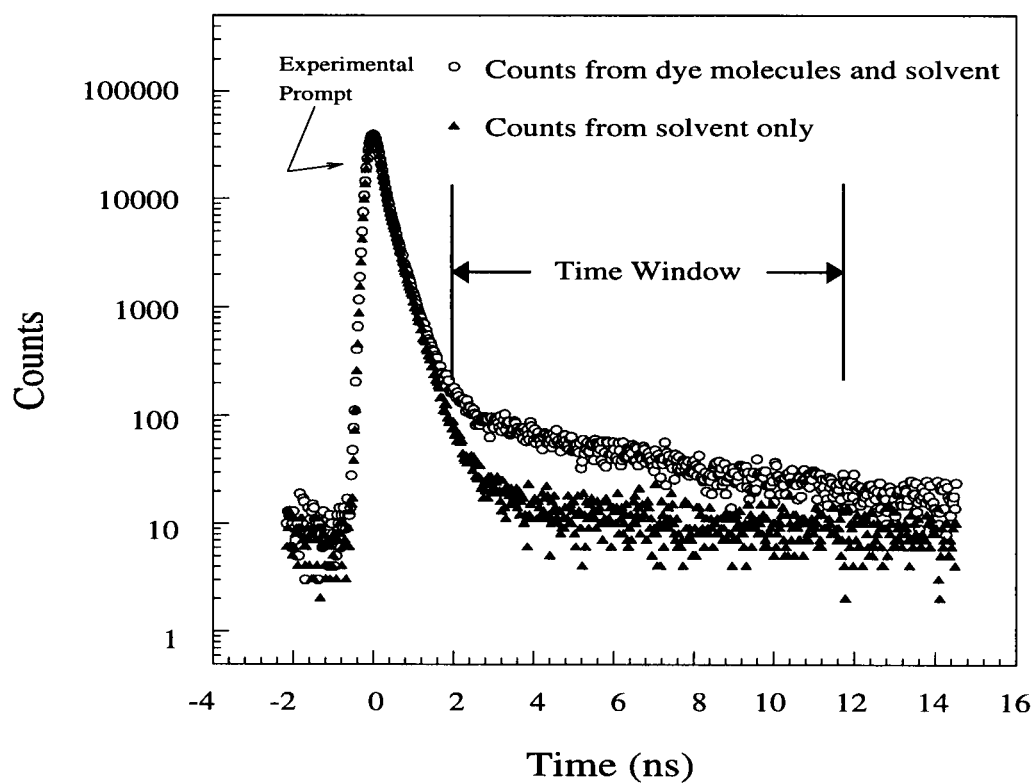


Figure 2.13: Time spectrum of Raman scatter (adapted from Reference [2]).

within the sample is linearly proportional to the intensity, $I(\mathbf{x}, \mathbf{y}, z)$, within the element. Thus the total detected scattered light, B_{Raman} , is proportional to the volume integral of the overlap between the focused laser beam profile and the object space collection efficiency function,

$$V = \iiint (I(\mathbf{x}, \mathbf{y}, z) \pi \bar{w}_{y0} \bar{w}_{z0} / 2P) \text{CEF}(\mathbf{x}, \mathbf{y}, z) d\mathbf{x} d\mathbf{y} dz. \quad (2.45)$$

It is also proportional to the laser power, P , and the maximum collection efficiency function, CEF_{max} . That is,

$$B_{\text{Raman}} = B_S G V P \text{CEF}_{\text{max}}, \quad (2.46)$$

where B_S is the proportionality constant and G is the proportion of the experimental prompt that passes through the time gate. (Note that $G \sim 0.01$ is different from the proportion of fluorescence that passes through the time gate.)

B_S has the dimensions of counts per second per watt per cubic meter. It accounts for the Raman cross section of a solvent molecule at the excitation wavelength, the number of solvent molecules per unit volume, the number of photons per watt at the laser excitation wavelength, and the overlap of the solvent Raman spectrum with the transmission of the spectral filters. B_S can be treated as an empirical constant, which may be experimentally determined by measuring the rate of background counts, B_{Raman} , for given experimental conditions, for which G , V , P , and CEF_{max} are measured.

Most of the Raman scattered photons (i.e., a fraction of $1 - G$) are rejected by the time gate and so do not contribute to the dead time of the TAC electronics, but they increase the dead time of the SPAD.

2.9.4 Single Photon Avalanche Diode Afterpulses

After each photon is detected by the SPAD there is a small probability of a SPAD afterpulse. It occurs at a random time after the original photon detection event and so causes a net increase in the effective rate of background photons that pass through the time gate. Thus SPAD afterpulses may be accounted for simply by increasing the proportionality constant B_S in Equation 2.46. In fact, the method described above for experimentally determining B_S is such that SPAD afterpulses are already accounted for.

2.9.5 Induced Fluorescence of Optical Components

When Rayleigh scattered excitation light from solvent molecules or specularly scattered laser light from the sample cell walls strikes the collection optics, either the microscope objective or the spectral filters, the optics themselves may fluoresce. If this occurs then a photon may be detected which was not generated by dark noise, Raman scatter, or a molecule in the sample volume. Unlike unrejected scattered light, induced fluorescence may possible vary nonlinearly with the laser power, P , the sample volume V , and the maximum collection efficiency, $CEF_{\max}$. Thus noise due to induced fluorescence can be difficult to account for in a simulation unless it has first been accurately characterized in a particular experiment. However, if induced fluorescence did occur and did vary linearly with $PVCEF_{\max}$, then its effects could be accounted for at the same time as the Raman scatter by adjustment of the proportionality constants, B_S and G .

2.9.6 Impurity Molecules in Solution

Impurities within the solution sample may create fluorescence photon bursts similar to those of dye molecules. However, in general, impurity molecules will have lower ab-

sorption cross-sections and fluorescence quantum efficiencies and higher photodegradation probabilities than the sample molecules. Therefore, they can be expected to produce smaller photon bursts and to generate poor statistics when attempting to identify the molecule type.

If impurity molecules are known to exist (for example, if the solvent blank gives detectable photon bursts) then they could be accounted for in the simulation by adding the presence of another molecule type with appropriate concentration and estimated fluorescence parameters.

However, this was not done for the conditions of the simulations reported in Chapter 5 because the corresponding experimental results indicated the rate of photon bursts from impurity molecules in the solvent blank was considerably lower than the rate of bursts from molecules in the sample.

Chapter 3

The SMD Simulation

3.1 Overview

Some analytic calculations of expected experimental results are possible if several approximations are made and many stochastic features are neglected [2]. However, the most practical way to incorporate all of the physical processes discussed in Chapter 2 is to develop a Monte Carlo simulation of the experiment. This chapter discusses the implementation of such a simulation written in the C language. Currently two versions of the simulation are maintained: a 16-bit DOS version using the Microsoft C compiler and Microsoft graphics for the PC and a 32-bit X Window version using VOGLE graphics libraries for a Silicon Graphics Indigo 2 workstation¹ with a MIPS R4000 processor and 64 MB of memory.

The chapter begins with a discussion of random number generators and routines used for the generation of random numbers with various distributions which are required in the simulation. Section 3.3 then introduces the many parameters, variables, and data storage elements that are used in the simulation. With these defined, Section 3.4 then presents and explains the overall flow diagram of the simulation. The details of the individual parts of the flow diagram are discussed in the subsequent

¹From NSF grant CTS-9512489.

sections.

3.2 Random Number Generators

As with any Monte Carlo simulation, there is a need for high quality random numbers, and the use of Monte Carlo integration heightens the importance for good random numbers. Several types have been explored for the simulation—all of which produce acceptable results. Table 3.1 presents a list of the random number generators and some characteristics for each of them.

Table 3.1: Random number generators used by simulation

Generator	Reference	Period, τ_R	Comments
RAN0	[6]	2^{31}	Fast, high correlation
RAN1	[6]	2^{31}	Slow, low serial correlation
RAN2	[6]	2^{62}	Very Slow, low correlation
MZРАН13	[7]	2^{125}	Very fast, very low correlation, will not work on 64-Bit machines

Throughout this chapter, uniform normalized random numbers are referred to as R_U , where $0 \leq R_U \leq 1$. A random number between $-\infty$ and ∞ with a normalized Gaussian distribution with a standard deviation of 1 is denoted by R_{GD} . *Numerical Recipes* provides a routine, `gasdev()`[6], which calculates this random normalized Gaussian deviate using R_U . The representation for a random number with an exponential distribution from 0 to ∞ is $R_{EX}(\text{mean})$, where *mean* is the average of the distribution. The subroutine that generates the exponential deviate calculates it as $R_{EX}(\text{mean}) = -\ln(\text{mean} \times R_U)$.

3.3 Initializations and Declarations

The parameters used in the simulation are listed in Tables 3.2 to 3.7. The parameters with values given in parenthesis may be altered during the simulation, and the others are constants defined in the file `smdsim.h`. Parameters labeled by the † symbol are used only in the extension of the simulation to identify a number, N , of different molecule types according to their fluorescence lifetimes, as discussed in Chapter 4.

Table 3.2: Laser parameters.

Constant Name	Description	Value
<code>lambda</code>	laser wavelength in microns	0.585
<code>p_energy</code>	photon energy in joules	3.4×10^{-19}
<code>rep_rate</code>	laser pulses per second	7.6×10^7
<code>w0y</code>	beam waist in y (dimensions in microns)	3.5
<code>w0z</code>	beam waist in z (dimensions in microns)	3.5
<code>My2</code>	$M^2(y)$ factor accounts for non-ideal beam	1.3
<code>Mz2</code>	$M^2(z)$ factor accounts for non-ideal beam	1.3
<code>power</code>	average laser power in watts	(0.05)

Table 3.3: Parameters for light collection efficiency and experimental geometry.

Constant Name	Description	Value
<code>pinhole</code>	diameter of spatial filter in microns	400.0
<code>magnification</code>	magnification of collection objective	60.0
<code>NA</code>	collection objective Numerical Aperture	0.85
<code>image_loss</code>	optical loss due to uncoated optics	0.75
<code>filter</code>	throughput of interference filter	0.5
<code>spad_eff</code>	detector quantum efficiency	0.65
<code>tgate</code>	time gate efficiency for passing fluorescence	0.6
<code>refract_index</code>	refractive index of aqueous solvent	1.33
<code>tip_radius</code>	input capillary tip radius in microns	0.5
<code>tip_to_beam</code>	distance from capillary tip to laser beam in microns	15.0

Table 3.4: Dead time parameters.

Constant Name	Description	Value
<code>ns_SPAD_dead_time</code>	SPAD dead time in nanoseconds	65
<code>ns_TAC_dead_time</code>	TAC electronics dead time in nanoseconds	800

Table 3.5: Background noise parameters.

Constant Name	Description	Value
<code>dark_in_window</code>	detector dark count (counts s ⁻¹)	50.0
<code>noise_in_prompt</code>	proportionality constant, B_S of Equation 2.46 (counts s ⁻¹ W ⁻¹ m ⁻³)	3.5×10^5
<code>noise_in_window</code>	proportionality constant, $B_S G$ of Equation 2.46 (counts s ⁻¹ W ⁻¹ m ⁻³)	3.48×10^3

Table 3.6: Molecular parameters for sulforhodamine 101 (molecule 0) and rhodamine 640 (molecule 1).

Constant Name	Description	Value of Molecule Type	
		0	1
<code>Fluor_QE[N]</code>	fluorescence quantum efficiency [8]	0.35	0.35
<code>SIGMA[N]</code>	absorption cross-section in μm^2	3.7×10^{-8}	3.7×10^{-8}
<code>PHI_D[N]</code>	photodestruction quantum efficiency [8]	5.5×10^{-5}	5.5×10^{-5}
<code>PHI_CROSS[N]</code>	the probability of triplet crossing	0.002	0.002
<code>CROSS_MEAN[N]</code>	the mean number of seconds before triplet relaxation occurs	4.0×10^{-6}	4.0×10^{-6}
<code>lifetime[N]</code> [†]	fluorescence lifetime in nanoseconds	4.6	2.2

Table 3.7: Other molecule and flow parameters.

Constant Name	Description	Value
<code>pulses_per_time_step</code>	number of laser pulses per iteration	1 – 5
<code>diffusion</code>	diffusion coefficient in $\mu\text{m}^2 \text{s}^{-1}$	4.5×10^2
<code>flow_rate</code>	sheath flow rate in $\mu\text{m} \text{s}^{-1}$	(8.0×10^3)
<code>concentration</code>	sample concentration in moles per liter	(1.0×10^{-11})
<code>ratio_0[†]</code>	fraction of molecules that are of type 0	0.5
<code>thres</code>	threshold for discrimination of peaks in WSS	(5 – 25)

Tables 3.8, 3.9, and 3.10 contain variables and structures that are used in the simulation for storage of information needed in later iterations. The structures described in Tables 3.8 and 3.9 are used by many different subroutines and for convenience are declared with global scope in the include file `smdglobe.h`.

Table 3.8 contains the data structure used for describing every molecule that may be in the simulation. The dimension of the structure array determines the maximum number of molecules in the system. This is currently 10, but could be easily increased to deal with more concentrated solutions or a larger sample volume.

Table 3.9 contains the data structure that keeps a record of the photons detected on the current and previous time steps. The structure is overwritten when the number of detected photons becomes greater than `h.size`. The information stored about each photon is: `delay`, which is the number of iterations since the previous photon was detected, `type`, which is the source of the photon, i.e., whether the photon originated from background noise or molecule type 0, molecule type 1, etc., and `nanosec`, which contains detailed nanosecond timing information about the photon, as discussed in Section 4.3.

Table 3.10 contains other variables which must be stored for use in later iterations. `TAC_dead_count` is used to simulate the TAC electronics dead time after a photon

Table 3.8: Structure containing information for molecules in simulation.

molecule[10]	structure array	
x	float	present x -coordinate of this molecule
y	float	present y -coordinate of this molecule
z	float	present z -coordinate of this molecule
dead	long int	remaining time steps that molecule will be dead
type[†]	short int	type of molecule (0, 1, etc.)

Table 3.9: Structure containing information on photons detected on the current and previous time steps.

h_size	#defined	65536 for Indigo 2, 1000 for PC
history[h_size]	structure array	history structure dimensioned to h_size
delay	long int	number of time steps since last detected photon
type[†]	short int	type of molecule or background from which this photon came
nanosec[†]	short int	the nanosecond bin in which the photon has fallen

Table 3.10: Other temporary storage space used by the simulation.

TAC_dead_count	long int	number of remaining time steps TAC is to be dead
SPAD_dead_count	long int	number of remaining time steps SPAD is to be dead
photon_number	unsigned int	incremented when photon detected
total_num_molec	unsigned int	incremented when molecule is injected
start[†]	unsigned int	value of photon_number at the beginning of a detected peak in the WSS

has been detected, while `SPAD_dead_count` is used to simulate the SPAD dead time. These two variables are discussed in Section 3.8. The parameter `photon_number` is the variable which is used to refer to the detected photons within the structure array `history[h_size]`. When `photon_number` reaches the value `h_size`, it is reset to 0. The parameter `total_num_molec` is the variable used to keep track of the number of molecules that have been introduced into the system.

Variables calculated from the parameters of Tables 3.2-3.7 are defined in Table 3.11. These are declared with their respective data types in the include file `smdglobe.h`. They are evaluated once at the beginning of the simulation or are recalculated if any of the adjustable parameters in parenthesis are changed. A description of how each variable is calculated is given in the listed equation, briefly in the table, or in the following text.

The duration of time it takes to complete one iteration in the simulation, which is represented by the parameter `time_step`, is calculated by dividing the integer parameter, `pulses_per_time_step`, by the repetition rate of the excitation laser, `rep_rate`,

$$\text{time_step} = \frac{\text{pulses_per_time_step}}{\text{rep_rate}}. \quad (3.1)$$

To correctly account for all processes, `pulses_per_time_step` should be set to 1, but for approximate simulations `pulses_per_time_step` may be set to a larger value. Under most conditions, the largest value of `pulses_per_time_step` that generates an accurate simulation is such that $\text{time_step} \leq \text{ns_SPAD_dead_time}$, which occurs when $\text{pulses_per_time_step} < \sim 5$.

The mean distance each molecule moves per time step due to the sheath flow is

$$\text{flow_step} = \text{time_step} \times \text{flow_rate}. \quad (3.2)$$

Table 3.11: Variables that are evaluated by the initialization routine.

Variable	Type	Description (Equation Number)
time_step	float	time step used by simulation (3.1)
flow_step	float	distance in microns the molecule moves per time step due to sheath flow (3.2)
t_between_molec	float	mean number of time steps between molecules entering simulation (3.3)
sdt	float	standard deviation of diffusion per time step (3.13)
zlimit	float	molecules exit experiment when $\text{molecule}[i].z > \text{zlimit} = w_0 z \times 2$
ry	float	Rayleigh range in y (2.24)
rz	float	Rayleigh range in z (2.24)
P	float	Peak photon flux per laser pulse (3.20)
ex[N]	float array	factor for determining molecule excitation (3.22)
cm[N]	long int array	mean triplet lifetime in time steps (3.26)
s0	double	size of the pinhole in object space (3.28)
s1	double	used to evaluate the CEF (3.30)
theta	double	half-angle of light collection (3.32)
s2	double	tangent of theta (3.31)
phi_C	float	maximum overall detection efficiency of fluorescence photons, CEF_{max} (2.34)
QE[N]	float array	fluorescence quantum efficiencies multiplied by CEF_{max} (3.35)
background	float	mean number of background counts per time step (3.37)
prompt	float	mean number of prompt photons detected per time step (3.38)
probe_volume	float	volume integral of intensity profile $\times \text{CEF}$ (2.45)
SPAD_dead_time	long int	number of time steps SPAD is dead (3.40)
TAC_dead_time	long int	number of time steps TAC is dead (3.41)
w[w_limit]	float array	weights for weighted sliding sum (3.48)
w_limit	long int	dimension of array w of weights
step	long int	number of time steps before WSS is recalculated (3.50)

3.4 Overall Flow Diagram

The presentation of the simulation given here is mainly in the form of flow diagrams starting with the overall diagram of the simulation, shown in Figure 3.1. This diagram has key process blocks labeled by the section number in which that process is discussed.

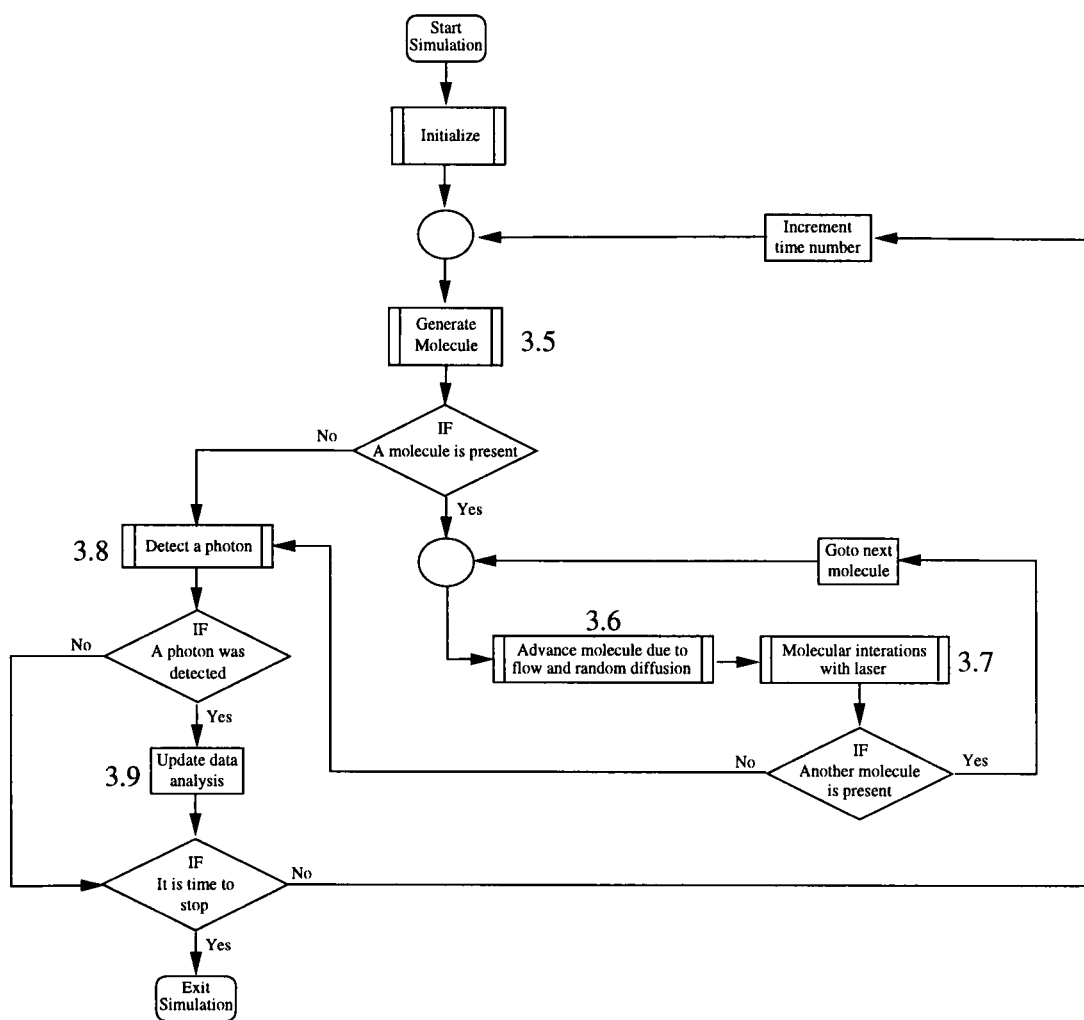


Figure 3.1: Flow diagram for the SMD simulation.

The simulation starts by calling an initialization function which evaluates all of the variables listed in Table 3.11 and initializes the graphics. Next, the simulation

enters the main loop at `time_step = 0`. The first function called within the main loop is the routine that generates molecules, as discussed in Section 3.5. Now, if at least one molecule exists within the simulation, then another loop is entered. The simulation accounts for multiple molecules by calculation of the physical interactions between the molecule, the surrounding sheath flow, and the laser beam within this loop. The first step is to advance the position of each molecule as it diffuses and passes through the sheath flow. This function is discussed in Section 3.6. Each simulated molecule may then interact with the laser beam, thus possibly becoming excited, possibly intersystem crossing and hence becoming unexcitable for a period of time, as discussed in Section 3.7, or possibly photodegrading. If no molecules are present or if all the molecules in the system have been considered, then a code segment is executed to determine if a photon was detected, as discussed in Section 3.8. If a simulated photon detection process occurs, then the data analysis routine is called to calculate the simple or weighted sliding sum, as discussed in Section 3.9. Within this analysis function are the algorithms used to detect bursts of photons and to identify the type of molecule that generated the photon burst by analyzing the nanosecond temporal information of the detected photons. This is the subject of Chapter 4. Finally, if the simulation is running in the graphical user interface mode and the user chooses to exit the simulation then it will stop at this time. Otherwise, if the simulation is running in batch mode without a graphical user interface, then it will stop when `total_num_molec` equals a preset total number of molecules that are to run through the simulation. If neither condition is met, then the simulation simply continues by incrementing the time number.

3.5 Injecting a Molecule into the System

This routine introduces molecules into the system and is called once each time step. Its flow diagram is shown in Figure 3.2. A static unsigned long integer counter,

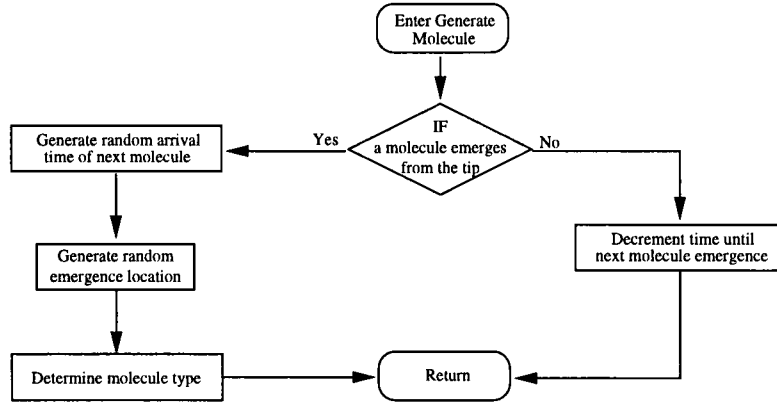


Figure 3.2: Flow diagram to generate a molecule.

`t_molec`, is used within the routine to keep track of the number of remaining time steps that must occur before the next molecule is to be injected. Each time the routine is called, `t_molec` is decremented by 1. When `t_molec` falls to 0 another molecule will be generated. At this time, `t_molec` is assigned a new random number with an exponential deviate and a decay constant which corresponds to the mean time between molecules, $t_molec = R_{EX}(t_between_molec)$. An exponential deviate is used because the molecules are randomly distributed in the flow, hence the time between successive arrival times has an exponential distribution. Accordingly, the number of molecules generated over a fixed interval will follow Poisson statistics. The parameter `t_between_molec` gives the average number of time steps between the introduction of each molecule into the system. It is calculated by the equation,

$$t_between_molec = 1/[\pi(\text{tip_radius})^2 N_A(\text{concentration})(\text{flow_step})], \quad (3.3)$$

where N_A is Avogadro's number. Equation 3.3 is calculated in the initialization routine at the beginning of the simulation.

Note that the simulation can produce no more than one molecule per time step. Because the solution in the experiment is ultra-dilute, the probability for more than one molecule emerging per time step is usually negligible. However, this will not be the case if `t_between_molec` is smaller than ~ 10 , as may occur if `pulses_per_time_step` is too large for the given concentration, flow rate, and injection tip cross-sectional area.

When a molecule emerges from the capillary tip (when `t_molec` = 0) it is assumed to emerge from a random location within the tip. Hence the radial distance of emergence is determined by,

$$r = \text{tip_radius} \sqrt{R_U} \quad (3.4)$$

and the angular component is determined by,

$$\phi = 2\pi R_U. \quad (3.5)$$

The number of molecules that may be simultaneously present in the simulation is determined by the dimension of the structure of Table 3.9. Molecules are labeled by the index i . The Cartesian components of the i -th molecule will then be,

$$\text{molecule}[i].x = r \cos \phi, \quad (3.6)$$

$$\text{molecule}[i].y = r \sin \phi, \quad (3.7)$$

and

$$\text{molecule}[i].z = -\text{tip_to_beam}. \quad (3.8)$$

Finally, the type of molecule produced is determined by

$$\text{molecule}[i].\text{type} = \begin{cases} 0, & \text{for molecule type 0 if } R_U \geq \text{ratio_0} \text{ or} \\ 1, & \text{for molecule type 1 otherwise.} \end{cases} \quad (3.9)$$

3.6 Advancing Molecules Through the Sheath Flow

Advancing a molecule through the sheath flow is accomplished by sending it on a random walk in accordance with the diffusion equation, Equation 2.8, together with a constant displacement per time step to account for the sheath flow. As was discussed in Section 2.4, for each molecule present, the solution to the diffusion equation represents a Gaussian probability distribution about the initial location.

The movement of the molecules through the sheath flow are calculated in Cartesian coordinates. The position is obtained by calculating a Gaussian deviate for x , y , and z , about the initial position. The change in position of the molecule between the current and previous time step of the i -th molecule in the system, is evaluated from the equations,

$$\Delta\text{molecule}[i].x = R_{GD} \times \text{sdt}, \quad (3.10)$$

$$\Delta\text{molecule}[i].y = R_{GD} \times \text{sdt}, \quad (3.11)$$

and

$$\Delta\text{molecule}[i].z = R_{GD} \times \text{sdt} + \text{flow_step}, \quad (3.12)$$

where sdt is defined in the initialization routine as,

$$\text{sdt} = 2\sqrt{\text{diffusion} \times \text{time_step}}, \quad (3.13)$$

in accordance with Equation 2.8.

3.7 Laser-Molecule Interactions

3.7.1 Overview

Figure 3.3 is the flow diagram representing the laser-molecule interactions. This code segment first checks to see if the molecule is in the triplet manifold, and if it is, the

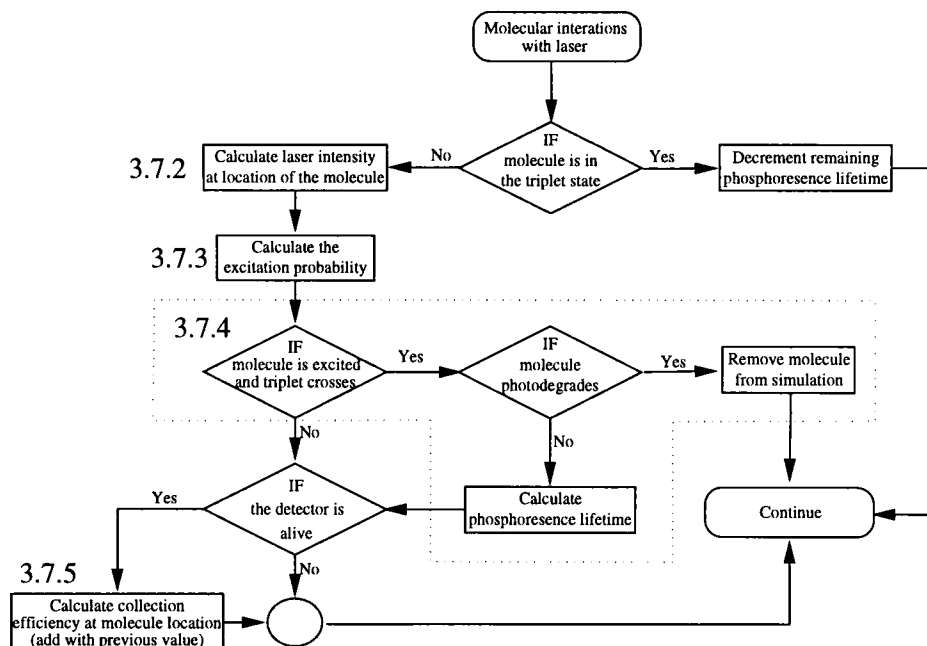


Figure 3.3: Flow diagram for the molecular interactions with the laser.

simulation decrements the number of iterations the molecule is to remain in this state and then continues with next iteration. Most of the time, however, the molecule will not be in the triplet state. In this case, the laser intensity at the location of the molecule relative to the peak intensity is calculated, as explained in Section 3.7.2, and this is then used to calculate the excitation probability, as explained in Section 3.7.3. The excitation probability, together with the probability of intersystem crossing given a molecule is excited, is used to stochastically decide if the molecule intersystem crosses. If it does cross into the triplet manifold, then the probability of photodegradation is used to stochastically decide if the molecule has photodegraded. If the photodegradation condition is satisfied, the molecule is removed from the simulation, otherwise, the phosphorescence lifetime is used to stochastically determine the number of time steps that the molecule is to spend in the triplet state. Details of the calculations pertaining to intersystem crossing and photodegradation are discussed in

Section 3.7.4. Finally, if both the SPAD and TAC are active, then the net collection efficiency at the location of the molecule is calculated, as discussed in Section 3.7.5. This, multiplied by the probability the molecule was excited, gives the “brightness”, that is, the probability that a fluorescence photon will be detected from the molecule during the present iteration. The brightness for each type of molecule present is summed, then used to stochastically determine if a photon is detected during the current iteration, as discussed in Section 3.8.

3.7.2 Calculation of Laser Intensity at a Molecule’s Position

For the i -th molecule present within the simulation, the probability of excitation is dependent on the intensity of the laser at the molecule’s position, which is calculated in accordance with the discussion in Section 2.5. First, the square of each coordinate of the molecule is calculated:

$$x2 = (\text{molecule}[i].x)^2, \quad (3.14)$$

$$y2 = (\text{molecule}[i].y)^2, \quad (3.15)$$

and

$$z2 = (\text{molecule}[i].z)^2. \quad (3.16)$$

These parameters are also used during the same iteration for calculating the collection efficiency of light from the location of the molecule, if this is needed. The size of the laser beam in the y and z dimensions is now calculated from Equation 2.23,

$$\bar{\omega}_y^2 = w0y^2 \times (1 + x2 \times ry), \quad (3.17)$$

and

$$\bar{\omega}_z^2 = w0z^2 \times (1 + x2 \times rz), \quad (3.18)$$

where ry and rz are calculated using Equation 2.24 upon initialization.

Next, the intensity at the location of molecule i relative to the intensity at the origin is calculated by

$$\text{rel_intensity}(i) = \frac{\exp[-2(y^2/\bar{w}_y^2 + z^2/\bar{w}_z^2)]}{\sqrt{2 + x^2(ry + rz)}}. \quad (3.19)$$

The actual photon flux per laser pulse at the location of the molecule is then $\text{rel_intensity}(i) \times P0$, where

$$P0 = \frac{2}{\pi} \times \frac{\text{power}}{\text{rep_rate}} \times \frac{1}{\text{p_energy}} \times \frac{1}{\sqrt{w0y^2 + w0z^2}}, \quad (3.20)$$

in accordance with Equation 2.22 and 2.26.

3.7.3 Calculating the Excitation Probability for the i -th Molecule

The probability of excitation per laser pulse may now be calculated from the photon flux defined in Equations 3.19 and 3.20 above and from Equation 2.30 as

$$\text{excite}(i) = 1 - \exp(-\text{ex}[\text{molecule}[i].\text{type}] \times \text{rel_intensity}(i) \times \mathbf{R}_{MO}). \quad (3.21)$$

Here,

$$\text{ex}[\mathbf{N}] = P0 \times \text{SIGMA}[\mathbf{N}] \quad (3.22)$$

is evaluated for each of the $\mathbf{N}$ molecule types upon initialization, and $\mathbf{R}_{MO}$, which accounts for the random orientation of the molecule as it passes through the laser beam, is given by

$$\mathbf{R}_{MO} = \sin^2(\pi \mathbf{R}_U) \cos^2(2\pi \mathbf{R}_U). \quad (3.23)$$

The expected number of excitations of the molecule per iteration is thus,

$$\mathbf{N}_E = \text{pulses_per_time_step} \times \text{excite}(i). \quad (3.24)$$

$\mathbf{N}_E$ is used for two purposes: accounting for intersystem crossing and photodegradation and accounting for fluorescence. Both are explained in the next two sections.

3.7.4 Accounting for Intersystem Crossing and Photodegradation

The first purpose for calculating N_E is to use it to determine if the molecule has intersystem crossed. N_E is multiplied by the probability of intersystem crossing for the molecule type and compared with a random number, $X = R_U$. If $X < N_E \times \text{PHI_CROSS}[\text{molecule}[i].\text{type}]$, then the molecule is assumed to have crossed to the triplet state. From here, one of two things may occur—photodegradation or relaxation back to the ground state (vibrational deactivation).

Photodegradation occurs if the same random number, X , satisfies the condition $X < \text{PHI_D}[\text{molecule}[i].\text{type}]$. When this rare condition happens, the molecule is removed from the simulation. Otherwise, the i -th molecule will remain within the triplet manifold for a number of time steps which is random with an exponential distribution. Therefore, the molecule is said to be “dead” for a number of time steps given by

$$\text{molecule}[i].\text{dead} = R_{EX}(\text{cm}[\text{molecule}[i].\text{type}]), \quad (3.25)$$

where

$$\text{cm}[N] = \text{CROSS_MEAN}[N]/\text{time_step}, \quad (3.26)$$

and $\text{CROSS_MEAN}[N]$ is the triplet lifetime for each of the N molecule types, which is estimated from the experimentally determined value for rhodamine 6G, given in Reference [4]. At this point, the molecule will not be excited by the simulation, and it behaves as if it had photodegraded. The variable $\text{molecule}[i].\text{dead}$ is a counter representing the number of time steps the molecule will not generate any fluorescence photons. The molecule will still continue its journey in the sheath flow until the calculated number of time steps from Equation 3.25 has elapsed, i.e. until $\text{molecule}[i].\text{dead} = 0$. Once this time has elapsed, the molecule returns to its ground

state and once again becomes excitable. Because the method by which the molecule returns to its original electronic state is much more likely to be by internal conversion, as discussed in Section 2.7.2, there is negligible contribution to the brightness by a phosphorescence photon.

3.7.5 Calculating the Probability of Fluorescence Detection

The second purpose for calculating N_E is for use in determining the total probability of a photon being detected from any of the molecules in the sheath flow. The probability of a photon from each molecule present is calculated from as the product of expected number of excitations, the fluorescence quantum efficiency of the molecule, the peak detection efficiency, which accounts for the filter throughput, the SPAD efficiency, the time gate efficiency, and the efficiency of the optics, and lastly the relative collection efficiency from the location of the molecule. The relative collection efficiency accounts for the optical throughput of the spatial filter and is implemented in the code as a function called `depth_of_focus(i)`.

The function `depth_of_focus(i)` is evaluated according to the discussion in Section 2.8. First, the normalized radial location of the molecule is calculated using

$$R = \sqrt{x^2 + z^2 / s_0^2}, \quad (3.27)$$

where

$$s_0 = \text{pinhole} / \text{magnification}, \quad (3.28)$$

and the normalized location of molecule along the axis of the collection optics is calculated using

$$Z = \text{molecule}[i].y \times s_1 \quad (3.29)$$

where

$$s_1 = s_2 / s_0, \quad (3.30)$$

$$s2 = \tan(\text{theta})/s0, \quad (3.31)$$

and

$$\text{theta} = \text{NA}/\text{refract_index}. \quad (3.32)$$

The quantities `theta`, `s0`, `s1`, and `s2`, are calculated in the initialization function. Next, if $Z < 1 - R$, then the image of the molecule is sufficiently in focus upon the spatial filter such that 100% of the light emitted by the molecule will be collected by the optics. When $Z > R - 1$, the molecule is outside the field of view of the pinhole such that none of the light will be collected by the optics. If $1 - R < Z < R - 1$, then the fraction of light collected will be between 0% and 100% and will be calculated according to Equation 2.36. First, $A(1, R, Z)$ is evaluated using Equations 2.42, 2.43, and 2.44. Then, the two remaining cases are considered—either $1 - R < Z < 1 + R$ or $1 + R < Z < R - 1$. If $1 - R < Z < 1 + R$, the relative collection efficiency is

$$\text{depth_of_focus}(i) = \frac{A(1, R, Z)}{\pi Z^2}. \quad (3.33)$$

Otherwise, if $1 + R < Z < R - 1$, the relative collection efficiency is

$$\text{depth_of_focus}(i) = \left[1 - \frac{Z}{\sqrt{s2^2 + Z^2}} \right] / (2\pi \text{CEF}_{\text{max}}), \quad (3.34)$$

where $s2^2$ is calculated upon initialization. Thus, the probability of light collection may be determined based upon the molecule's position with respect to the collection optics within the simulated flow system.

The resulting multiplication of $\text{depth_of_focus}(i)$ by the other factors mentioned in the first paragraph of this section is the probability for a photon to be detected in one time step from the i -th molecule in the system. This is called the “brightness” of molecule i , and is given by $N_E \times \text{QE}[\text{molecule}[i].\text{type}] \times \text{depth_of_focus}(i)$, where

$$\text{QE}[N] = \text{Fluor_QE}[N] \times \text{phi_C}. \quad (3.35)$$

In the simulation, the net brightness for each of the N types of molecules, `brightness[N]`, is accumulated for each iteration, i.e., where

$$\text{brightness}[N] += N_E \times \text{QE}[\text{molecule}[i].\text{type}] \times \text{depth_of_focus}(i), \quad (3.36)$$

and `brightness[N]` is set to 0, at the beginning of the time step.

3.8 Background Noise, Photon Detection, and Inactive Times

3.8.1 Adding Background to the Total Brightness

After the probabilities for detection of a fluorescence photon from each type of molecule have been accumulated, as discussed in Section 3.7.5, the total photon detection probability must be determined. This total “brightness” includes the brightnesses accumulated from Equation 3.36, background photons, and the experimental prompt. The probability for detection of a background photon per time step is given by

$$\begin{aligned} \text{background} = & (\text{dark_in_window} \\ & + \text{power} \times \text{noise_in_window} \times \text{probe_volume}) \\ & \times \text{time_step}, \end{aligned} \quad (3.37)$$

where `dark_in_window` is the number of detector dark counts per second that fall within the time window as given by B_{Dark} of Section 2.9, and `noise_in_window` is the background noise due to unrejected Raman scatter and detector afterpulses, as given by $B_S G_{\text{CEF}_{\text{max}}}$ of Equation 2.46. The `probe_volume` is calculated during the initialization routine from Equation 2.45 by Monte Carlo integration, as discussed in *Numerical Recipes* [6].

The probability per time step for detection of a prompt photon, which would be removed by the time gate but which would give rise to SPAD detector dead time, is

$$\begin{aligned} \text{prompt} = & \text{power} \times \text{noise_in_prompt} \\ & \times \text{probe_volume} \times \text{time_step}, \end{aligned} \quad (3.38)$$

where `noise_in_prompt` is given by $B_{sCEF_{\max}}$ of Equation 2.46. Hence the total photon detection probability per time step is

$$\text{total_bright} = \sum_{j=0}^N \text{brightness}[j] + \text{background} + \text{prompt}, \quad (3.39)$$

where N is the number of different molecule types. Note that for the simulation to work properly, the time step should be sufficiently small so that `total_bright` $\ll 1$.

3.8.2 Detection of a Photon and Dead Times

Figure 3.4 is the flow diagram used for determining if a photon is detected during the current time step. First, a photon can only be detected if the SPAD detector is not dead, i.e., if `SPAD_dead_count` = 0. If the SPAD is dead, the number of iterations the SPAD will remain dead is decremented. If the SPAD is not dead, then a random number, $X = R_U$, is generated. If $X \leq \text{total_bright}$ then some kind of photon has been detected and `SPAD_dead_count` is set equal to `SPAD_dead_time`, where

$$\text{SPAD_dead_time} = \frac{\text{ns_SPAD_dead_time}}{\text{time_step}}. \quad (3.40)$$

The SPAD will be dead for this number of subsequent time steps. Next, if $X > \text{total_bright} - \text{prompt}$, then the detected photon was a prompt photon, that is, it falls outside the time gate. In this case the photon is basically thrown away and the simulation continues with the next iteration. Otherwise, the photon will only be registered only if the TAC electronics are alive, i.e., if `TAC_dead_count` = 0. If the photon is registered, `TAC_dead_count` is set equal to `TAC_dead_time`, where

$$\text{TAC_dead_time} = \frac{\text{ns_TAC_dead_time}}{\text{time_step}}, \quad (3.41)$$

The TAC will be dead for this number of subsequent time steps. Also, the counter, `photon_number`, which is used to index a structure array of previous photon detection

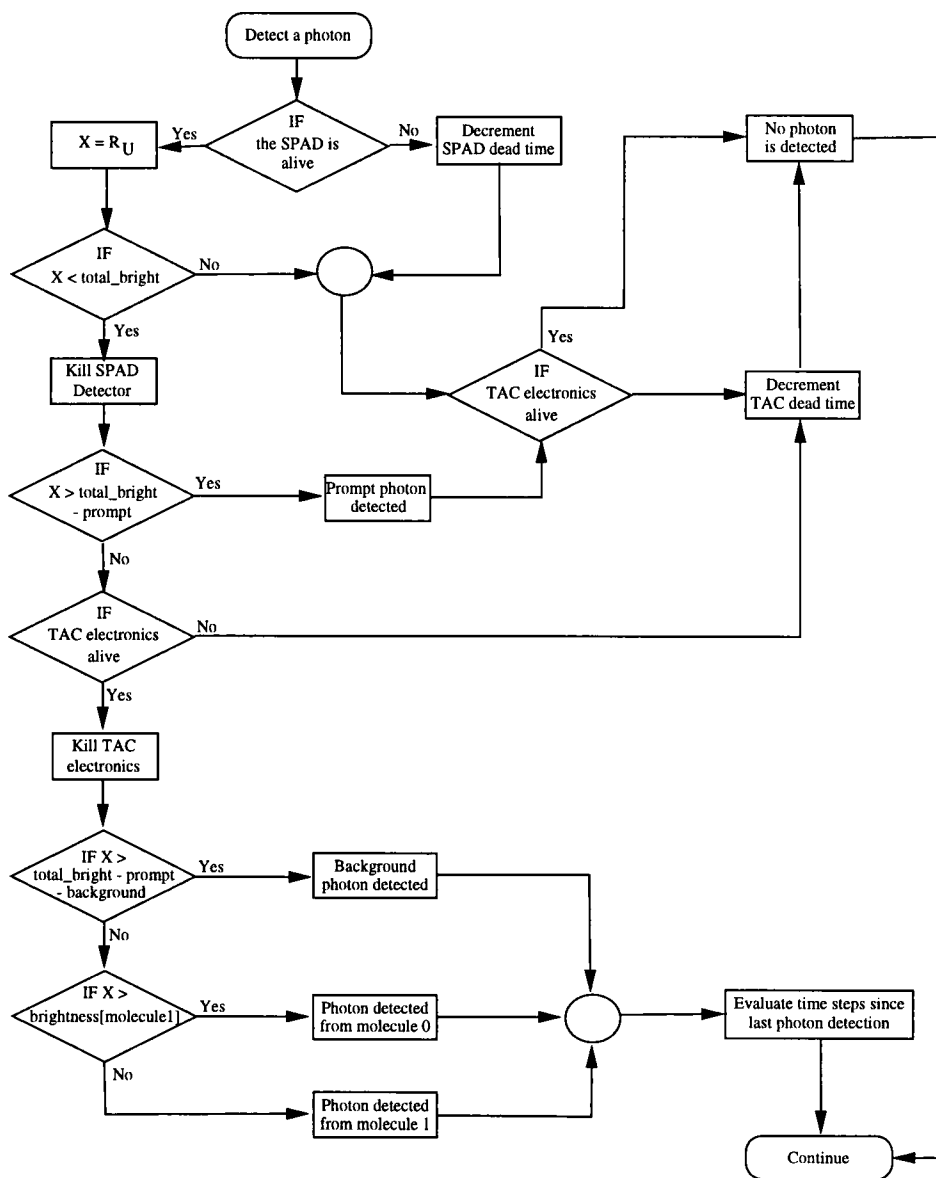


Figure 3.4: Flow diagram to illustrate the model of the photon detection process.

events (Table 3.9), is incremented until `photon_number` equals `h_size` at which point it is reset to 0. Next, if $X > \text{total_bright} - (\text{prompt} + \text{background})$, then the detected photon was due to background, and thus

$$\text{history}[\text{photon_number}].\text{type} = \text{BACKGROUND} = 2. \quad (3.42)$$

Now, if $X > \text{total_bright} - (\text{prompt} + \text{background} + \text{brightness}[0])$, then the detected photon was due to a molecule of type 0, and thus

$$\text{history}[\text{photon_number}].\text{type} = 0. \quad (3.43)$$

Otherwise the detected photon was due to a molecule of type 1, and thus

$$\text{history}[\text{photon_number}].\text{type} = 1. \quad (3.44)$$

Finally, the number of iterations that have elapsed since the previously detected photon is recorded as `history[photon_number].delay`. This is the main variable used by the data analysis algorithms.

3.9 Data Processing Schemes

The analysis of the data is in real time, meaning it is updated whenever a photon detection event occurs in the simulation. The process by which the data is analyzed is either by a simple sliding sum or a weighted sliding sum digital filter.

3.9.1 Simple Sliding Sum

The simplest way to differentiate a fluorescence photon bursts from background noise is to filter the data stream using a simple sliding sum (SSS) over an interval, Δt , equal to the molecule transit time through the laser beam. The interval is slid along the time axis as time increases, as illustrated in Figure 3.5. The output from the filter,

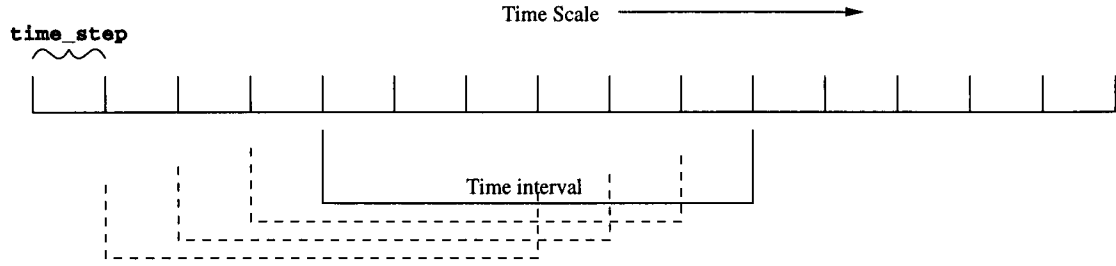


Figure 3.5: The interval over which the sliding sum is calculated.

which represents the total number of photons within the interval Δt , is determined by

$$S(t) = \sum_{t'=t-\Delta}^t s(t'), \quad (3.45)$$

where $s(t')$ is the photon signal at time t' and has the value

$$s(t') = \begin{cases} 1, & \text{if a photon is counted at } t', \\ 0, & \text{otherwise.} \end{cases} \quad (3.46)$$

$S(t)$ will peak at the time when photon counts are close together in time so that there are a large number of counts within the interval Δt . The photon bursts caused by molecules can be distinguished from fluctuations in the background noise by considering only those peaks in the SSS above a preset threshold, `thres`, which is set above the the background fluctuation level.

3.9.2 Weighted Sliding Sum

Improved distinction between photon bursts caused by molecules and those due to background fluctuations can be obtained by using a weighted sliding sum (WSS) rather than the SSS. This is because photon bursts caused by molecules passing through the Gaussian laser beam on average have the same Gaussian profile, with a

higher probability for photon detection occurring when the molecule is at the center of the beam. In contrast, background noise fluctuations occur with a wide range of profiles. The overall molecule detection efficiency will be improved, and there will be less falsely detected molecules if Equation 3.45 is multiplied by a weighting factor. This weighting factor has Gaussian weights that are based upon the expected intensity profile of a molecule passing through the Gaussian laser beam profile. The interval over which the sum is calculated is taken to be 2 standard deviations on either side of the peak. Thus,

$$S(t) = \sum_{t'=t-4\sigma}^t s(t')w(t'+2\sigma), \quad (3.47)$$

where

$$w(x) = \exp[-x^2/2\sigma^2], \quad (3.48)$$

and

$$\sigma = w0z/(2 \times \text{flow_step}). \quad (3.49)$$

The plot of $S(t)$ overlaid with the signal, $s(t')$, is presented in Figure 3.6. In the simulation, the WSS is re-evaluated in increments of `step` time steps, where

$$\text{step} = \sigma/2. \quad (3.50)$$

As in the case with the SSS, a molecule is said to be detected when the WSS passes above the preset threshold, `thres`. Overall molecule detection efficiencies are obtained as follows. The start and end of the burst are taken to be the times at which the weighted sliding sum falls to a minimum on either side of the peak. if any of the photons between the start (i.e., `photon_number = start`) and the end of the peak originated from a molecule, then `history[photon_number].type` will indicate the corresponding molecule type. In this manner, it may be determined whether a detected

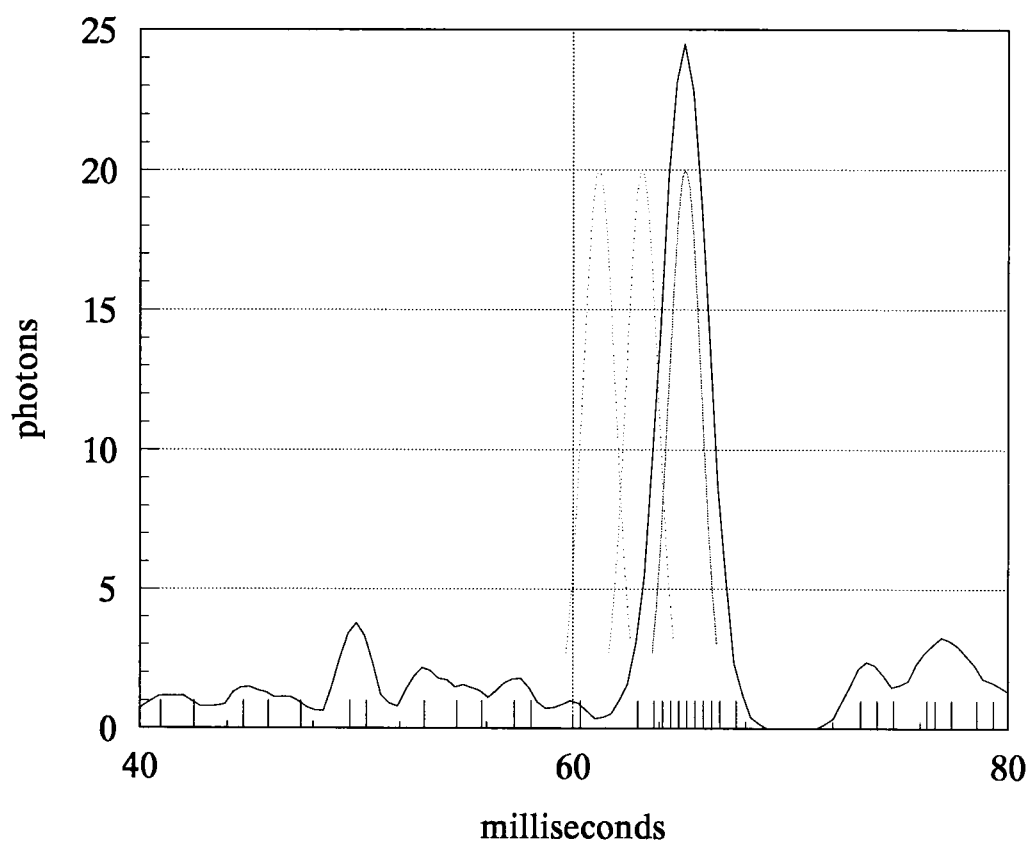


Figure 3.6: Gaussian weights superimposed upon the signal.

photon burst originated solely from background fluctuations (a false detection event), from a molecule of type 0, or type 1, or from two different molecules passing simultaneously. The simulation keeps track of the total number of detected photon bursts due to these various causes, the amplitudes of the peaks in the weighted sliding sum, and the total number of each type of molecule injected into the simulation. From these numbers, statistics such as the overall detection efficiency, the false detection percentage, and peak amplitude distributions may be generated.

3.9.3 Autocorrelation Function

In the simulation, the average transit time of a molecule, 2σ , is known from the beam waist `w0z` and `flow_rate`. However, in the experiment, the average transit time of the molecule through the laser beam, which is needed to calculate the weights for the weighted sliding sum, is not readily known. The autocorrelation function resolves this problem by estimating the average transit time, thus enabling the weights for the WSS to be calculated.

For a continuous function, $s(t)$, the autocorrelation function is defined by

$$g(\tau) = \int_0^{\infty} s(t)s(t+\tau)dt, \quad (3.51)$$

where τ is introduced as a phase shifting factor which will produce a shadow affect as illustrated in Figure 3.7. Equation 3.51 is a correlation integral, which calculates the area of overlap between $s(t)$ and $s(t+\tau)$. From this, the mean width of the peaks may be determined.

In the experiment, the photon signal $s(t)$, is not continuous. The digital equivalent to Equation 3.51 is

$$g(mT) = \sum_{n=0}^{\infty} s(nT)s((n+m)T), \quad (3.52)$$

where mT is the current time step and nT is the n -th time step. In the experiment,

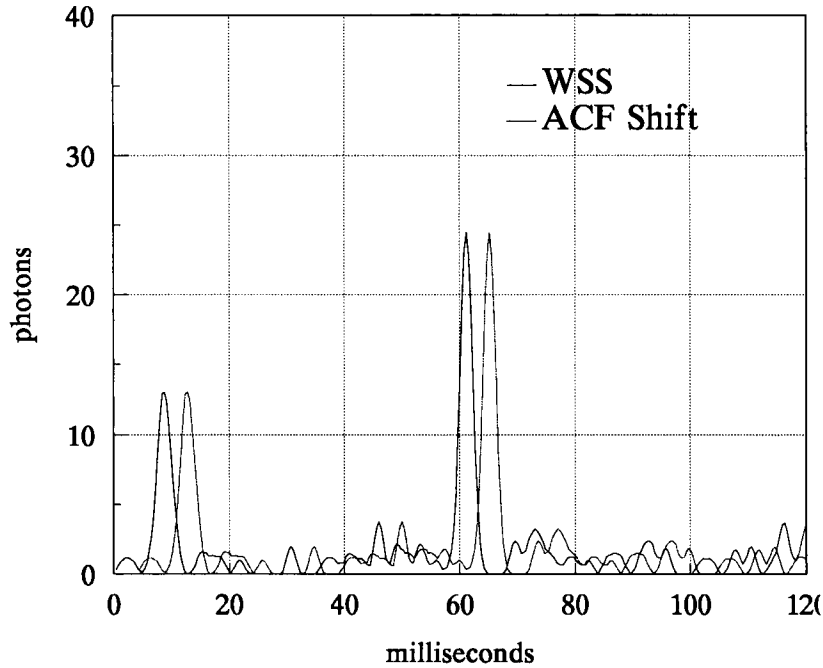


Figure 3.7: The weighted sliding sum with the phase shift $s(t + \tau)$.

Equation 3.52 is simple to calculate because $s(t) = 0$ if no photon was detected during the time step and $s(t) = 1$ if a photon was detected during the time step, as was previously expressed by Equation 3.46. Therefore, only when both $s(nT)$ and $s((n + m)T)$ are 1 will there be any contribution to the autocorrelation function.

When Equation 3.52 is plotted versus mT , for given experimental conditions, it will produce a graph similar to Figure 3.8. The mean transit time for the Gaussian weights may be determined by curve fitting the experimental autocorrelation function with a Gaussian function. The autocorrelation function from the experiment of Li is shown in Figure 3.9 [9]. The data shows an exponential curve superimposed at the tip of the Gaussian autocorrelation function. This effect was originally thought to have been caused by intersystem crossing and photodegradation, however the simulation did not reproduce this effect under the same conditions. While frequently changing the flow rate in the simulation so as to simulate flow rate fluctuations, the autocor-

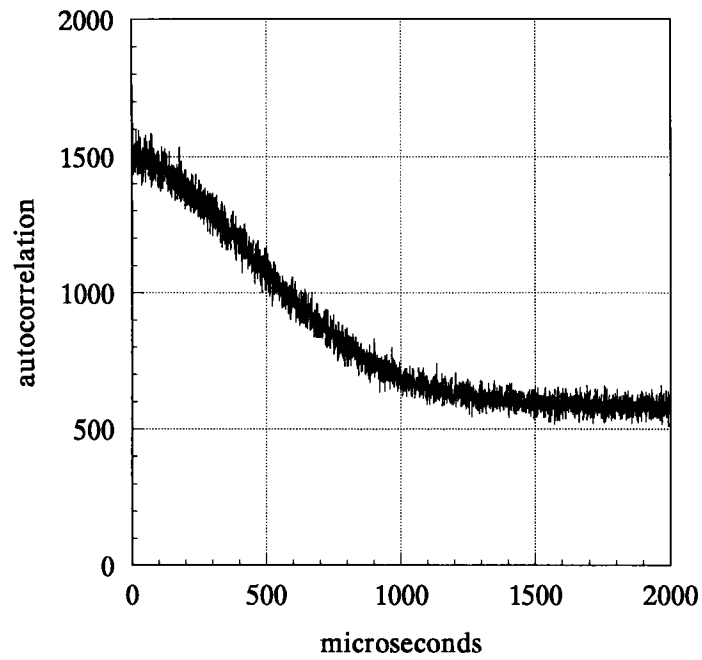


Figure 3.8: The autocorrelation function.

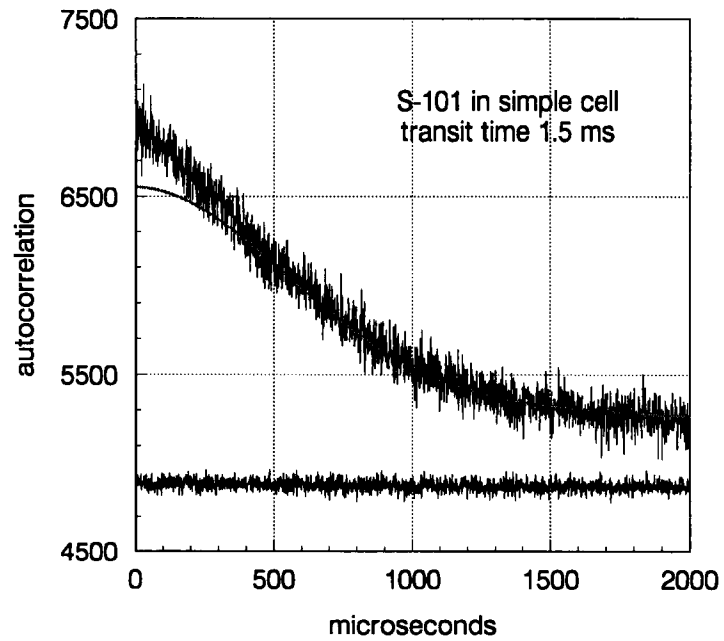


Figure 3.9: An anomalous experimental result of the autocorrelation function (taken from Reference [9]).

relation did reproduce a result similar to that of the experiment. It was determined that the flow system in the experiment was not producing a steady flow, and thus it was changed from a pressure driven system to a gravity fed system.

Chapter 4

Molecule Identification

4.1 Overview

Spectroscopic identification of individually detected molecules is a requirement for several future single molecule detection applications, including rapid DNA sequencing. This chapter explains the simulation of experiments for molecule identification based upon fluorescence lifetime measurements. Although at least two experiments on the measurement of fluorescence lifetimes of individual molecules in solution have been reported in References [10] and [11], and although such measurements, if sufficiently precise, could be used to distinguish between molecules with different lifetimes, to date no such experiments have been reported. Several methods for distinguishing between molecules with different fluorescence lifetimes are developed in this chapter, and in the next chapter the results of simulations indicating the feasibility of experiments to distinguish between sulforhodamine 101 and rhodamine 640, with lifetimes of 4.6 and 2.2 nanoseconds respectively, are presented.

Before discussing the methods for identification, Section 4.2 outlines the experimental acquisition of fluorescence decay data and the corresponding generation of simulated data. The generated time data from each detected photon burst is histogrammed into bins. The optimization of the widths of the time bins is explained in

Section 4.3. Next, Section 4.4 presents an intuitive approach to the estimation of a molecule's lifetime for the simplest case in which background and the finite extent of the decay profile are negligible. This results in the weighted averaging method (WAM) for lifetime estimation. Section 4.5 introduces the general method of the maximum likelihood estimation (MLE) techniques that are used for more rigorous analysis of the fluorescence decay data. Section 4.6 applies the MLE method to the estimation of the fluorescence decay rate, which is the reciprocal of the molecule's lifetime, in the case of a finite known background level and finite decay profile extent. Once the lifetime is estimated from the experimental or simulated data, identification between two different types of molecules with known lifetimes can be easily made according to which of the known lifetimes is closer to the estimated lifetime. An alternate approach is to assume from the outset that the detected molecule has one or the other of the known lifetimes. Following this approach, Section 4.7 discusses the application of a multinomial distribution (MND) to the problem of directly distinguishing between two molecule types with different lifetimes without prior estimation of the lifetime.

4.2 Generation of Simulated Fluorescence Decay Data

As discussed in Section 2.7, if a molecule is excited and a fluorescence photon is emitted, the nanosecond time interval between excitation and emission is random with an exponential distribution and a mean determined by the fluorescence lifetime of the molecule. In an experiment, this time interval can be measured for each detected photon using a TAC electronic module. The signals from the TAC can be digitized and sorted by the computer into a small number of bins, which may have equal or different time widths. When a molecule passes through the laser beam and generates a burst of photons, each of the detected photons is sorted according to its

nanosecond arrival time and a histogram of the binned times is accumulated. Ideally, the histogram will have a profile similar to an exponential decay profile, as shown in Figure 4.1, where the widths of the bins are represented to be equal.

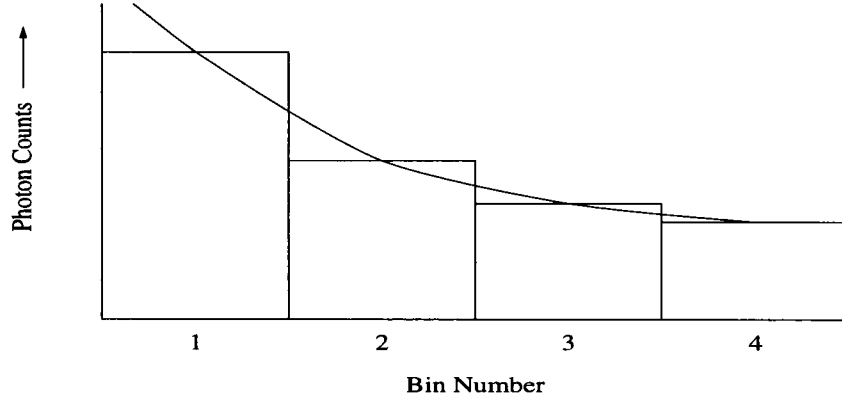


Figure 4.1: Exponential decay profile for histogram produced for binned data.

In the simulation, the nanosecond bin in which each photon falls must be decided, and it is then recorded in the structure element, `history[photon_number].nanosec`, previously described in Table 3.10. The nanosecond bin is random with a probability density dependent upon the origin of the photon, that is, whether the photon came from a molecule of type 0, type 1, or from background noise. This is known and available in the simulation from the structure array element `history[photon_number].type` and is used for generating statistics on the identification accuracy of the various estimators.

If a detected photon originated from background noise, then the arrival time with respect to an excitation pulse is completely random and the probability density is flat. The probability for the photon to fall in the j -th bin is then

$$p_j^b = \frac{w_j}{\sum_{k=0}^{N_B} w_k}, \quad (4.1)$$

where w_j is the width in nanoseconds of the j -th bin and N_B is the number of bins (i.e., $N_B = \text{NUM_OF_BINS}$). For example, if there were 4 bins and the width of those bins were all 2.5ns, then each bin would have a probability of 0.25. A more accurate determination of the probability density for background photons may be made from the experimental prompt time spectrum, as depicted in Figure 2.13. If the time window is divided into bins, the tail of the prompt extends into the first bin. Thus, the actual density is not completely flat but has a higher probability for the first bin.

If a detected photon originated from a molecule of type γ , with a known lifetime τ_γ and fluorescence decay rate $\Gamma_\gamma = 1/\tau_\gamma$, the density will have non-uniform probabilities because of the exponential decay behavior of the photon arrival time. If the time window extends from time t_0 to time t_{N_B} and is divided into bins of width $w_j = t_j - t_{j-1}$, then the probability for a photon to fall in the j -th bin is

$$p_j^\gamma = \frac{\Gamma_\gamma \int_{t_{j-1}}^{t_j} e^{-\Gamma_\gamma t} dt}{\Gamma_\gamma \int_0^T e^{-\Gamma_\gamma t} dt}, \quad (4.2)$$

The denominator is a re-normalization factor to take into account the finite extent of the time window which truncates the exponential tail of the decay profile. Evaluating Equation 4.2 yields

$$p_j^\gamma = e^{-\Gamma_\gamma(t_j - t_0)} \left(\frac{e^{\Gamma_\gamma w_j} - 1}{1 - e^{-\Gamma_\gamma T}} \right) \quad (4.3)$$

$$= e^{-\Gamma_\gamma(t_{j-1} - t_0)} \left(\frac{1 - e^{\Gamma_\gamma w_j}}{1 - e^{-\Gamma_\gamma T}} \right), \quad (4.4)$$

where $T = t_{N_B} - t_0 = \sum_{j=1}^{N_B} w_j$.

The normalized probabilities of Equations 4.1 and 4.3 are calculated in the initialization routine of the simulation. Also, the cumulative probabilities

$$P_j^\gamma = \sum_{k=1}^j p_k^\gamma \quad (4.5)$$

are calculated upon initialization for the background ($\gamma = 2$) and each molecule

type ($\gamma = 0, 1$), and these are used during the simulation to determine within which nanosecond bin each photon falls.

Whenever a photon is detected in the simulation, if the TAC electronics are not dead, i.e., if `TAC_dead_count = 0`, then the bin is determined by a routine called `decay( $\gamma$ )`, where $\gamma = \text{history}[\text{photon_number}].\text{type}$. Within this routine a random number $X = R_U$ is generated. The photon is determined to be in bin j if $X < P_j^\gamma$, where j runs from 1 to `NUM_OF_BINS` until the condition is met.

Whenever a photon burst is detected, that is, whenever the weighted sliding sum exhibits a peak of amplitude greater than the threshold, `thres`, a histogram of `history[photon_number].nanosec` values is accumulated from the start to the end of the photon burst, i.e., from `photon_number = start` to the current `photon_number`. This histogram is stored within the array `net_accumulation`, dimensioned to the value `NUM_OF_BINS`, and it is then passed to the various molecule identification routines.

4.3 Selection of the Number of Bins and Their Widths

The greater the number of bins that are used for storing information on the nanosecond photon arrival times, the greater will be the stored information content and hence the more precise will be the molecule identification. However, the increase in the information content is asymptotic—considerably more is gained by increasing the number of bins from 3 to 4 than from 100 to 101. Reference [12] considers this issue in detail and shows that good precision can be achieved with only `NUM_OF_BINS = 4`. The anticipated error in a lifetime measurement when using 4 equal bins is theoretically shown to be approximately only twice that when using an infinite number of bins. The use of a small number of bins has advantages of experimental simplicity,

and therefore, for this work all simulations consider the case of 4 bins.

However, because the resultant time histograms are sparse, and because the density of nanosecond photon arrival times is expected to decay exponentially according to the fluorescence lifetime, more information may be gained by shortening the bins near the beginning of the decay profile and widening the bins near the tail. This will produce a more even number of photons in each bin, and it will enable the molecule identification algorithms to make more accurate predictions. Variable bin widths have the added effect of reducing the bias of an estimator. A bias is caused because sometimes the statistics lead to a count histogram which indicates an exponential growth rather than an exponential decay [13]. The smaller the population of the histogram, the greater the probability of having a distribution with exponential growth. Also, molecules with longer lifetimes would have flatter distributions than those of molecules with short lifetimes. As a result, the statistics can more easily throw the bin distributions into an exponential growth situation. Hence, the longer the lifetime of the molecule the greater the bias.

To find the optimum bin widths for distinguishing two different molecule types, the goal is to produce a time spectrum in which the mean, or expected, count histograms for each type of molecule differ the most from one another, as illustrated in Figure 4.2. If a hypothetical molecule had spectral properties that were exactly intermediate between the two actual molecule types, i.e., a fluorescence lifetime equal to the average of the lifetimes of molecule type 0 and molecule type 1, then the bin widths should be such that the expected histogram from this molecule is flat.

It is not practical to calculate the optimum bin widths so as to take into account background because the duration of the burst, and hence, the probability of background noise for each bin is not known until after the burst. Unfortunately, a circular argument begins because to find the probability of background noise, the widths of

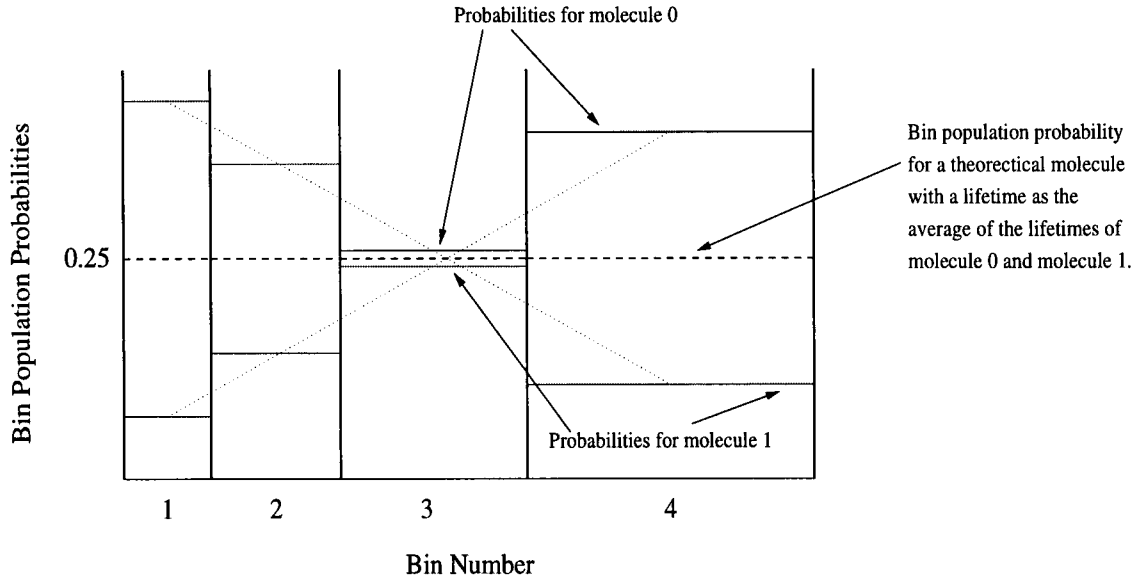


Figure 4.2: Representation of bin widths which produce the most distinct distributions for each molecule type.

the bins must be known, but to find the optimum bin widths, the probability of background noise must be known. Therefore, the optimum bin widths shall be defined by neglecting background and assuming that all photons within a burst are due to molecular fluorescence. In this case, Equation 4.4 may be used for the expected histogram probabilities and the optimum bin widths are obtained by solving $\mathbf{p}_j^\gamma = 1/N_B$ with $\Gamma_\gamma = \bar{\Gamma} = (\Gamma_0 + \Gamma_1)/2$. This yields a recursive formula for the optimum widths

$$\mathbf{w}_j = \frac{1}{\bar{\Gamma}} \left[\frac{e^{\bar{\Gamma}(t_{j-1}-t_0)}}{N_B} (1 - e^{-\bar{\Gamma}T}) \right], \quad (4.6)$$

where $t_{j-1} = t_0 + \sum_{k=1}^{j-1} \mathbf{w}_k$.

4.4 An Intuitive Approach to Molecule Lifetime Estimation

An intuitive approach to the problem is to first consider a distribution over a hypothetical time window of infinite duration ($T/\tau \rightarrow \infty$) in which just one photon

is detected at time t_1 with respect to $t_0 = 0$. The most likely value for the fluorescence lifetime, τ , based upon this very sparse information is $\tau = t_1$. Expanding upon this idea, if two photons were detected at times t_1 and t_2 with respect to subsequent excitations, then the most likely value for τ would be $(t_1 + t_2)/2$. In general, for n detected photons, the best estimate for the lifetime would be the mean of all the detection times.

If photons are sorted into successive bins of widths w_j according to the interval between excitation of detection, then by this approach the best estimate for the fluorescence lifetime, $\hat{\tau}$, would be an average of the mean time that each bin represents weighted by the number of detected photons in that bin, n_j , i.e.,

$$\bar{\tau} = \sum_{j=1}^{N_B} n_j \left(\frac{t_j - t_{j-1}}{2} \right) / \sum_{j=1}^{N_B} n_j. \quad (4.7)$$

Equation 4.7, which is the lifetime estimate by the weighted average method (WAM), has compromised accuracy for of the following two reasons. First, it assumes the bins extend out to an infinite time, whereas in practice the decay profile is collected only over a finite interval. Photons that fall beyond this interval occur but are never seen so that the estimate is biased. Note further, if all detected photons happen to fall within either the first or the last bins, then the estimated lifetime is either $(t_1 - t_0)/2$ or $(t_{N_B} - t_{N_B-1})/2$. The estimated lifetime will always be within those limits. Second, the WAM assumes all photons within a given burst are due to molecular fluorescence whereas in fact a certain proportion are expected to be due to background. These two factors are considered in the MLE method for estimating the lifetime explained in Section 4.6.

4.5 Maximum Likelihood Estimation

The other two algorithms used for identification of molecules in the simulation are based upon maximum likelihood estimation (MLE) techniques, which are explained in general terms in this section. Suppose a set of N data, $\mathbf{Y} = \mathbf{y}_1, \dots, \mathbf{y}_N$, are to be fit to a model that has M adjustable parameters $\mathbf{A} = \mathbf{a}_1, \dots, \mathbf{a}_M$. The model provides the net probability for obtaining the particular data set $\mathbf{Y}$ given the parameter set is $\mathbf{A}$. This probability, $f(\mathbf{Y}; \mathbf{A})$, is called the likelihood function. Suppose in a particular experiment the measured data set is $\mathbf{Y} = \mathbf{Y}_M$. Then the MLE method affirms that the best estimate for the parameter set $\mathbf{A}$ is that for which $f(\mathbf{Y}_M; \mathbf{A})$ is a maximum [14].

For the purpose of this work, the set $\mathbf{A}$ contains just one parameter, $\mathbf{a}$. Depending on the model, $\mathbf{a}$ may take on a continuous range of values, and the corresponding likelihood function, $f(\mathbf{Y}_M; \mathbf{a})$, which corresponds to a probability density function, may be continuous and differentiable with respect to $\mathbf{a}$. In this case, the point at which it is a maximum may be found by solving

$$\frac{\partial f(\mathbf{Y}_M; \mathbf{a})}{\partial \mathbf{a}} = 0 \quad (4.8)$$

to find the stationary points and then checking to ensure that the stationary point is a maximum.

Another method by which the best estimate, $\hat{\mathbf{a}}$, may be found is to maximize the natural logarithm of the likelihood function, referred to as the log-likelihood function, $L(\mathbf{Y}; \mathbf{a})$. Since the entropy of a physical system with degrees of freedom represented by parameters $\mathbf{A}$ is proportional to the natural logarithm of the probability function, $\ln f(\mathbf{Y}; \mathbf{A})$, maximization of the log-likelihood function corresponds to maximizing the entropy, or information content, of the system. Taking the derivative of $L(\mathbf{Y}; \mathbf{a})$ with respect to the parameter $\mathbf{a}$ and equating this to zero will find the stationary

points of this function, that is,

$$\frac{\partial L(\mathbf{Y}; \mathbf{a})}{\partial \mathbf{a}} = \frac{\partial}{\partial \mathbf{a}} \ln[f(\mathbf{Y}; \mathbf{a})] = \frac{1}{f(\mathbf{Y}; \mathbf{a})} \frac{\partial f(\mathbf{Y}; \mathbf{a})}{\partial \mathbf{a}} = 0. \quad (4.9)$$

The model may be such that the parameter $\mathbf{a}$ may possibly only take on a finite set of possible values, $\mathbf{a} = \mathbf{a}^0, \mathbf{a}^1, \dots, \mathbf{a}^s$. In this case, the best estimate for the parameter $\mathbf{a}$ would be found by simply evaluating $f(\mathbf{Y}_M; \mathbf{a})$ for each possible value of $\mathbf{a}$ and then choosing the particular $\mathbf{a}$ that gives the maximum.

4.6 Maximum Likelihood Estimation of the Lifetime of a Molecule

Now consider an example of the MLE technique to the task of estimating the lifetime of a molecule by analyzing an experimental or simulated fluorescence decay profile. The data set $\mathbf{Y}^M = \mathbf{y}_1^M, \dots, \mathbf{y}_{N_B}^M$ corresponds to the number of histogrammed photons, n_j , within each of the N_B bins. The likelihood function, $f(\mathbf{Y}^M; \Gamma)$, which is the net probability for obtaining the data set $\mathbf{Y}^M$, given the decay rate is Γ , is obtained from the probabilities for finding a photon in each of the bins, $p(\mathbf{y}_j; \Gamma)$. If a given photon burst contains N photons, then those photons will be apportioned amongst the N_B bins according to a multinomial probability distribution. The net probability for obtaining the particular distribution of $\mathbf{y}_1^M, \dots, \mathbf{y}_{N_B}^M$ photons in bins $1, \dots, N_B$ is

$$f(\mathbf{Y}^M; \Gamma) = N! \prod_{j=1}^{N_B} \frac{[p(\mathbf{y}_j^M; \Gamma)]^{n_j}}{n_j!}. \quad (4.10)$$

The log-likelihood function is

$$L(\mathbf{Y}; \Gamma) = \ln N! - \sum_{j=1}^{N_B} \ln n_j! + \sum_{j=1}^{N_B} n_j \ln p(\mathbf{y}_j; \Gamma). \quad (4.11)$$

Setting $\frac{\partial L}{\partial \Gamma} = 0$ yields

$$\sum_{j=1}^{N_B} \frac{n_j}{p(\mathbf{y}_j; \Gamma)} \frac{\partial p(\mathbf{y}_j; \Gamma)}{\partial \Gamma} = 0, \quad (4.12)$$

in accordance with Equation 4.9.

If all photons within a burst are assumed to be due to molecular fluorescence, $p(\mathbf{y}_j; \Gamma)$ is given by Equation 4.3 with $\Gamma_\gamma = \Gamma$. In this case,

$$\frac{1}{p} \frac{\partial p(\mathbf{y}_j; \Gamma)}{\partial \Gamma} = -(t_j - t_0) + \frac{w_j}{1 - e^{-\Gamma w_j}} - \frac{T}{1 - e^{\Gamma T}}, \quad (4.13)$$

and the maximum likelihood estimate for the decay rate, Γ , is the root of the equation

$$\bar{t} - \frac{1}{N} \sum_{j=1}^{N_B} \frac{n_j w_j}{1 - e^{-\Gamma w_j}} + \frac{T}{e^{\Gamma T} - 1} = 0, \quad (4.14)$$

where

$$\bar{t} = \frac{1}{N} \sum_{j=1}^{N_B} n_j (t_j - t_0), \quad (4.15)$$

and $N = \sum_{j=1}^{N_B} n_j$ is the total number of photons in the burst.

Equation 4.14 is for the case where background noise is neglected, but background may be accounted for as follows. Individual photon bursts are detected as a peak in the weighted sliding sum and the duration of the burst, which may differ from molecule to molecule because of diffusion and possible photodegradation, may be taken to be the number of time steps between the minima on either side of the peak, that is, **duration** is the number of time steps between that at the start of the peak (i.e., when **photon_number** = **start**) and the current time step, as explained in Section 3.9.2. The expected number of photons in the burst that are due to background is then

$$\bar{b} = \text{duration} \times \text{background}, \quad (4.16)$$

where **background**, the mean number of background counts per time step, is given in Equation 3.37. Therefore, if a photon burst contains N photons and lasts for a measured duration, the proportion of photons that are expected to be due to fluorescence is $(N - \bar{b})/N$. Here it is assumed the threshold, **thres**, for detection of peaks in the weighted sliding sum is such that $N > \bar{b}$.

With background included, the probability for a detected photon to fall into the j -th bin becomes

$$\bar{p}(y_j; \Gamma) = \frac{\bar{b}}{N} p_j^b + \frac{N - \bar{b}}{N} p(y_j; \Gamma), \quad (4.17)$$

so that

$$\frac{1}{\bar{p}} \frac{\partial \bar{p}}{\partial \Gamma} = \left(\frac{N - \bar{b}}{N} \right) \left(\frac{p}{\bar{p}} \right) \left(\frac{1}{p} \frac{\partial p}{\partial \Gamma} \right) \quad (4.18)$$

$$= \frac{p(y_j; \Gamma)}{1 + \frac{\bar{b}}{N - \bar{b}} p_j^b} \left(\frac{1}{p} \frac{\partial p}{\partial \Gamma} \right) \quad (4.19)$$

and Equation 4.12 gives

$$\sum_{j=1}^{N_B} n_j \left[-(t_j - t_0) + \frac{w_j}{1 - e^{-\Gamma w_j}} - \frac{T}{e^{\Gamma T} - 1} \right] / \left[1 + \left(\frac{\bar{b} p_j^b}{N - \bar{b}} \right) e^{\Gamma(t_j - t_0)} \left(\frac{1 - e^{-\Gamma T}}{e^{\Gamma w_j} - 1} \right) \right] = 0. \quad (4.20)$$

This equation, although cumbersome, can be readily solved numerically to find the root, Γ . In the simulation, the MLE algorithm uses a *Numerical Recipes* implementation of Ridders' Method [6] root solver to find the value of Γ , which is then inverted to obtain an estimate for the lifetime, τ . For each detected burst of photons, the molecule that gave rise to the photon burst is identified according to this estimated lifetime.

4.7 Direct Distinction Between Molecule Types

A method has been developed to directly identify which type of molecule gave rise to a particular photon burst. Again, the data set $\mathbf{Y}^M = \mathbf{y}_1^M, \dots, \mathbf{y}_{N_B}^M$ corresponds to the number of histogrammed photons within each of the N_B bins. However, the assumed model now contains one parameter that may take just one of two discrete values. Each detected photon burst is assumed to originate either from a molecule of type 0, with a decay rate of Γ_0 , or from a molecule of type 1, with a decay rate of Γ_1 . Thus, if the parameter, α , is taken as the decay rate, it may only take the values Γ_0

or Γ_1 . The likelihood function $f(\mathbf{Y}_M; \mathbf{a})$, which is the net probability of obtaining the experimentally measured distribution, is again given by Equation 4.10, but is now only defined at two points, $\Gamma = \Gamma_0$ and Γ_1 .

The molecule may be directly distinguished by evaluating the ratio of the multinomial distribution functions at these two points

$$Q = f(\mathbf{Y}_M; \Gamma_0) / f(\mathbf{Y}_M; \Gamma_1) \quad (4.21)$$

$$= \prod_{j=1}^{N_B} \left(p(\mathbf{y}_j^M; \Gamma_0) / p(\mathbf{y}_j^M; \Gamma_1) \right)^{n_j}. \quad (4.22)$$

If $Q > 1$, then the accumulated time spectrum from the photon burst is determined to be from a molecule of type 0; whereas if $Q < 1$ the accumulated time spectrum is determined to be from a molecule of type 1. Determination of the ratio rather than each likelihood individually eliminates the requirement for the calculation of the factorial terms and hence avoids the requirement for scaling to avoid numerical underflow. In the simulation, Equation 4.22 is calculated using the probabilities with expected background, given by Equations 4.3 and 4.17.

In addition to determining which of the two molecules types is more likely, the relative confidence of the prediction may also be determined. The confidence a given experimental distribution $\mathbf{Y}_M$ is due to a molecule of type k relative to the confidence it is due to the type 0 or type 1 is

$$c_k = \frac{f(\mathbf{Y}_M; \Gamma_k)}{f(\mathbf{Y}_M; \Gamma_0) + f(\mathbf{Y}_M; \Gamma_1)}. \quad (4.23)$$

Thus, $c_0 = 1/(1 + 1/Q)$ and $c_1 = 1/(1 + Q)$. For the small number of cases in which the statistics are such that the experimental distribution is intermediate between the expected distributions from each molecule type, $c_0 \approx c_1 \approx 0.5$, and the prediction is clearly seen to be unreliable.

Chapter 5

Results and Discussion

5.1 Overview

Results of the simulation are reported under three conditions, I, II, and III, as characterized in Table 5.1.

Table 5.1: Experimental conditions for the simulation.

Parameter	units	Condition I	Condition II	Condition III
ns_SPAD_dead_time	ns	800	65	65
ns_TAC_dead_time	ns	2500	1	1000
power	W	0.014	0.05	0.100
w0y	μm	9.6	3.5	4.5
w0z	μm	4.8	3.5	4.5
pinhole	μm	800	600	600
flow_rate	$\mu\text{m s}^{-1}$	9.3×10^3	3.5×10^4	1.0×10^4
concentration	M	3.08×10^{-11}	6.0×10^{-12}	1.0×10^{-11}
probe_volume	μm^3	1309	262	431
noise_in_window	$\text{s}^{-1} \text{W}^{-1} \text{m}^{-3}$	2870	3480	3480
noise_in_prompt	$\text{s}^{-1} \text{W}^{-1} \text{m}^{-3}$	1.4×10^5	3.5×10^5	3.5×10^5

Condition I is the parameters of past SMD experiments conducted by Li, which were published in Reference [9]. Since these experiments, there have been several improvements and changes in the experimental setup, particularly a decrease in the excitation sample volume, a decrease in the SPAD dead time at the expense of an

increase in background due to SPAD afterpulses, and the use of different interference filters, which allow a larger fraction of the fluorescence to pass (0.5 rather than 0.4) at the expense of an increase in the throughput of background. Because of the improvements, faster single molecule detection is possible using a faster flow rate and a higher laser power. Thus, Condition II is intended to simulate current and future experiments on very rapid single molecule detection, without identification of the molecule type. In this situation, the TAC is not needed, and therefore the TAC dead time is set to 1 in the simulation. Condition III is intended to simulate a future experiment in which the experimental conditions are altered in order to achieve a higher number of photon counts per molecule, so that the molecules may be more reliably identified by spectroscopic measurements.

5.2 Simulation of a Previous Experiment (Condition I)

Figure 5.1 is a plot of experimental results presented in Reference [9]. In this figure, the photon burst with a peak of ~ 42 is thought to have been caused by two molecules passing through the system simultaneously. The bottom graph in this figure is of the background noise obtained by running pure solvent through the system. The parameters under which the experiment was run, as listed in Table 5.1, were applied to the simulation to produce Figure 5.2. When compared to Figure 5.1, in the time period of 100 milliseconds, both the simulation and experiment produced 13 photon bursts. However, the average peak height is about 24 in the simulation and about 18 in the experiment. There is reasonable agreement between the results of the simulation and those of the experiment, certainly within the 25% error in the experimental estimate of the optical net throughput quoted in Reference [9], and this agreement lends credence to the simulation.

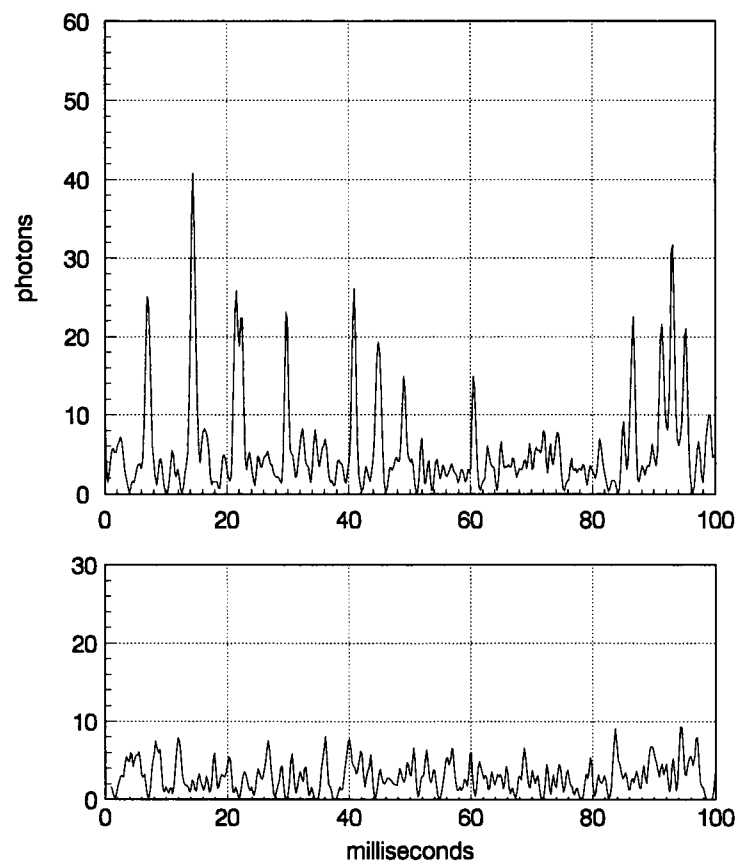


Figure 5.1: Experimental photon burst signals from sulforhodamine 101 under Condition I (taken from Reference [9]).

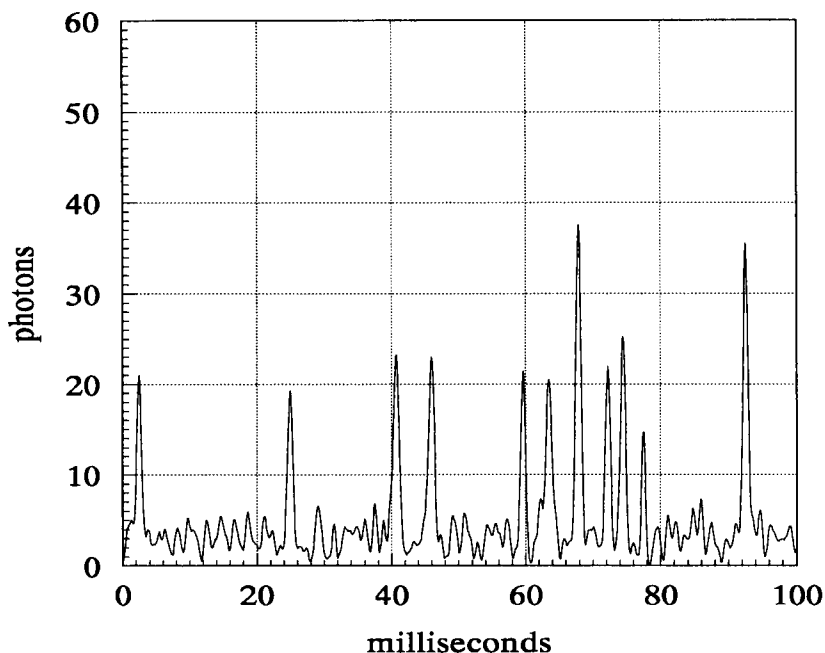


Figure 5.2: Simulated photon burst signals from sulforhodamine 101 under Condition I.

5.3 Simulation of Rapid Detection (Condition II)

The simulation of the experiment under Condition II is intended to determine whether an experiment on rapid detection, with molecule transit times of $\sim 200 \mu\text{s}$ would be feasible. Experiments are currently ongoing, but as of yet, there are no experimental data to compare with this simulation. However, experimental measurements indicate that for pure solvent and 20 mW of laser power, the count rate within the time window is 2100 s^{-1} and that within the prompt is $212,000 \text{ s}^{-1}$. These values together with the probe volume and the maximum collection efficiency of the 0.85 N.A. objective are used in Equation 2.46 to yield the values of `noise_in_window` and `noise_in_prompt` listed in Table 5.1. With this level of background, the results of the simulation in Figure 5.3 indicate that rapid detection of single molecules should be achievable with the parameters of Condition II.

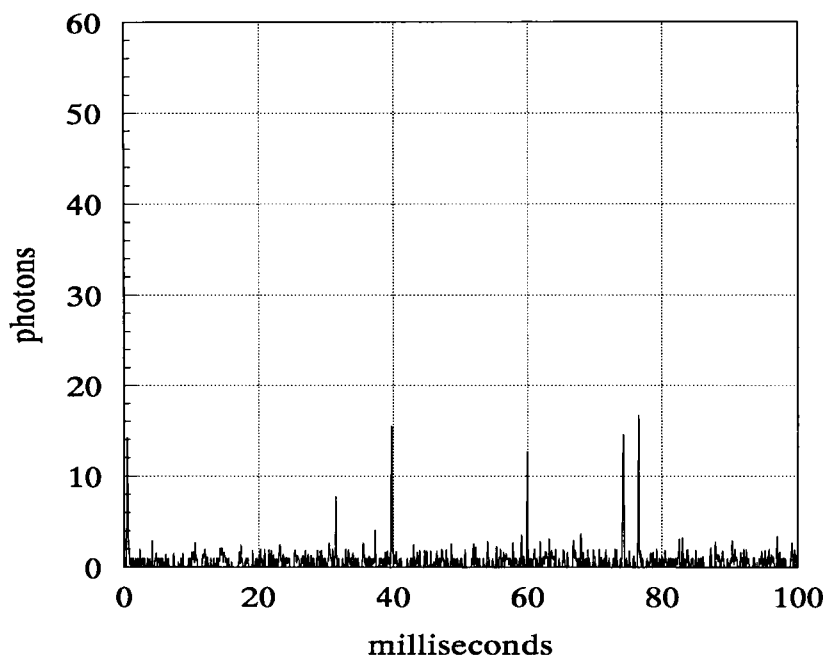


Figure 5.3: Simulated photon burst signals under Condition II.

In the simulation, the source of each photon burst, i.e., from a molecule or background, is known. The heights of the peaks in the weighted sliding sum that are due to molecular fluorescence and due to background noise fluctuations may thus be separately histogrammed. These are displayed in Figure 5.4 for simulation results in which the total number of generated molecules is $M_G = 20000$. To generate the histograms, a low threshold value, `thres` ~ 2 , is initially used. The optimum threshold value, `thres`, may then be set according to the figure at the point at which the two histograms cross. For the situation of Condition II, the optimum threshold is 6, i.e., all photon peaks that are > 6 are considered to indicate the detection of a molecule.

The overall detection efficiency of molecules is determined by taking the ratio of M to M_G , where M is the number of peaks above the threshold that are due to molecules, i.e.,

$$D_M = \frac{M}{M_G}. \quad (5.1)$$

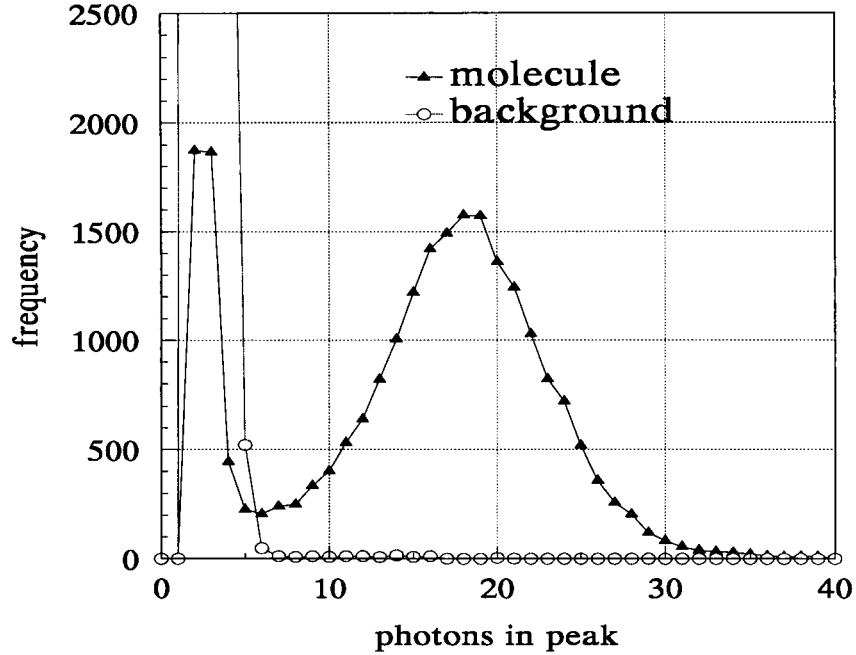


Figure 5.4: Efficiency analysis for the parameters of Condition II.

The molecule detection efficiency determined from Figure 5.4 and Equation 5.1 is $\sim 92.31\%$, which is higher than that achieved in the experiment of Reference [9]. Loss of detection efficiency is caused mostly by photodegradation of molecules, which results in peak heights smaller than the threshold.

A small number of the background peaks are of amplitude > 6 , and these lead to false detections. The fraction of detection events that are false is given by

$$D_F = \frac{B}{B + M}, \quad (5.2)$$

where B is the number of peaks above the threshold that are due to background. The percentage of false detections is determined from Figure 5.4 and Equation 5.2 to be $\sim 0.63\%$.

The autocorrelation function generated for Condition II produced an interesting result. Figure 5.5 shows an exponential tip superimposed upon the Gaussian autocorrelation function. However, the tip is on a much faster time scale than that of the

anomalous result given in Figure 3.9. Because of the fast time scale, the exponential tip in Figure 5.5 was thought to be due to intersystem crossing of molecules into the triplet manifold, as discussed in Section 2.7.3. This was confirmed by observing that the tip does not occur if the triplet relaxation lifetime is set to zero.

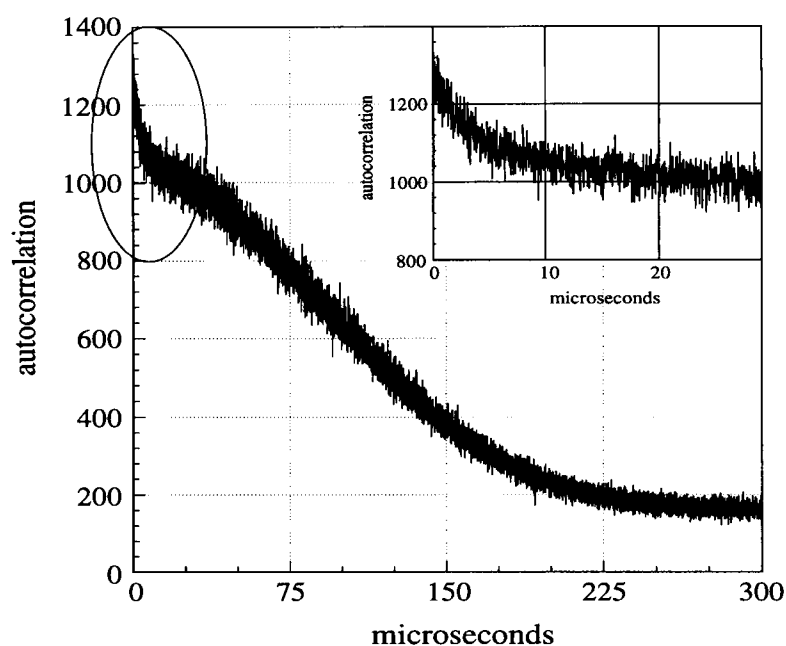


Figure 5.5: Autocorrelation function generated by Condition II.

5.4 Simulation of Molecule Identification (Condition III)

For molecule identification algorithms to make accurate predictions, photon bursts must contain a sufficiently large number of photons. Condition III attempts to increase the number of photons obtained per molecule for this reason. Figure 5.6 shows the weighted sliding sum analysis of simulated data, in which relatively large amplitude photon burst signals are generated. This graph includes bursts from each of the 2 molecule types, molecule 0, with a fluorescence lifetime of 2.2 ns, and molecule

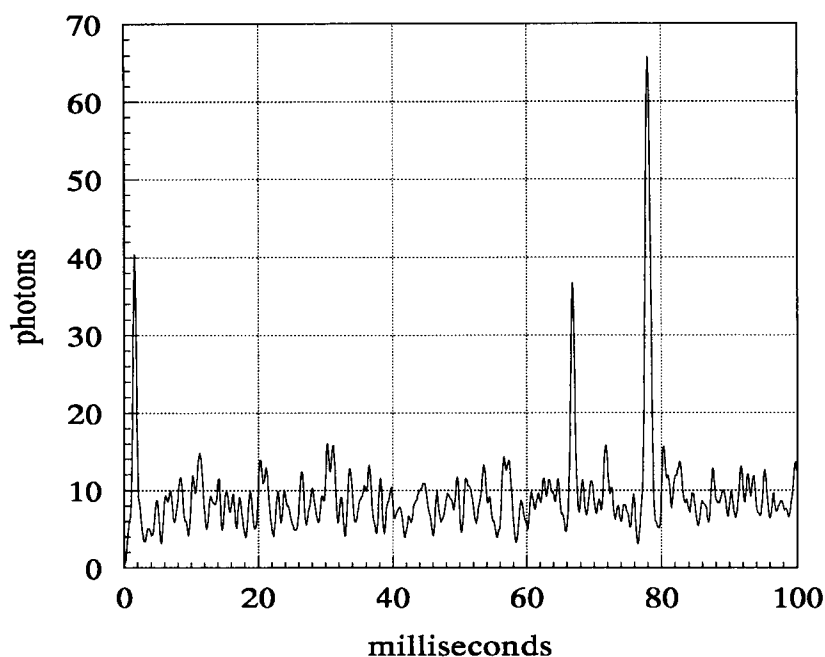


Figure 5.6: Simulated photon burst signals under Condition III.

1, with a lifetime of 4.6 ns.

Figure 5.7 shows the histograms of amplitudes of peaks due to molecules or to background fluctuations from a simulation run in which 20000 molecules were generated. The optimum threshold obtained from the point at which the two histograms cross is 19 and the overall molecule detection efficiency for this value of the threshold is $\sim 84.1\%$. A large fraction of molecules go undetected and a wide range of peak amplitudes is obtained because most molecules photodegrade as they pass through the laser beam, due to the relatively high laser power and moderately slow flow rate.

According to Figure 5.7 the proper threshold for the simulation under Condition III should be `thres = 19`. However, in order to achieve accurate predictions, the molecule identification algorithms require fluorescence photon bursts whose amplitudes are considerably greater than background photon burst amplitudes. Thus, the threshold is set to a value such that the background bursts have a minimal in-

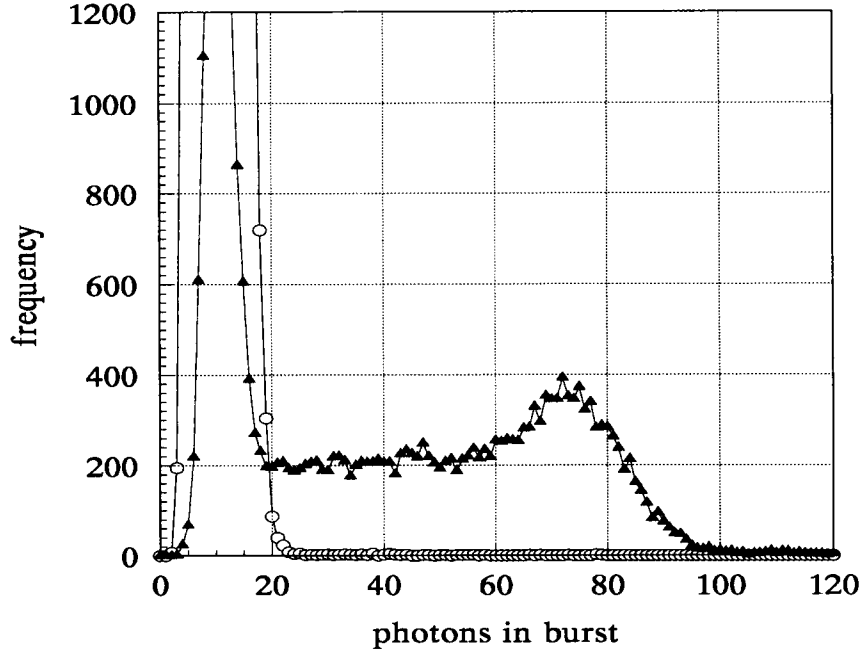


Figure 5.7: Peak height frequency under Condition III.

fluence on the molecule identification algorithms. For testing the identification of molecules according to their fluorescence lifetimes, the threshold is set to a value of `thres` = 25 so that only photon bursts with an amplitude > 25 are considered. Also, in order to produce less cluttered graphs, the simulation was executed for a smaller data set, in which a total of 512 photon bursts of amplitude > 25 were detected, 249 known to be from molecule 0, 241 from molecule 1, 1 from background noise, and 21 from the simultaneous passage of both types of molecules. This same data set was used for generating fluorescence decay profiles under the two conditions of equal and optimal bin widths, as discussed in Section 4.3. The results of the analysis of the binned decay profiles for molecule identification by the three methods discussed in Sections 4.4, 4.6, and 4.7, the weighted averaging method (WAM), the maximum likelihood estimator (MLE), and the multinomial distribution (MND) are summarized in Table 5.2.

While Table 5.2 contains considerable detail, an overall comparison between the different methods for analysis may be taken from the last three lines. First, all three methods improve in their accuracy of prediction when optimal bin widths are used. Second, the WAM predictor is seen to be clearly inferior to the other two methods. On closer inspection, this is seen to be due to an almost consistent misidentification of molecules of type 1, which have the longer fluorescence lifetime of 4.6 ns. This in turn is due to biased estimation of lifetimes by the WAM, i.e., due to the neglect of background and of the truncation of the tail of the decay profile, as discussed in Section 4.4.

The bias in the lifetime estimates can be seen in Figures 5.8 and 5.9, which plot the lifetime predictions made by the WAM versus the number of photons per burst, when using equal and optimal bin widths respectively. In principle, the bias could be taken into account by moving the dividing line between the predictions, shown as a vertical solid line in the figures, to a value less than the mean of the actual lifetimes. However, the separation between the points on the graphs corresponding to either type of molecule is still not as distinct as that obtained using the MLE predictor. This can be seen by comparing Figures 5.8 and 5.9 with Figures 5.10 and 5.11, which plot the lifetime predictions made by the MLE versus the number of photons per burst, when using equal and optimal bin widths.

Whereas the summary of comparisons in Table 5.2 provides no information on the dependence of the prediction reliability on the number of photons in the burst, Figures 5.10 and 5.11 show that bias in the estimated lifetime and misidentification of the molecule type occur more frequently when the number of photons in the burst is small. Also, the simultaneous passage of both types of molecules invariably generates bursts with a large number of photons and the peak in the weighted sliding sum caused by background fluctuations is seen to contain a very small number of photons.

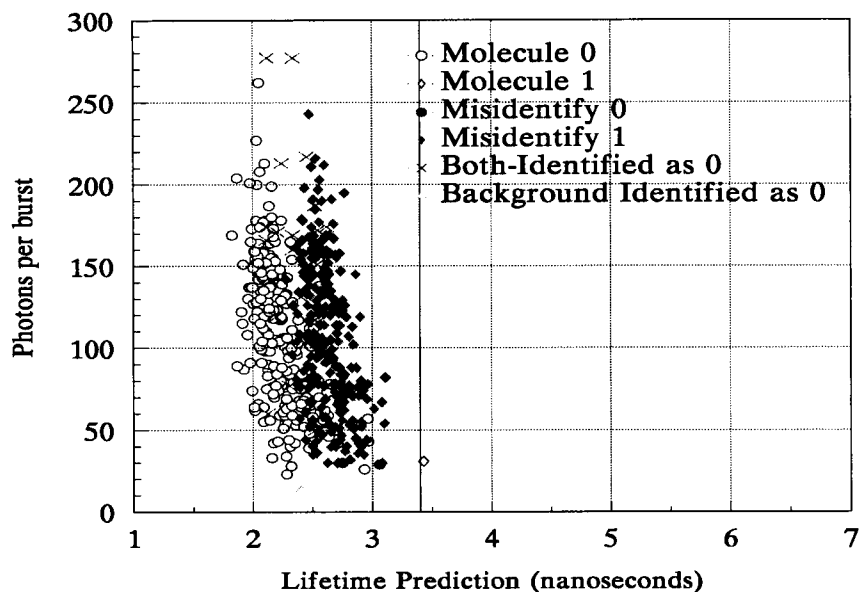


Figure 5.8: Lifetime estimations by the weighted averaging method using equal bin widths.

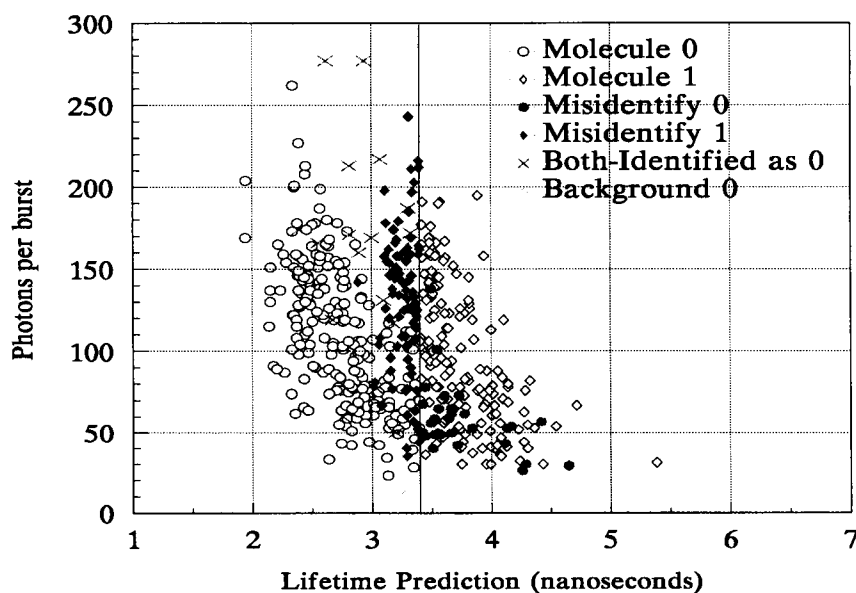


Figure 5.9: Lifetime estimations by the weighted averaging method using optimal bin widths.

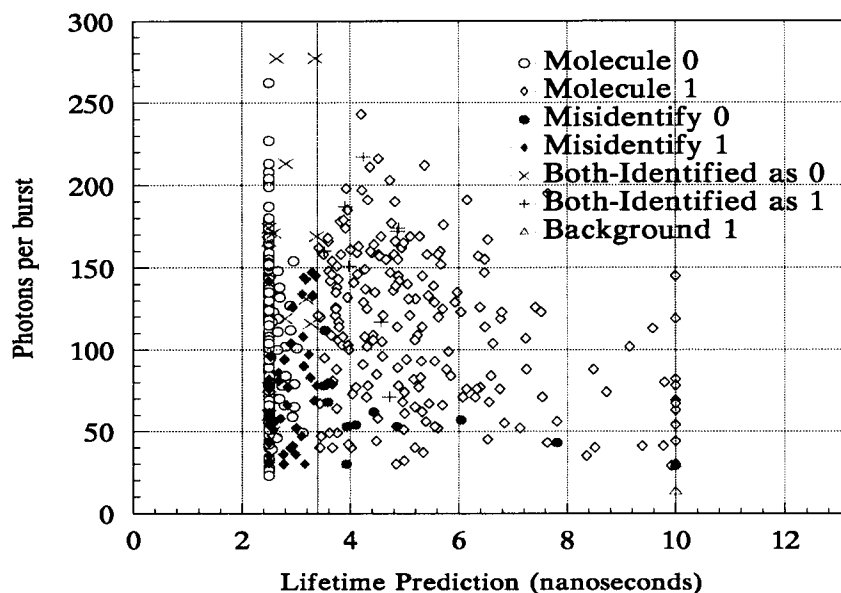


Figure 5.10: Lifetime estimations by the maximum likelihood estimator using equal bin widths.

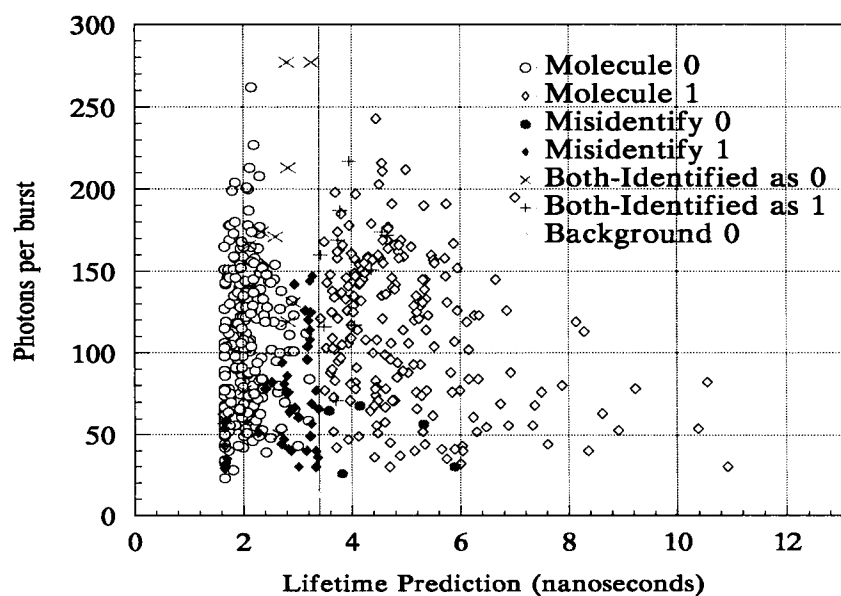


Figure 5.11: Lifetime estimations by the maximum likelihood estimator using optimal bin widths.

A third overall feature evident from the last three lines of Table 5.2 is that the MND method for distinguishing between two types of molecules is slightly better than the MLE method, yielding a higher fraction of correct predictions and a lower fraction of incorrect predictions. This can also be seen by comparing Figures 5.10 and 5.11 with Figures 5.12 and 5.13, which plot the confidence of molecule 0 prediction, as given by Equation 4.23, versus the number of photons in the burst. Also, incorrect identifications seldom yield a confidence level $< 5\%$ or $> 95\%$, whereas in the MLE method, incorrect identifications are sometimes associated with predicted lifetimes that are on the opposite side of the incorrect lifetime.

In addition to being a superior predictor of the molecule type, the MND method is numerically more efficient. By contrast, in the MLE method, one must first iteratively find a root, which is the decay rate of the molecule, invert the result to yield the fluorescence lifetime, and then determine which molecule belongs to that lifetime. The MND method directly calculates the ratio of two multinomial probabilities and then is able to immediately predict the molecule type.

Finally, the improvement in the MND method obtained with the use of optimal bin widths, although evident in Table 5.2, is best appreciated by close comparison of Figures 5.12 and 5.13. The use of optimal bin widths gives rise to shifts in the confidences of the predictions. Molecules known to be of type 1 (represented by open or filled diamonds on the figures) become shifted to the left, while those molecules known to be of type 0 (represented by open or filled circles) become shifted to the right. In particular, correct predictions shift to better confidence levels, to the left for molecule type 1 and to the right for molecule type 0, thereby creating a higher level of clustering on both sides of the graph. Specifically, molecule predictions with confidences $> 95\%$ for type 0 and $< 5\%$ for type 1 increases by 2% and 47% respectively.

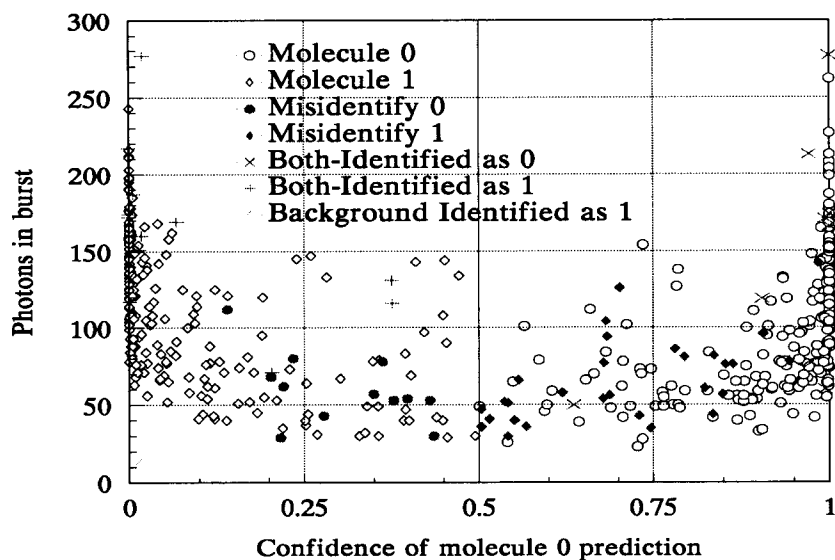


Figure 5.12: Multinomial distribution predictions using equal bin widths. A confidence level < 0.5 indicates a prediction of molecule type 1.

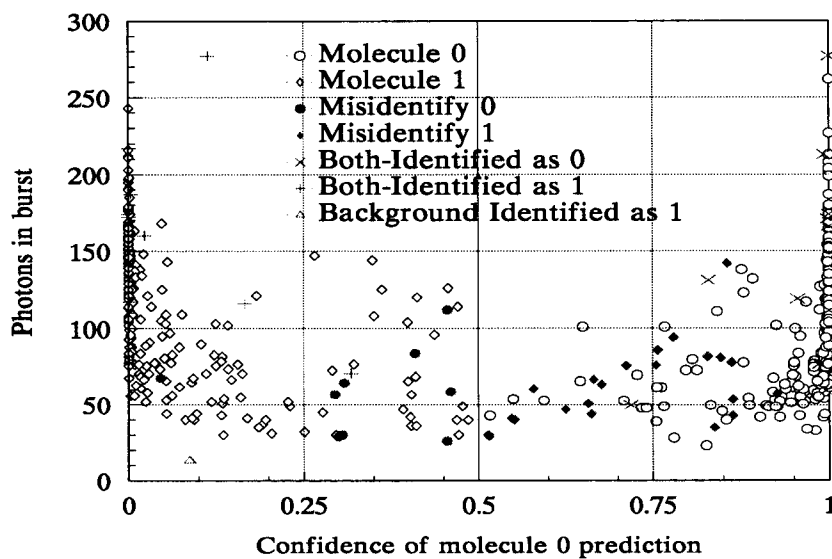


Figure 5.13: Multinomial distribution predictions using optimal bin widths.

Chapter 6

Summary

The results in this thesis have successfully demonstrated the simulation of a single molecule detection experiment. The simulation is useful for helping one to understand the physical aspects of single molecule detection. Also, the simulation assists in overcoming problems encountered in the experiment, such as the autocorrelation anomaly explained in Section 3.9.3. It also has been shown that rapid single molecule detection should be possible with high detection efficiency. The simulation is an effective way to compare different experimental setups as well as the viability of an experiment under given operational parameters. It has enabled the development and efficient testing of molecule identification algorithms which could be incorporated into a data analysis program of an experiment.

In future work, this simulation could be expanded to include more than only two types of molecules. This would be an effective method for accounting for higher levels of impurity molecules in a solution of two or more dye molecules. Also, a neural network algorithm could be developed to efficiently identify molecules directly with no bias. The simulation could be used to create a training set consisting of the molecule's type and the time bin spectrum. Then, the neural network trained from the simulated data would be used in real time on experimental data to identify molecules with a high degree of accuracy and speed.

BIBLIOGRAPHY

Bibliography

- [1] E.B. Shera, N.K. Seitzinger, L.M. Davis, R.A. Keller, and S.A. Soper. "Detection of single fluorescent molecules,." *Chem. Phys. Lett.*, 174:553–557, 1990.
- [2] L.Q. Li. *Single Molecule Detection in Solution*. Ph.D. Dissertation, The University of Tennessee, 1994.
- [3] L.M. Davis and L.Q. Li. "Monte Carlo model of a single molecule counting experiment." *Laser Applications to Chemical Analysis-Topical Meeting*, page 206, 1994.
- [4] S. Nie, D.T. Chiu, R.N. Zare "Probing individual molecules with confocal fluorescence microscopy." *Science*, 266:1018–1021, 1994.
- [5] H. Qian and E.L. Elson. "Analysis of confocal laser-microscope optics for 3-D fluorescence correlation spectroscopy." *Applied Optics*, 30(10):1185–1195, 1991.
- [6] W.H. Press, B.P. Flannery, S. A. Teukolsky, and W.T. Vetterling. *Numerical Recipes in C*. Cambridge University Press, 1992.
- [7] G. Marsaglia and A. Zaman. "Some portable very-long-period random number generators." *Computers in Physics*, 8(1):117–121, 1994.

- [8] S.A. Soper, H.L. Nutter, R.A. Keller, L.M. Davis, and E.B. Shera. "The photophysical constants of several fluorescent dyes pertaining to ultrasensitive fluorescence spectroscopy." *Photochemistry and Photobiology*, 57(6):972–977, 1993.
- [9] L.Q. Li and L.M. Davis. "Rapid and efficient detection of single chromophore molecules in aqueous solution." *Applied Optics*, 34(18):3208–3217, 1995.
- [10] S.A. Soper, L.M. Davis, and E.B. Shera. "Detection and identification of single molecules in solution." *J. Opt. Soc. Am. B*, 9(10):1761–1769, 1992.
- [11] C.W. Wilkerson, Jr., P.M. Goodwin, W.P. Ambrose, J.C. Martin, and R.A. Keller. "Detection and lifetime measurement of single molecules in flowing sample streams by laser-induced fluorescence." *Appl. Phys. Lett.*, 62(17):2030–2032, 1993.
- [12] M. Kollner and J. Wolfrum. "How many photons are necessary for fluorescence-lifetime measurements?" *Chemical Physics Letters*, 200(1,2):199–205, 1992.
- [13] J. Tellinghuisen and C.W. Wilkerson, Jr. "Bias and precision in estimation of exponential decay parameters from sparse data." *Anal. Chem.*, 65:1240–1246, 1993.
- [14] A. Papoulis. *Probability and Statistics* Prentice Hall, 1990.
- [15] G.M. Barrow. *Physical Chemistry*, Fifth Edition, McGraw-Hill, 1988.

APPENDIX

Appendix A

Derivation of Fick's Second Law

Consider a box of unit cross sectional area with uniform concentration within any cross section, as shown in Figure A.1. The rate of diffusional transport along the x -

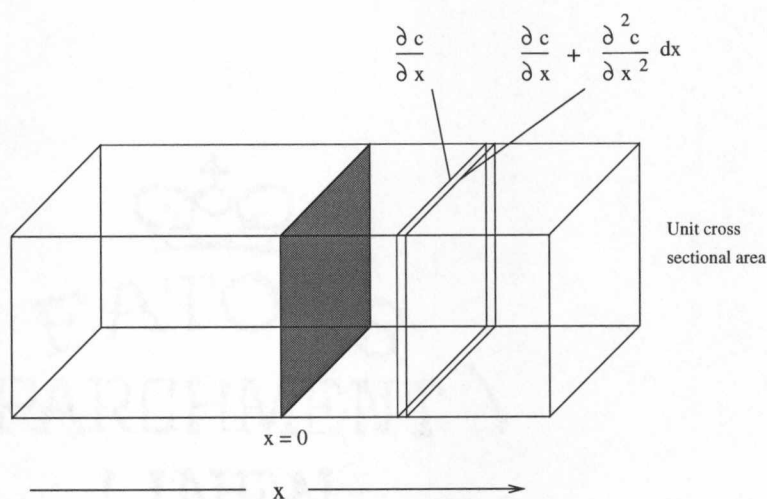


Figure A.1: Concentration change due to diffusion from a plane source.

direction is proportional to the concentration gradient, $\partial c / \partial x$. This leads immediately to Fick's first law of diffusion in one dimension,

$$\text{Rate of diffusion in } x\text{-direction} = -D \frac{\partial c}{\partial x}. \quad (\text{A.1})$$

D , the diffusion coefficient, is introduced as the proportionality constant, and the negative sign allows D to be positive. Fick's second law is obtained from the rate

of accumulation, which is determined by the rate of entry at the cross section at $\mathbf{x}$, $-D(\partial \mathbf{c} / \partial \mathbf{x})$, and the rate of leaving at the cross section at $\mathbf{x} + d\mathbf{x}$, $-D[(\partial \mathbf{c} / \partial \mathbf{x}) + (\partial^2 \mathbf{c} / \partial \mathbf{x}^2) d\mathbf{x}]$. The difference between these terms leads to the second order differential equation,

$$\text{Rate of accumulation} = -D \frac{\partial \mathbf{c}}{\partial \mathbf{x}} - \left[-D \left(\frac{\partial \mathbf{c}}{\partial \mathbf{x}} + \frac{\partial^2 \mathbf{c}}{\partial \mathbf{x}^2} d\mathbf{x} \right) \right] \quad (\text{A.2})$$

$$= D \frac{\partial^2 \mathbf{c}}{\partial \mathbf{x}^2} d\mathbf{x}. \quad (\text{A.3})$$

Dividing the rate of accumulation by $d\mathbf{x}$ yields the rate of change of concentration,

$$\frac{\partial \mathbf{c}}{\partial t} = D \frac{\partial^2 \mathbf{c}}{\partial \mathbf{x}^2}. \quad (\text{A.4})$$

This is the concentration equation, known as Fick's second law. Expanding the concentration equation to three dimensions gives Equation 2.6 [15].

VITA

Dennis Bunfield was born May 8, 1972 in Pompton Plains, New Jersey. At the age of two he and his parents moved to Colorado, where he later attended Homestead Elementary School in Englewood. In 1984, he moved back to New Jersey with his mother and new step father, Rick, where he spent the next two years at Eisenhower Middle School in Wyckoff. Upon graduation from middle school, Dennis moved to Timonium, Maryland, where he attended Dulaney High School. There, he participated in wrestling, learned how to play the saxophone at the Peabody Conservatory of Music, and later joined the band.

In 1990, Dennis attended Western State College in Gunnison, Colorado. At Western, he participated in the many band activities and spent many very cold, clear nights staring at the stars in the astronomy club. In May of 1994, he received his baccalaureate degree in physics with a computer science emphasis and a minor in chemistry.

The following fall, Dennis went to The University of Tennessee Space Institute as a self-supporting student to work on his Master's degree in physics. After his first year, he was awarded a graduate research assistantship by Dr. George Shi and began work on his thesis under the guidance of Dr. Lloyd Davis. Among several activities in which Dennis participated at UTSI include the soccer club, the cheap rocket society, president of the paintball club, and the UTSI softball team, who won the team spirit award two years running. He received his Master of Science degree in physics in May, 1997.

Dennis now is planning to pursue a career as a Nuclear Propulsion Officer in the United States Navy.