Fluorescence Lifetime Discrimination of Near-Infrared Dye-Labeled Oligonucleotide Fragments Separated by Capillary Gel Electrophoresis for Base-Calling Applications in DNA Sequencing.

Benjamin Leighton Legendre Jr
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/6630
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700  800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
FLUORESCENCE LIFETIME DISCRIMINATION OF NEAR-INFRARED DYE-LABELED OLIGONUCLEOTIDE FRAGMENTS SEPARATED BY CAPILLARY GEL ELECTROPHORESIS FOR BASE-CALLING APPLICATIONS IN DNA SEQUENCING

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Chemistry

by

Benjamin L. Legendre Jr.
B.S. in Chemistry, Nicholls State University, 1992
B.S. in Biology, Nicholls State University, 1990
May, 1998
Acknowledgments

This manuscript is dedicated to all of the people that have touched my soul dearly, both family and friends.

God: For making me the person that I am and surrounding me with the most supportive family and friends that any one person could ever imagine.

Mom and Dad: The most wonderful parents that anyone could imagine having. I will never forget the love and support, and I hope that I will have the love and patience for others that you have shown. I love you.

Lisa, Dean, Jake, Nicole, and Stephanie: I know that I forgot how to be a brother (and friend) for a while. I know I do not have to ask for forgiveness, for I am already forgiven. I thank you for the patience, for I would have never been able to get through this without your constant support and love. Never forget that I will always be there for you.

Rusty and Stacy: What can I say, you guys are the greatest. I knew I always had someone that would listen to me about life and not offer any advice. I needed that. Thank you.

Dr. Soper: A personal thanks. I know I did not believe in myself at times. Thank you for the words of encouragement and praise. You went beyond the bounds of just being my advisor, you are my friend.

My advisory committee: Thank you for the support and encouragement.

Dr. Soper's research team: Daryl and James, I'm glad we started together: I'm glad we finished together. I know I wouldn't have made it
through without our friendship. We made a pretty good team. To the rest of my group and colleagues, you are always in my thoughts for the support and encouragement.

**Lanny:** The one that help me make it through Nicholls with a little bit of sanity. I appreciate everything that you have done for me.

**Chris and Rich:** Thanks for all the cold beer, good conversation, and a couch to sleep on, and, oh yeah, the great friendship that we developed.

**Fairway View connection: Mandi, Michelle, Karen, Delia, Meredith, Meg:** Thanks for listening to all my stories, for sharing in some good times, and for just being there. Most of all, thanks for all the beautiful smiles.

**Chally:** You know, Neil Diamond and fireworks rule, eh!

**Shelly:** What you have taught me about life, love, and family will never be forgotten. I wish you the best. (Hi Grammy, Grandpa, Grandma, and the rest of the William’s clan of Welch, OK).

**In special remembrance:** To Grandma, for teaching me the will to survive any adversity and how important it is to be at peace with yourself and God. To Grandpa, for teaching me that hard work and dedication does pay off. To Mommie, for teaching me that a sense of humor will make life more bearable. To Paw-Paw, for teaching me that any goal is obtainable. I LOVE YOU, and I miss you!
TABLE OF CONTENTS

ACKNOWLEDGMENTS..................................................................................ii

LIST OF TABLES..................................................................................vii

LIST OF FIGURES..................................................................................viii

ABSTRACT..............................................................................................xi

CHAPTER

1 NEAR-INFRARED DYE-LABELED OLIGONUCLEOTIDE FRAGMENTS SEPARATED BY CAPILLARY GEL ELECTROPHORESIS AND DETECTED BY LASER INDUCED FLUORESCENCE FOR SEQUENCING APPLICATIONS

1.1. Introduction.................................................................................. 1
1.2. Separation Methodologies.......................................................... 2
1.2.1. Slab Gel Electrophoresis.......................................................... 3
1.2.2. Capillary Gel Electrophoresis................................................... 4
1.3. Detection...................................................................................... 8
1.4. DNA Sequencing Strategies....................................................... 9
1.5. Research Focus........................................................................... 10
1.6. References................................................................................... 13

2 TIME-CORRELATED SINGLE PHOTON COUNTING : AN OVERVIEW

2.1. Introduction................................................................................. 16
2.1.1. Fluorescence............................................................................. 17
2.1.2. Fluorescence Lifetimes............................................................ 20
2.2. Factors Influencing the Time-Resolved Behavior of Fluorescent Molecules......................................................................................................................... 22
2.2.1. Near-IR Fluorescence............................................................. 23
2.3. Methods for Measuring Fluorescence Lifetimes......................... 27
2.3.1. Phase-Resolved Spectroscopy.................................................. 27
2.3.2. Time-Resolved Spectroscopy................................................. 28
2.3.2.1. TCSPC Instrumentation...................................................... 33
2.3.2.1.1. Light Sources................................................................. 34
2.3.2.1.1.1. The Ti:sapphire Laser.................................................. 42
2.3.2.1.1.2. The Semiconductor Laser....................................... 44
2.3.2.2. Detectors............................................................................. 48
2.3.2.2.1. Photomultiplier Tubes................................................... 49
2.3.2.2.2. Multichannel Plate Photomultipliers............................. 50
2.3.2.2.3. Single Photon Avalanche Diodes................................. 51
2.3.2.2.4. Timing and Jitter.......................................................... 53
3 ERROR ANALYSIS OF SIMPLE ALGORITHMS FOR DETERMINING FLUORESCENCE LIFETIMES IN ULTRADILUTE SOLUTIONS

3.1. Introduction ........................................................................ 67
3.2. Experimental ........................................................................ 73
3.2.1. Instrumentation ................................................................ 73
3.2.2. Reagents and Chemicals .................................................. 78
3.3. Results and Discussion ....................................................... 80
3.4. Conclusions ....................................................................... 91
3.5. References ........................................................................ 93

4 ON-LINE FLUORESCENCE LIFETIME DETERMINATIONS IN CAPILLARY ELECTROPHORESIS

4.1. Introduction ....................................................................... 95
4.2. Experimental ...................................................................... 99
4.2.1. Instrumentation ................................................................ 99
4.2.2. Free Solution Capillary Electrophoresis ......................... 100
4.2.3. CE Lifetime Determinations ......................................... 101
4.2.4. Preparation of Gel Columns and DNA Sequencing Fragments .......................................................... 103
4.3. Results and Discussion ...................................................... 104
4.3.1. Comparison of Simple Algorithms in Free Solution CE .......................................................... 104
4.3.2. Identification via Fluorescence Lifetime Discrimination .......................................................... 113
4.3.3. Effect of Neighboring Components on Lifetime Determination .................................................. 116
4.3.4. Lifetime Determination of Near-IR Dye-Labeled DNA Fragments Separated via Capillary Gel Electrophoresis .......................................................... 120
4.4. Conclusions ..................................................................... 122
4.5. References ....................................................................... 124
LIST OF TABLES

2.1. Absorption maxima, molar absorptivities, fluorescence lifetimes, and fluorescence quantum yields for the near-IR dye IR-125 in a various solvent systems ................................................... 26

3.1. Fluorescence lifetimes for IR-125, IR-132, and DTTCI in ethanol as calculated by the MLE algorithm ........................................ 89

4.1. Fluorescence lifetime determinations in capillary electrophoresis using the MLE and RLD methods for the fluorescent dyes DTTCI and IR-125 ....................................................... 107

5.1. Calculated fluorescence lifetimes for the near-IR dye-labeled DNA sequencing primers ...................................................... 140
### LIST OF FIGURES

1.1. A simplified block diagram of a CGE system for DNA analysis .................... 5

1.2. Chemical structures of tricarbocyanine, near-IR heavy-atom modified dyes along with the calculated lifetimes ................................................................. 12

2.1. Jablonski diagram showing radiative and nonradiative pathways for molecular relaxation ........................................................................................................ 18

2.2. Absorption and emission spectra of IR-125 in water and methanol .......................................................................................................................... 24

2.3. Block diagram of a TCSPC device ........................................................................ 29

2.4. Schematic of a three-level and four-level laser .................................................. 38

2.5. Timing errors associated with leading edge discriminators .............................. 56

2.6. Schematic diagram of the function of a constant fraction discriminator .............. 57

3.1. Decay profile for IR-125 showing the RLD method for lifetime determination .................................................................................................................. 70

3.2. Structures of the near-IR commercial dyes, DTTCI, IR-132, and IR-125 ............. 74

3.3. Diagram of our near-IR TCSPC instrument ....................................................... 75

3.4. Decay profiles for instrumental response, DTTCI, IR-132, and IR-125 .................. 79

3.5. Calculated lifetimes as a function of the background-to-fluorescence ratio for the near-IR dyes DTTCI (diamonds), IR-132 (squares), and IR-125 (circles) via the MLE (closed symbols) and the RLD (open symbols) ............................................................................. 81

3.6. Calculated fluorescence lifetimes for the dyes IR-125 (circles) and DTTCI (squares) as a function of the start channel in the calculation at a B/F ratio of 50% via the MLE (open symbols) and RLD (closed symbols) ............................................. 85
3.7. Decay profiles for IR-125 and DTTCI at [DYE] = 5 \times 10^{-12} \text{M} and 1 \text{s integration period} ..............................................................................................................86

4.1. Chemical structures of Hexamethylindotricarbocyanine iodide (HITCI), IR-140, and IR-144......................................................................................................................102

4.2. Free solution capillary electropherogram and the associated decay profiles for DTTCI and IR-125..................................................................................105

4.3. Capillary electropherogram of six near-IR fluorescent dyes with peak identification via fluorescence lifetime matching.............................................................................................114

4.4. Decay profiles for the six near-IR dyes in Figure 4.2........................................................................115

4.5. CE separation of three cationic near-IR dyes (a) and lifetimes (b) as a function of the integration time ................................................................................................................117

4.6. Decay profiles for IR-140 only at various integration times corresponding to t_1, t_2 and t_3 from Figure 4.5...........................................................................118

4.7. Capillary gel electropherogram of C-terminated DNA fragments (a) and an expanded view (b) showing the integration times over which the decay profiles were accumulated. In (c) is shown the decay profile for dye-labeled oligonucleotides in the detection zone (circles) and of the gel only (squares)........................................................................121

5.1. Diagram of our diode-based near-IR TCSPC instrument..................................................................................131

5.2. Block diagram of the electronics for the pulsed diode laser and the TCSPC board..........................................................................................................................132

5.3. Chemical structures and properties of near-IR dye-labeled primers.................................................................................................................................136

5.4. Capillary gel electropherogram of dye-labeled DNA primers (a) and time-resolved decay profiles (b) of the gel matrix, IRD40 and IRD41 dye-labeled primers. ........................................................................138

5.5. Steady-state fluorescence intensity electropherogram of A- and C-terminated DNA fragments separated by capillary gel electrophoresis........................................................................142

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
5.6. Time-resolved decay profiles for the gel matrix and two bands taken from Figure 5.5, which are indicated with an anterisk ................................................................. 143

5.7. Expanded view of the intensity electropherogram shown in Figure 5.5 with bases identified via lifetime discrimination .............................................................................. 145

5.8. Time-resolved decay profiles for a series of 2 s electrophoresis intervals as collected by the TCSPC board ................................................................................................... 146

5.9. Expanded view of Figure 5.5 showing the inability of the counting board to effectively integrate over only one peak in the electropherogram .................................................. 148

6.1. Flowchart of the computer program for Monte Carlo simulations ................................................................. 154

6.2. Simulated electropherograms containing two generated Gaussian peaks (resolution = 0.53) with the lifetimes in 1 channel (0.2 s) intervals calculated via the MLE method ..................................................................... 162

6.3. Simulated electropherograms containing two generated Gaussian peaks (resolution = 0.53) with the lifetimes in 1 channel (0.2 s) intervals calculated via the RLD method ..................................................................... 163

6.4. Simulated electropherograms containing two generated Gaussian peaks (resolution = 0.53) with the lifetimes in 10 channel (2 s) intervals (t1, t2, t3) calculated via the MLE (circles) and RLD (squares) method ..................................................................... 167

6.5. Decay profiles from times t1, t2, and t3 from Figure 6.3 ................................................................. 170

6.6. Simulation of two generated Gaussian peaks with a resolution of 0.23 .................................................................................................................. 172

6.7. Simulation of two generated Gaussian peaks with a resolution of 1.0 .................................................................................................................. 173

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
ABSTRACT

The research presented in this dissertation involves the construction of a near-infrared time-correlated single photon counting system which utilizes a mode-locked Ti:sapphire or pulsed diode laser as the excitation source and single photon avalanche diode as the photodetector and the subsequent study of two simple algorithms, the Maximum Likelihood Estimator (MLE) and the Rapid Lifetime Determination (RLD) methods, for fluorescence lifetime determination. The purpose of this research is to aid in the development of a low cost, highly efficient, and high throughput device for DNA sequencing which utilizes near-IR fluorophores which have similar absorption and emission properties but possess unique fluorescence lifetimes, which will allow the identity of the terminal base to be accomplished by lifetime discrimination.

The performance of the MLE and RLD for measuring nanosecond and subnanosecond fluorescence lifetimes were evaluated in static solutions in the limits of low concentrations and high backgrounds from scattered photons generated from the solvent using experimental and Monte Carlo simulation results. The conclusions from these results were used in the on-line determinations of fluorescence lifetimes of components separated via capillary electrophoresis. For example, a capillary gel electrophoretic separation of near-IR dye-labeled C-terminated fragments was done with decay profiles of the separated fragments collected. The MLE lifetime

xi
value for decay profiles collected from 35 individual peaks was $581 \pm 9$ ps, which agreed with the high concentration value ($\tau_f = 581$ ps). Also, a pulsed-diode laser based instrument with the counting electronics situated on a PC board was constructed and its performance evaluated by a CGE separation of A- and C-terminated DNA fragments. The results indicated lifetime values of $669 \pm 42$ ps and $528 \pm 68$ ps for the A-terminated and C-terminated fragments, respectively, for the MLE.

In order to study the effects of peak resolution on the fluorescence lifetime determination, Monte Carlo simulations were used to study the effect of electrophoretic resolution between peaks on the calculated lifetime using both the MLE and RLD algorithms. In addition, accuracy and precision were analyzed for the calculated lifetimes during the electrophoresis as a function of integration time.
CHAPTER 1

Near-Infrared Dye-Labeled Oligonucleotide Fragments Separated by Capillary Gel Electrophoresis and Detected by Laser Induced Fluorescence for Sequencing Applications

1.1. Introduction.

There is a tremendous effort, known as the Human Genome Project (HGP),\(^1\) to sequence the entire code of the human genetic material in a reasonable time frame, with high efficiency (> 99.9\%) and at an economical cost. To put the project in perspective, Venter and his group sequenced the \(H.\text{Influenza}\) virus, which is composed of 11.8 Mbp using 14 commercially available automated DNA sequencers.\(^2\) The sequencing required 13 months and costs exceeding $300,000 in reagents alone. Therefore, using the same manpower and technology, sequencing the human genome, which is comprised of three billion nucleotide bases, would require 275 years at a cost of $82.5 million for reagents only. Therefore, a primary goal of the HGP is to develop new technologies in which cost reduction and increased speed and high accuracies in the base-calling are priorities. Also, automation of the sample handling and preparation and multiplexing the electrophoresis devices are important in building a high throughput DNA sequencing device.

DNA sequencing involves five major steps; (1) preparation of the DNA template whose sequence is to be determined; (2) construction of the DNA sequencing ladders using Sanger chain termination methods; (3) separation of the constructed DNA ladders; (4) calling or identifying the bases during the
electrophoresis and; (5) reassembly of the DNA sequence. For the preparation of
the DNA template, the genomic DNA is digested into smaller fragments and then
amplified via recombinant or polymerase chain reaction (PCR) techniques. After
amplification, a series of oligonucleotides, which differ in the number of bases, are
prepared using a chain termination method developed by Sanger. In this
approach, deoxynucleotides and dideoxynucleotides are mixed with a polymerase
enzyme and the DNA molecule whose sequence is to be determined. Since the
dideoxynucleotides lack a -OH group at the 3'-position of the deoxyribose sugar
unit, once incorporated, the chain extension ceases. The result is the production
of a series of oligonucleotides with a common terminal base, adenine, thymine,
cytosine, or guanine. After these have been synthesized, the DNA fragments are
separated by gel electrophoresis, which can be performed by using a slab gel or
capillary gel format. The next step is to identify the terminal bases during the
electrophoresis and the common method is to use laser-induced fluorescence with
a unique spectral marker for each of the four nucleotide bases. The final step is to
reassemble the various pieces of sequenced DNA into a continuous strand.

1.2. Separation Methodologies.

Modern applications for DNA sequencing involve electrophoretic
separations, in which charged molecules are separated by their movement through
a fluid or gel media under the influence of an applied electric field. There are two
major types of electrophoretic techniques for DNA separations; the traditional slab
gel electrophoresis (SGE) method and capillary gel electrophoresis (CGE). The separation is based upon charge and mass differences in the analytes. Capillary gel electrophoresis offers many distinct advantages as a separation technique over slab gel electrophoresis, including speed, quantitation, reproducibility and automation. However, slab gel is more conducive to multiplexed operation since many samples can be run on a single gel.

1.2.1. Slab Gel Electrophoresis.

Slab gel electrophoresis (SGE) involves the separation of charged molecules within a semisolid gel matrix which is sandwiched between glass plates and inserted into a Plexiglass chamber. The gel in DNA sequencing applications serves as a sieving medium, which differentiates the DNA fragments according to size. The gel composition is adjusted to define specific pore sizes, each for a nominal range of molecular sizes. A carrier buffer is also required for electrophoresis, which maintains the requisite pH and provides sufficient conductivity to allow the passage of current necessary for the separation.

The gel, such as polyacrylamide or agarose, serves many important functions other than acting as the sieving mechanism, including the reduction of diffusion and convection which decreases band-broadening and physical stabilization. The production of heat, known as Joule heating, by the applied electric field induces conductive movement of the electrolyte buffer. This movement results in band-broadening that reduces the efficiency of the separation.
Joule heating arises from the frictional collisions between mobile ions and buffer molecules and fortunately the gel helps to minimize these effects. Three problems that can result from Joule heating are temperature changes due to ineffective heat dissipation, development of thermal gradients across the capillary, and breakdown of the gel matrix.

The viscous gel inhibits the movement of the buffer in the electric field. Since the separation involves a highly viscous gel, molecular diffusion is reduced as well, further enhancing the efficiency of the separation. The gel also must be sufficiently viscous to provide physical support. Low viscosity solutions or gels would flow if the plates were not held horizontal.

1.2.2. Capillary Gel Electrophoresis.

CGE operates under the same principles as does SGE, except that the separation is performed in a capillary instead of between glass plates. Figure 1.1 shows a simple block diagram of a typical CGE instrument. The separation is performed in a gel-filled narrow bore capillary tube with an inner diameter range of 5-100 μm. The ends of the capillary are immersed in two reservoirs, one held at a high voltage (cathode) and the other at ground (anode). For DNA separations, the dye-labeled fragments are injected at the cathode. The detection is then carried out on-column.
Figure 1.1. A simplified block diagram of a CGE system for DNA analysis.
In electrophoretic separations, the apparent electrophoretic mobility, $\mu_{app}$, can be calculated from:

$$\mu_{app} = \frac{L_d L_t}{tV}$$

(1.1)

where $L_d$ is the length of the capillary from injector to detector (cm), $L_t$ is the total length of the capillary (cm), $t$ is the migration time (s), and $V$ is the applied voltage (V).

The efficiency in CGE is determined by calculating the number of theoretical plates ($N$) generated during the separation and can be determined by:

$$N = \frac{\mu_{app} E^2 t}{2D}$$

(1.2)

where $E$ is the separation field strength (V/cm), $t$ is the migration time (s), and $D$ is the diffusion coefficient (cm²/Vs). In CGE applications involving DNA sequencing, the efficiency of the separation is very high due to the high viscosity gel matrix and the inherently small diffusion coefficient associated with DNA. It is not atypical to see plate numbers approaching $10^6$/$m$ in DNA separations via CGE. As can be seen from Equation 1.2, increases in $E$ can increase $N$. While $N$ increases with the square of $E$, at certain field strengths, excessive Joule heating is produced in the column which can destroy the gel matrix as well as lower the
plate numbers. However, higher field strengths can be used in CGE applications compared to SGE because the Joule heat dissipation is more effective in CGE due to the greater surface area to volume ratio.

Driven by the electric field, the DNA fragments migrate through the gel toward the appropriate electrode. The resolution in the separation is determined primarily by the size of the pores, since DNA fragments with base lengths > 20 have the same free solution electrophoretic mobility. Since the gel networks contain pores of different sizes, the DNA is separated via differences in the number of base pairs within the oligonucleotide. One of the most common gel matrices used to separate DNA is polyacrylamide because it is electrically neutral and, under appropriate preparation conditions, contains pore sizes sufficient for DNA fractionations from 10-10000 bases in length. The gel composition is designated by the %T, the total amount of acrylamide and %C, the amount of crosslinker (bisacrylamide). The %T is calculated from:

\[
%T = \frac{\text{acrylamide}(g) + \text{bisacrylamide}(g)}{100 \text{ ml}} \times 100\% \tag{1.3}
\]

and %C is:

\[
%C = \frac{\text{bisacrylamide}(g)}{\text{bisacrylamide}(g) + \text{acrylamide}(g)} \times 100\% \tag{1.4}
\]
The pore size is controlled by the %T and %C. The selection of %T is based on the molecular size to be separated, with low %T useful for long DNA lengths and high %T giving enhanced separation efficiency for short DNAs. Typical gel conditions for separation of DNA fragments for sequencing are 3%T/3%C polyacrylamide gel matrix.\textsuperscript{5}

1.3. Detection.

Fluorescence as a detection scheme in DNA sequencing was first demonstrated by Smith et al.\textsuperscript{7,8}, Ansorge et al.\textsuperscript{9}, and Prober et al.\textsuperscript{10} Due to its ease of automation, sensitivities comparable to autoradiography, and the ability to provide real time detection during the acquisition of the electropherogram, fluorescence has become the detection methodology of choice for large-scale genomic sequencing. For DNA sequencing involving fluorescence detection, the fluorescent dyes are attached to either the 5'-end of a sequencing primer or to a chain terminating dideoxynucleotide at a non-hydrogen bonding site. In all cases, labeling of the oligonucleotide with a fluorescent tag is required due to the poor intrinsic fluorescence quantum yields associated with the nucleotide bases and the need for deep UV excitation. The fluorescent tags that are typically associated with DNA sequencing applications are fluorescein or rhodamine derivatives, since they possess absorption maxima which match with the 488 nm and 514 nm lines of the Ar ion laser and favorable quantum yields.
1.4. DNA Sequencing Strategies.

For base-calling in DNA sequencing applications, numerous strategies have been implemented to determine the terminating nucleotide base of DNA fragments separated via CGE using standard Sanger dideoxy chain-terminating methods and fluorescence detection. One method is to use a single chromophore covalently linked to the 5'-end of the sequencing primer followed by four sequential electrophoretic separations. The four electropherograms can then be overlaid and the sequence of the template can be determined. The difficulty in this approach when implementing CGE is that gel breakdown during each run may result in mobility shifts, making it difficult to effectively overlay sequential CGE separations. Also, this method reduces the throughput of the system due to the time required to perform four sequencing runs.

A single chromophore detection scheme employing a single electrophoretic run was accomplished by Tabor et al.\textsuperscript{11,12} In this method, a single dye-labeled primer is used along with the T7 DNA polymerase enzyme. This enzyme incorporates dideoxynucleotides (ddNTPs) uniformly, therefore, the terminal nucleotide can be determined by adjusting the molar ratios of the ddNTPs.\textsuperscript{13,14} Base calling is then accomplished by monitoring the fluorescence intensity of the electrophoretic peaks.\textsuperscript{15,16} The base-calling accuracy of this technique has been determined to be 90% to read lengths 250 bases from the primer.

Another method for base-calling involves the use of four different labels which possess distinct spectral properties. The advantage of this scheme is the
need for only a single separation lane, however, the difficulty with this approach results from the need for four separate detection channels and in some cases, the need for multiline laser excitation, which can add complexity and cost to the system.

1.5. Research Focus.

Our approach for base-calling in DNA sequencing involves the use of a single-lane CGE separation of near-infrared (near-IR) dye-labeled DNA fragments with the terminal bases identified via fluorescence lifetime discrimination. This method employs the use of heavy-atom modified tricarbocyanine dyes which possess similar absorption and emission maxima as well as similar electrophoretic mobilities, but they have unique fluorescence lifetimes. The chemical structures along with their corresponding fluorescence lifetimes of these tricarbocyanine, near-IR dyes which we have prepared are shown in Figure 1.2.

Near-IR fluorescence has recently been shown to be an attractive alternative to visible fluorescence in a number of bioanalytical applications, such as high performance liquid chromatography, fluoroimmunoassays, and free solution capillary electrophoresis. Near-IR detection offers many advantages over visible detection including the intrinsically smaller backgrounds associated with this region of the electromagnetic spectrum due to the fact that few molecules fluoresce in the near-IR and smaller Raman cross sections. In addition, near-IR
fluorescence allows the use of simple instrumentation to carry out the detection\textsuperscript{28-31} such as diode lasers and avalanche diode detectors.

The dyes typically used in near-IR applications are the tricarbocyanine dyes. This family of dyes possess heteroaromatic fragments linked by a polymethine chain. A decisive drawback of tricarbocyanine dyes is the intrinsically short upper state lifetime, which can range between 200-1000 ps, and the poor photophysical properties displayed by these dyes in predominately aqueous solvents\textsuperscript{29,32}. The short lifetimes place certain criteria on the instrumentation needed for the measurement, including subnanosecond pulses associated with the excitation source and a short transit time spread associated with the detector.

The dye series synthesized in our lab for this application contain a heavy-atom modification, which enhances the intersystem crossing rate through spin-orbit coupling, but does not alter the absorption and emission properties of the dye. However, since the fluorescence lifetime is perturbed through this modification, a set of dyes can be determined through lifetime discrimination. An advantage of lifetime discrimination over spectral discrimination is that the lifetime values are independent of concentration differences, which can cause problems in spectral discrimination methods. Additionally, simple algorithms for the calculation of fluorescence lifetimes can be implemented for base calling in the limit of low photocounts (i.e., low concentration).
Fluorescence lifetime (ps)

H     H     906
I     H     906
Br    H     882
Cl    H     858
F     H     831
Br    Br    702
Cl    Cl    708

* Lifetimes were calculated using a nonlinear least squares algorithm.

Figure 1.2. Chemical structures of tricarbocyanine, near-IR heavy-atom modified dyes along with the calculated lifetimes.
The focus of this work was the development of a capillary electrophoresis system which incorporated a near-infrared laser-induced time-correlated single photon counting detection system for the detection of dye-labeled oligonucleotide fragments. For calculation of the fluorescence lifetimes from experimental data, two simple algorithms, the maximum likelihood estimator and the rapid lifetime determination methods, were evaluated on near-IR fluorescent dyes in the limits of low concentrations and short integration times in static solutions and capillary electrophoretic separations and were compared to Monte Carlo simulation results. In addition, the ability to determine fluorescence lifetimes in capillary gel electrophoretic separations of near-IR dye-labeled oligonucleotide fragments was determined with base identification accomplished via fluorescence lifetime discrimination. Since there is the possibility of peak overlap in DNA separations, Monte Carlo simulations were also performed on generated Gaussian peaks to study resolution effects on the calculated lifetime.

1.6. References.


CHAPTER 2

Time-Correlated Single Photon Counting: An Overview

2.1. Introduction.

Fluorescence emission contains both spectral and temporal information, with the former being traditionally associated with routine applications of fluorescence spectroscopy. Advances in fluorescence spectroscopy over the past two decades have been focused on the development and application of new time-resolved techniques. Time-resolved fluorescence spectroscopy has become an important tool for studying various photophysical phenomena in chemistry and biochemistry, including the structure and dynamics of proteins, rotational diffusion in restricted environments, and excited state proton transfer reactions. In addition, fluorescence lifetime determinations have been used in many analytical applications such as liquid chromatography, fluorescence microscopy, determination of adsorption modes on chromatographic stationary phases, and measurements of fluorescence lifetimes for single molecular events. There are many advantages of time-domain measurements as compared to steady-state techniques, with the major advantage being that fluorescence measurements in the time-domain possess information about the reaction rates of intra- and intermolecular processes. In addition, fluorescence lifetime measurements provide a method to probe the local environment of the fluorophore. In addition, under
appropriate conditions, lifetimes can be determined with higher precision than intensity-based measurements.

2.1.1. Fluorescence.

Fluorescence emission involves the absorption of a photon of light and subsequent depopulation of the excited state through the release of a photon. Figure 2.1 shows a Jablonski diagram which describes fluorescence and other processes which depopulate an electronic state. The absorption of a photon excites a molecule from the ground singlet state ($S_0$) into a vibrational state of an excited singlet state ($S_1$). The absorption rate occurs on the order of $10^{-15}$ s. Relaxation processes from $S_1$ competing with fluorescence are intersystem crossing, internal conversion, and several different types of photochemical reactions. Internal conversion involves the relaxation from the lowest vibrational level of $S_1$ to an excited vibrational energy state of $S_0$. Relaxation via intersystem crossing involves the transition from the lowest vibrational state of $S_1$ to the first excited triplet state ($T_1$). While the transition to the triplet state is a quantum mechanically spin-forbidden transition, spin-orbit coupling relaxes this restriction. From the triplet state, relaxation to the singlet ground state may occur through a radiative process, phosphorescence, or a nonradiative process. The main process that typically competes with fluorescence is intersystem crossing to the triplet state.\textsuperscript{14} Vibrational relaxation is usually complete before electronic relaxation occurs since this process occurs in $\approx 10^{-12}$ s.
**Figure 2.1.** Jablonski diagram showing radiative and nonradiative pathways for molecular relaxation.
In quantum mechanical terms, fluorescence can be described as the electric dipole transition from an excited singlet state to a lower singlet state, usually the ground state, through the release of energy in the form of a photon. Mathematically, the probability of fluorescence emission is proportional to \( R_{ij}^2 \), where \( R_{ij}^2 \), the transition moment integral between the excited state \( i \) and the relaxed state \( f \), is given by:\(^4\)

\[
R_{ij}^2 = \int_{-\infty}^{\infty} \Psi_{ef} \tilde{M} \Psi_{el} dt_e \int_{-\infty}^{\infty} \Psi_{nf} \Psi_{nl} t_n
\]  

(2.1)

where \( \Psi_e \) represents the electronic wavefunction, \( \Psi_n \) is the vibrational wavefunction, \( \tilde{M} \) is the electronic dipole moment operator and the Born-Oppenheimer principle of separability of electronic and vibrational wavefunctions has been invoked. According to the Franck-Condon factor, the electronic transitions of a molecule occur in a time frame in which the positions of the nuclei do not change, therefore, all electronic transitions are vertical. The Franck-Condon factors describing transition probabilities are usually equally probable for the absorption and emission processes; therefore, a mirror image relationship is observed between the excitation and emission spectra in fluorescence. If the mirror image relationship is not observed, there are usually geometric differences (nuclear reorganization) between the ground state and excited state of the molecule.
The steady-state fluorescence \( I_f \) signal can be expressed by,\(^{15}\)

\[
I_f = 2.303 I_0 b C \Phi_f \varepsilon F(\Theta) G(\lambda) \tag{2.2}
\]

which gives the spectral information of the fluorophore where \( I_0 \) is the incident photon flux (photons/cm\(^2\)), \( b \) is the pathlength of the sample cell (cm), \( C \) is the concentration of the fluorescent species (M), \( \Phi_f \) is the fluorescence quantum yield of the fluorescing species, \( \varepsilon \) is the molar absorptivity of the chromophore (cm\(^{-1}\) M\(^{-1}\)), \( F(\Theta) \) is the instrumental collection and transmission efficiencies of fluorescent photons, and \( G(\lambda) \) is the quantum efficiency of the detector at the observation wavelengths. This equation also provides information on how the photophysical and instrumental parameters affect the fluorescence signal. Optimization of these parameters of the chromophore would allow for increased sensitivity in the measurement.

2.1.2. Fluorescence Lifetimes.

The fluorescence lifetime of a molecule can be defined as the average relaxation time from the excited singlet state to the ground singlet state. The expression for a simple exponential decay can be described by,\(^{13,14,16}\)

\[
I(t) = \sum_{i=1}^{n} A_i e^{-\alpha_i t} \tag{2.3}
\]
where \( n \) represents the number of components in the decay, \( A \) is the preexponential factor, \( t \) is the time bin, and \( \tau_f \) is the fluorescence lifetime. Because the fluorescence lifetime is proportional to the fluorescence quantum intensity, the fluorescence lifetime can be determined experimentally by measuring the time taken for the fluorescence intensity to fall to \( 1/e \) of its initial value following the excitation of the molecule. This forms the basis of time-correlated single photon counting (TCSPC) whereby a time distribution of individual fluorescence photons are analyzed.

The use of the fluorescence lifetime, \( \tau_f \), of a molecule depends upon the radiative and nonradiative decay processes involved with the molecule and can be expressed through\textsuperscript{13,14}

\[
\tau_f = \frac{1}{k_r + k_{nr}} = \frac{1}{k_m} \tag{2.4}
\]

where \( k_r \) is the radiative rate constant (s\(^{-1}\)), \( k_{nr} \) is the nonradiative rate constant (s\(^{-1}\)), and \( k_m \) is the total decay rate constant (s\(^{-1}\)).

From steady-state analysis, the fluorescence quantum yield, \( \Phi_f \), is given by\textsuperscript{13,14}

\[
\Phi_f = \frac{k_r}{(k_r + k_{nr})} = \frac{k_r}{(k_r + k_{isc} + k_{ic} + k_d + k_{se})}. \tag{2.5}
\]
where $k_{isc}$ is the intersystem crossing rate, $k_{ic}$ is the internal conversion rate, $k_d$ is the photodecomposition rate, and $k_{se}$ is the solvent dependent rate. The $\Phi_f$ is expressed as a percentage (0 - 100%) or as a number between 0 and 1.

The fluorescence lifetime, $\tau_f$, is therefore given by:

$$\tau_f = \frac{1}{(k_r + k_{isc} + k_{ic} + k_d + k_{se})}.$$ \hspace{1cm} (2.6)

As can be seen from Equations 2.5 and 2.6, rationalization of the photophysics and photochemistry of any singlet state molecular species in terms of absolute rate constants for the various competing decay processes can not be obtained with the sole knowledge of the quantum yields.\(^{13}\)

2.2. Factors Influencing the Time-Resolved Behavior of Fluorescent Molecules.

As can be seen from Equations 2.2 and 2.6, there are many factors that influence the fluorescence and fluorescence lifetime. The fluorescent molecule’s microenvironment, such as the solvent properties of polarity and viscosity, probably plays the most important role in determining the decay kinetics.
2.2.1 Near-IR Fluorescence.

The major nonradiative pathway for most near-IR polymethine dyes is internal conversion based primarily upon the nonplanar structure of the molecule arising from a conformationally "loose" polymethine backbone linking the heteroaromatic fragments, with the efficiency of this deactivation pathway related, in part, to the degree of steric hindrance between the heteroaromatic fragments. Increases in the polymethine chain length in a series of dyes with similar structures have been shown to result in increases in the fluorescence properties of these dyes.\textsuperscript{16-18} Additionally, the restriction of the molecule to a planar conformation through covalent bridging of the heteroaromatic fragments can drastically reduce the internal conversion rate.

Another proposed method for nonradiative deactivation in carbocyanine dyes is cis/trans photoisomerization within the polymethine chain.\textsuperscript{19-21} Photoisomerization in several di- and tricarbocyanine dyes has been illustrated through observation of the transient absorption spectra originating from the photoisomer, a second component in the fluorescence decay spectrum, and a large viscosity effect on the fluorescence lifetime.\textsuperscript{16} Properties of the solvent also play a key role in determining the photophysics of the near-IR dyes such as the polarity and viscosity are responsible for spectral shifts in the absorption and emission spectra. Figure 2.2 shows the absorption and emission spectra for IR-125 in water and methanol. Note that the absorption and emission maxima are both red-shifted when the solvent is switched from water to methanol.
Figure 2.2. Absorption and emission spectra of IR-125 in water and methanol. 

\[ [\text{DYE}] = 5 \times 10^{-6} \text{ M}. \]
Additionally, Table 2.1 shows the absorption maxima, molar absorptivity, fluorescence lifetime, and fluorescence quantum yield for IR-125 in various solvent systems.\textsuperscript{16} The progressive red shift in the absorption maxima for IR-125 as a function of alkyl chain length in the alcohol series is consistent with previous research on polymethine dyes, which attributed the bathochromic shift in the absorption spectra to the nucleophilic solvation of the cationic center of the dye.\textsuperscript{16} Solvation results in a lowering of the excited state energy as Hückel calculations show that the positive charge is more localized in the excited state, making it more susceptible to nucleophilic solvation. As the alkyl chain length of the alcohols increase, the nucleophilicity of the solvent increases giving rise to the bathochromic shift. Table 2.1 also shows the effects of changing the polarity of the solvent on the fluorescence lifetime of IR-125.\textsuperscript{16} As can be seen from the table, the fluorescence lifetimes increase with increasing nucleophilicity of the solvent.

The viscosity of the medium may also play an important role in the decay kinetics. For example, Table 2.1 shows the dependence of the viscosity on the fluorescence lifetime of IR-125 in different binary glycerol/methanol mixtures. As can be seen from this data, the fluorescence lifetime does increase with increasing viscosity, indicating that conformational reorganization is involved in the nonradiative depopulation of the excited state.
Table 2.1. Absorption maxima, molar absorptivities, fluorescence lifetimes, and fluorescence quantum yields for the near-IR dye IR-125 in a various solvent systems.\textsuperscript{16}

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\varepsilon \times 10^4$ (cm$^{-1}$M$^{-1}$)</th>
<th>$\tau_f$ (ps)</th>
<th>$\Phi_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>779</td>
<td>3.01</td>
<td>$^d$</td>
<td>0.01</td>
</tr>
<tr>
<td>Methanol</td>
<td>782</td>
<td>19.53</td>
<td>470 (1.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>Ethanol</td>
<td>786</td>
<td>19.44</td>
<td>570 (1.4)</td>
<td>0.05</td>
</tr>
<tr>
<td>Proponol</td>
<td>788</td>
<td>19.52</td>
<td>690 (3.0)</td>
<td>0.06</td>
</tr>
<tr>
<td>Butanol</td>
<td>790</td>
<td>19.28</td>
<td>720 (3.0)</td>
<td>0.07</td>
</tr>
<tr>
<td>SDS</td>
<td>792</td>
<td>16.63</td>
<td>340 (1.4)</td>
<td>0.05</td>
</tr>
<tr>
<td>Triton</td>
<td>799</td>
<td>19.49</td>
<td>570 (1.6)</td>
<td>0.06</td>
</tr>
<tr>
<td>% Glycerol$^e$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td>470 (1.3)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>490 (1.3)</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
<td>500 (1.3)</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
<td>520 (1.3)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Molar absorptivity calculated using $\lambda_{\text{max}}$.

$^b$ The relative standard deviations in all fluorescence lifetime determinations ranged from 1-3%.

$^c$ The relative standard deviations in all the quantum yield measurements was found to be 10-20%, as determined by three replicate measurements in each solvent system.

$^d$ Lifetime could not be determined with the resolution of the near-IR TCSPC instrument.

$^e$ Measurements performed at 25 °C.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
2.3. Methods for Measuring Fluorescence Lifetimes.

2.3.1. Phase-Resolved Spectroscopy.

Phase or modulation spectroscopy incorporates a modulated excitation source such that the finite fluorescence lifetime of the sample causes the fluorescence emission waveform to be phase-shifted and of different amplitude when the signal is demodulated. If a sinusoidally modulated excitation signal, \( E(t) \), is used, a modulated fluorescence signal, \( F(t) \), is produced in the form of:

\[
E(t) = D_{\text{ex}} + A_{\text{ex}} \exp(\imath \omega t),
\]

is used, a modulated fluorescence signal, \( F(t) \), is produced in the form of:

\[
F(t) = D_F + A_F \exp(\imath \omega t + \phi).
\]  

where \( D_{\text{ex}} \) is the dc intensity component of the excitation beam, \( D_F \) is the dc intensity component of the fluorescence signal, \( A_{\text{ex}} \) is the amplitude of the excitation signal, \( A_F \) is the amplitude of the fluorescence signal, and \( \omega \) is the angular frequency (\( \omega = 2\pi f \) where \( f \) is the frequency). By substitution of Equation 2.8 into Equation 2.3, the phase relationship is given by:

\[
\tan \phi = \omega \tau
\]
and the expression for the demodulation factor is:

\[ m = \frac{A_F}{D_F} / \left( \frac{A_{Ex}}{D_{Ex}} \right) = \frac{1}{(1 + \omega^2 \tau^2)^{1/2}} \]  \hspace{1cm} (2.10)

Traditionally, phase spectroscopy has been carried out using electro-optic (Kerr or Pockels cell) or acousto-optic modulation of continuous wave lamp or laser excitation. The upper limit of the modulation frequency is important in determining the shortest lifetime which can be measured. Current modulation methods operate at a maximum frequency of approximately 200 MHz allowing \( \tau \) values as short as 1-100 ps to be measured.\(^{23,24} \)

2.3.2. Time-Resolved Spectroscopy.

Time-resolved spectroscopy involves the excitation of a molecule with a narrow pulse of light and subsequent measurement of the time interval of the emission from the excited state. In time-correlated single photon counting (TCSPC), the time evolution of individual photon events are processed and upon processing many such events, a histogram can be constructed which represents the decay kinetics of the excited state. The resolution and the precision of the lifetime determination is dictated by the width of the excitation pulse, the reproducibility of the pulse, and the instrumental response of the photodetector.

Figure 2.3 shows a typical layout of a time-correlated single photon counting instrument. The instrument consists of a pulsed light source, typically a

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 2.3. Block diagram of a TCSPC device: CFD, constant fraction discriminator; TAC, time-to-amplitude converter; MCA, multichannel analyzer.
mode-locked laser, which generates an excitation pulse train which stimulates absorption in the sample molecules. At low levels of excitation power, each sample molecule absorbs one photon at most, on a time scale which is effectively instantaneous. The subsequent relaxation of the molecules from the excited state to the ground state via the emission of fluorescence photons occurs with a distribution of time delays as described by Equation 2.3. The single photon counting technique records this distribution by measuring the time delays of the individual fluorescence photons with respect to the arrival time of the excitation pulse. The delay time between the excitation pulse and the fluorescence photon from the sample reaching the detector, typically a photomultiplier tube (PMT) or a single photon avalanche diode (SPAD), is measured by a time-to-amplitude converter (TAC). The excitation pulse serves as the “start” signal for charging a capacitor within the TAC. The voltage on the capacitor increases linearly until a “stop” fluorescence photon is detected. The “start”-“stop” time interval generates a proportional voltage across this capacitor. The voltage pulse is stored based upon the amplitude using an analog-to-digital converter (ADC) within a multichannel analyzer (MCA) and thereby placed into the appropriate channel number. On repeating the “start”-“stop” cycle many times, a histogram is formed representing the fluorescence decay profile. Discriminators are used to aid in removing random noise pulses resulting from background photons and to ensure that the timing definition of the “start” and “stop” pulses is largely independent of the signal pulse height.
TAC operation requires the ability to register the first “stop” pulse detected after a “start” pulse. Accordingly, the “stop” pulse rate must be low enough so that the probability of detecting more than one photon is negligible. In effect, the “start” pulse rate must be much greater than the “stop” pulse rate. Failure to adhere to this means that the TAC will preferentially detect photons which occur at shorter times, and the fluorescence decay time distribution will be biased, resulting in decay times that will appear to be faster than it really is. This photon pileup effect makes the single photon-counting technique inefficient when the fluorescence signal is intense due to the fact that the available signal has to be wasted in order to ensure the absence of photon pileup.

The time-correlated single-photon counting technique is a digital rather than an analog technique as is the case for phase-resolved methods. Advantages of TCSPC include a high dynamic range and the measured decay profile is independent of fluctuations in the excitation pulse intensity. Moreover, single photon detection theory is based upon well documented statistics for which the precision, data weights, goodness-of-fit, etc., can be easily calculated.

If, following one excitation pulse, the recording of a coincident event in channel $i$ corresponds to an average number of photons, $\bar{n}_i$, reaching the photodetector and liberating an average number of $\bar{x}_i$ photoelectrons, then$^{13,14}$

$$\bar{x}_i = \bar{n}_i q$$

(2.11)
where \( q \) is the photodetector quantum efficiency. If there are a large number of excitation pulses for every count registered in channel \( i \), then the probability, \( P_x(i) \), of liberating other than \( \bar{x}_i \) photoelectrons is given by the Poisson distribution:\(^{13,14}\)

\[
P_x(i) = \frac{(\bar{x}_i)^x e^{-\bar{x}_i}}{x!}
\]

(2.12)

with

\[
\sum_{x=0}^{\infty} P_x(i) = 1.
\]

(2.13)

The probability of at least one photoelectron pulse detected per excitation pulse can be determined via,\(^{13,14}\)

\[
P_{x \geq 1}(i) = 1 - e^{-\bar{x}_i} = (1 - \bar{x}_i + \frac{x^2}{2} + ...)
\]

(2.14)

when, at low excitation powers, \( \bar{x}_i \gg \bar{x}^2/2 \), gives the following relationship;

\[
P_{x \geq 1}(i) \approx \bar{x}_i \approx \bar{n}_i q.
\]

(2.15)

Therefore, under these conditions, the probability of detecting a coincident fluorescent photon in channel \( i \) is proportional to the fluorescence intensity at a delay time \( t \). This is the general condition for accurate single photon counting.
measurements. Moreover, the probability of detecting two fluorescence photons per excitation pulse is further diminished provided that:

\[ \frac{S_p}{S_t} \leq 0.01 \] (2.16)

where \( S_p \) is the photodetector pulse due to the fluorescence photons over all delay times and \( S_t \) is the repetition rate of the excitation source. For pulsed excitation, the number of counts, \( Y_i \), accumulated in channel \( i \) after excitation, in a specified measurement time \( T \), for a single exponential decay time \( \tau_f \), can be determined by:

\[ Y_i = \alpha S_i T \frac{\Delta t}{\tau_f} e^{-\frac{\Delta t}{\tau_f}} \] (2.17)

where \( \Delta t \) is the time difference between the detected photon and the excitation pulse. Equation 2.17 provides another inherent advantage of the single photon counting method, where the measurement precision can be enhanced by increasing the run time of the experiment, thus allowing more photons to accumulate within the decay profile.

2.3.2.1. TCSPC Instrumentation.

The two most important criteria to determine the overall system performance of a TCSPC device are the timing resolution and the sensitivity of the
device. These characteristics vary from system to system and are based largely upon the performance of the individual components of the device, with the choice of detector usually dictating the overall response of the system. A TCSPC system typically includes the following components: a pulsed light source such as a flashlamp or mode-locked laser, a photodetector, such as a PMT or SPAD, and the counting electronics, including the CFD, TAC, and the multichannel analyzer with an ADC.

2.3.2.1.1. Light Sources.

There are basically three choices for pulsed excitation in TCSPC measurements. The choices are flashlamps, storage ring or synchrotron sources, or mode-locked lasers. Flashlamps and mode-locked lasers are the most commonly used. Since the experiments in this document utilize a mode-locked laser as the excitation source, a detailed description of lasers is given here.

In general, a laser, which is an acronym for light amplification by the stimulated emission of radiation, is an optical oscillator which creates a very highly directed (coherent) beam of light at a precise wavelength or frequency. There are three important components associated with all lasers, which include the high reflector, the gain medium, and the output coupler.

For lasing to occur, the light passing through the gain medium will be amplified. The high reflector at one end of the laser and the output coupler serves as the laser cavity, in which the amplified light will return through the gain medium.
for further amplification. The output of the laser occurs when a fraction of the light is transmitted through the output coupler.

In order for lasing to occur, there are three types of energy exchanges needed: absorption, spontaneous emission, and stimulated emission. All three rely on the transitions from one energy level to another within the gain medium, with the difference between the two energy levels given as $\Delta E$.

Absorption of a photon of energy $\Delta E$ involves the promotion of the molecule of the gain medium from the ground state ($E_1$) to the excited state ($E_2$). Upon excitation, the molecule can relax back to $E_1$ via release of a photon of energy $\Delta E$ by either spontaneous or stimulated emission. Spontaneous emission is necessary for initiating lasing, but this process does not have the directional properties of stimulated emission and therefore represents a loss in the laser cavity.

In stimulated emission, an incident photon interacts with a molecule in $E_2$ and induces the emission of a photon with a transition to $E_1$, therefore the energy of the emitted photon is $\Delta E$. Stimulated emission is required for lasing to occur. The photons generated by stimulated emission have two important properties; the first is that the photons have the same direction as the incident radiation and the second is that the photons are in phase with the excitation light, which is responsible for the coherent nature of laser light. Both absorption and stimulated emission initially require the presence of radiation and have rates proportional to the radiation density, $\rho_v$ (J s/m$^3$), at frequency $v$. The first order rates of absorption and stimulated emission can be expressed by $\rho_v B_{12}$ and $\rho_v B_{21}$, respectively, where
A\textsubscript{21}, B\textsubscript{12}, and B\textsubscript{21} are referred to as Einstein coefficients for spontaneous emission, absorption, and stimulated emission.

The relationship between the three rate constants can be related via Boltzmann statistics. At thermal equilibrium, the populations $N_1$ and $N_2$ of levels $E_1$ and $E_2$ can be expressed by,

$$\frac{N_2}{N_1} = g_2 \exp\left(-\frac{E_2}{kT}\right) \frac{g_1}{g_1 \exp\left(-\frac{E_1}{kT}\right)}$$  \hspace{1cm} (2.18)

where $g_1$ and $g_2$ are the degeneracies of states 1 and 2, $k$ is Boltzmann's constant, and $T$ is the absolute temperature. Additionally, the number of transitions from level $E_1$ to $E_2$ must be equal to the number of transitions from level $E_2$ to $E_1$ at equilibrium,

$$g_1 \exp\left(-\frac{E_1}{kT}\right) \rho_\nu B_{12} = g_2 \exp\left(-\frac{E_2}{kT}\right) (\rho_\nu B_{21} + A_{21}).$$  \hspace{1cm} (2.19)

Einstein postulated that $\rho_\nu$ will increase to infinity with $T$ which leads to;

$$g_1 B_{12} = g_2 B_{21}. \hspace{1cm} (2.20)$$

Equation 2.20 implies that, at best, the rate of absorption would equal the rate of emission; therefore, light amplification does not occur. In order for light
amplification, it is necessary to create a population inversion, where the population of molecules in the excited state $E_2$ is greater than the population of the molecules in the ground state $E_1$ and is given by the expression,$^{13,14}$

$$\frac{N_2}{g_2} > \frac{N_1}{g_1}$$

(2.21)

A population inversion is generated by pumping and can only occur in systems which possess more than two levels which are involved in the lasing transition. The degree of population inversion, and therefore the efficiency of a laser, can be enhanced by either increasing the population of the upper metastable state or by decreasing the population of the lower state reached after stimulated emission. The metastable state is formed when the pump bands, or excited energy levels, of the laser rapidly relax via radiationless transitions ($\approx 50$ ns) to a longer-lived metastable state ($\approx 5$ ms). The initial nonradiative drop is called an idler transition. This can be seen in Figure 2.5 for a three-level and a four-level laser.$^{13}$

A three-level laser functions by the excitation of the medium to the pump bands, relaxation to a metastable state via an idler transition, and then stimulated emission of a photon of energy $hv$ for relaxation to the ground state. The major drawback of three-level laser systems is that the only means of depopulating the lower level is by the same relatively inefficient pumping process; thus to excite more molecules, additional pumping energy is needed. Some molecules can be pumped to the higher energy level, but a large number will remain in the ground
Figure 2.4. Schematic of a three-level and four-level laser.\cite{13}
A high number of atoms in the ground state diminishes the population inversion and leads to losses due to absorption of the laser beam.

Most lasers, however, depend on transitions from the metastable state to another short-lived energy level which is still higher in energy than the ground state and are called four-level lasers. Molecules in the lower short-lived energy level of the lasing transition quickly decay to the ground state by another idler transition. This greatly increases the degree of population inversion, and therefore the efficiency of the laser without expenditure of pumping energy.

In the optical resonator of a laser, the light travels back and forth between the output coupler and the high reflector mirror through the gain medium, reinforcing itself to the form of a standing wave with nodes at the surface of each mirror which has the form of:

$$E_a(t)=E_0\cos\omega t \sin(ks)$$  \hspace{1cm} (2.22)

where $E_0$ is the maximum amplitude, $\omega$ is the angular frequency (radians/s) and is equal to $2\pi v$, where $v$ is the frequency, $t$ is the time (s), and $k=2\pi/\lambda$ is the propagation constant. The points of maximum amplitude as well as the nodes between them of the light wave, do not move.

Since the resonator supports waves with an integral number of half wavelengths between the mirrors in the laser cavity of length $L$, resonance occurs only at discrete wavelengths, $\lambda_n$, given by:
\[ \lambda_n = 2L/n \]  

(2.23)

where \( n \) is an integer. This is called the longitudinal resonance condition. The wavelengths which oscillate in the laser are called the longitudinal modes. Only those wavelengths that satisfy Equation 2.23 will be amplified in the laser cavity. The frequency of light \( \nu_n \) is equal to \( c/\lambda_n \), can be expressed by;

\[ \nu_n = nc / 2L \]  

(2.24)

and the frequency difference, \( \Delta \nu \), between the longitudinal modes can be determined through;

\[ \Delta \nu = \nu_{n+1} - \nu_n = c/2L. \]  

(2.25)

The longitudinal modes of a laser are very close in wavelength, and typically the more longitudinal modes oscillating within the laser, the narrower the pulse widths that can be obtained.

Lasers will oscillate in different transverse modes as well, designated by the term \( \text{TEM}_{ij} \) (transverse electromagnetic), where \( i \) and \( j \) specify the number of nodes in two directions. These transverse modes are apparent in the cross-section of the oscillating light. Operation in \( \text{TEM}_{00} \) exclusively reduces the overall
intensity of the laser, since the higher order modes have wider spatial distributions and can make use of more of the population inversion in the lasing medium. The intensity across the TEM$_{00}$ mode is Gaussian in shape, permitting the focusing of higher intensities into smaller volumes.

It was mentioned that the formation of a pulse for TCSPC by a laser was formed by a process known as mode-locking. Laser cavities can support numerous longitudinal modes, each at different frequencies as determined by Equation 2.24. For a mode-locked laser, a periodic loss is introduced into the cavity by some modulator. This modulator acts as a shutter, opening once every round trip for the light in the resonator. When the modulator is fully open, the light can pass and aligns the phases of the different longitudinal modes with one another. When the amplitudes align, constructive interference reinforces the intensity of the pulse. Between the pulses, destructive interference eliminates the light. There are two ways to mode-lock a laser, either by passive mode-locking or active mode-locking.

Passive mode-locking involves the addition of some material or mechanism within the laser cavity that would automatically open to allow light pulses through and subsequently close otherwise. Passive mode-locking involves the pulse formation by using the properties of the light itself.

Active mode-locking involves the addition of an optical shutter within the laser cavity. In order to initiate the pulse, an optical shutter is opened, closed, and then reopened at precisely the same rate to allow a pulse of light to pass through
the shutter as it passes back and forth between the output coupler and high reflector. Only light that arrives at precisely the correct time is allowed to pass through without being blocked will be amplified. In other words, the modulator frequency must be precisely equal to the reciprocal of the round trip time in the laser cavity.

For the excitation source used in the TCSPC measurements in this document, two laser systems were used. The first was a passively mode-locked Ti:sapphire laser pumped by the all lines output of an Argon ion laser and the second was an actively mode-locked Gallium-Aluminum-Arsenide (GaAlAs) semiconductor laser. The Ti:sapphire laser and the Ar ion laser are both four-level laser systems, while the diode laser is a three-level laser system.

2.3.2.1.1. The Ti:sapphire Laser.

The Ti:sapphire laser (Coherent Model 900 Mira) pumped by an Ar laser (Coherent Innova 300) is a mode-locked ultrafast laser system that uses a Ti:sapphire crystal (Ti:SiO$_3$) as the gain medium for the near-IR and is tunable from 720 to 990 nm. The repetition rate of the laser is 76 MHz, producing a round trip transit time of light pulses within the cavity of 13.2 ns.$^{25}$

For the Ti:sapphire laser system to operate for TCSPC experiments, light pulses must be generated. The Ti:sapphire accomplishes this through passive mode-locking, in which the optical properties of light were used to form the narrow mode-locked pulses. More specifically, the beam diameter at certain
locations within the cavity is large when the laser is operating in continuous mode but diminish when the laser is producing the high intensity mode-locked pulses. The addition of a slit with a certain width before the output coupler introduces a loss to the large diameter laser beam associated with continuous operation. A high intensity beam, such as that for the mode-locked pulses, will pass unhindered through the slit since this beam is smaller.

In order to change the beam diameter in the presence of high intensity pulses, the electric fields associated with the light can distort the atoms to produce a mode-locking mechanism by altering its index of refraction forming a Kerr lens, which is a gradient lens. The Kerr lens is only formed if the intensity of light is extremely high therefore, the intensity of the mode-locked pulses are sufficient to form this lens and the weak intensity of the continuous mode is not. The lens is formed only upon the arrival of a mode-locked pulse, thus, the mode-locked beam is narrowed as compared to the continuous beam.

The Ti:sapphire laser operates in a TEM$_{00}$ mode, and the output pulses are nearly transform limited as dictated by the time bandwidth product of the pulses which is determined by the Heisenberg uncertainty principle. The Heisenberg uncertainty principle states that:\textsuperscript{13,14}

\[ \Delta E \Delta t = \hbar \]  \hspace{1cm} (2.26)
where $\Delta E$ is the bandwidth of the laser pulse and $\Delta t$ is the temporal width of the laser pulse. The greater the uncertainty in the energy of the pulse, i.e. the bandwidth, the less the uncertainty in the time of the pulse. Using Fourier transform analysis for the Heisenberg uncertainty principle, the narrowest pulse that may be obtained from a perfectly mode-locked laser is limited by this Fourier transform relationship, and a pulse which approaches this fundamental limit is called transform-limited. A pulse is nearly transformed limited if the time-bandwidth product of the Heisenberg uncertainty principle ($h$) is determined to be $< 0.4$. For the Ti:sapphire laser, the bandwidth is 11 nm and the pulse width is 150 fs (full width at half maximum), thus giving a time-bandwidth product of 0.4, near the transform limit.

2.3.2.1.1.2. The Semiconductor Laser.

Semiconductor diode lasers offer the advantages of low cost, relatively high powers, stable output, and long lifetimes (>40,000 hours). These lasers have also been shown to be adequate excitation sources for TCSPC measurements. Imasaki et al. were the first to demonstrate the use of diode lasers for single-photon counting studies of polymethine dyes.

Diode lasers use a semiconductor material as the gain medium for light production. A common semiconductor material being used is GaAlAs. The light production from semiconductor lasers occurs when electron-hole pairs рекомбинация
across the band gap of the semiconductor, with the energy of the emitted photon ($E_g$) dictated by the band gap.

The electronic band structure of semiconductors determines the optical properties of the material. The energy diagrams of semiconductor materials predict that an electron can be promoted from the electron rich valence band to the electron depleted conduction band by absorbing a photon of at least the band gap energy ($h\nu > E_g$), leaving behind a hole in the valence band.28 Each photon absorbed creates one electron-hole pair.

Light is emitted from semiconductors when these electron-hole pairs recombine across the band gap. The wavelength of light emitted is determined by $\lambda = hc / E_g$. The use of solid solutions or alloy semiconductors, such as Ga$_{(1-x)}$Al$_x$As, with the variable band gap energies, provides a means for choosing the wavelength of the emitted light. The recombination of the electron-hole pairs explains simple emission from a semiconductor, but it does not provide a means for obtaining a population inversion needed for laser action. Semiconductor junctions are needed to confine the spatial distribution of the electron-hole pairs to obtain the population inversion.

When a junction is formed between an n-type and p-type semiconductor, electrons flow from the electron rich n-type semiconductor to the p-type semiconductor until equilibrium is reached between the two materials. The transfer of the charge at the junction leaves a small area of net positive charge at
the n-type side and a small region of net negative charge at the p-type side of the junction.

The application of an external voltage source, which is negative relative to the voltage applied to the p-type side, induces a current, called forward biasing, in the semiconductor and subsequently disrupts the equilibrium condition. Thus, electrons must be provided with enough energy to move over the potential energy barrier from the n-type side to the p-type side, while the holes must be forced to move under the barrier from the p-type side to the n-type side during current flow in order for light production.

Under the influence of a forward bias voltage, electrons and holes recombine in the junction, emitting light in the process. The wavelength of the emitted light is dictated by the band gap energy, which is determined by the composition of the semiconductor material. A p-n junction, used in this manner, is called a light emitting diode and emits continuous wave light. Stimulated emission is not involved in this process; therefore, lasing does not occur.

In order for lasing to occur, the addition of a laser cavity for amplification and a means of producing a population inversion are necessary. The diode laser cavity is typically constructed by having at least one pair of opposite faces that are flat and parallel to one another. Parallel faces can be constructed by mechanical cleaving that occurs naturally in a particular crystallographic direction. Due to the change in the index of refraction at the boundary between the crystal and the surrounding air, these parallel surfaces act as mirrors that bound the diode laser.
A p-n junction constructed in this manner can support lasing when the cavity length is equal to a half-integral number of wavelengths, as seen from Equation 2.24.

The formation of the active layers of semiconductor laser requires a p-n junction. However, layers above and below the p-n junction will be other semiconductor layers that function to aid in optical confinement of the emitted light and to improve the efficiency of the recombination of the electron-hole pairs, thus increasing the lasing capability. For optical confinement, semiconductor layers with a lower index of refraction are situated adjacent to the active region. The probability of stimulated emission is increased due to the increased confinement of the light through the active region.

Placing materials with higher band gap energies next to the lasing region can improve the current confinement. The higher band gap energy can cause the formation of potential energy barriers between the materials. The electrons located in the lasing region are confined by this potential energy barrier, causing a local buildup of the local electron and hole concentrations in the active region. Increases in the concentration of electrons and holes improves the chances for stimulated emission.

A disadvantage of diode lasers is that the beam-shape is elliptical due to the longitudinal separation between the diode laser's emission points parallel and perpendicular to the junction. The separation of emission points is caused by a directional dependence on the refractive index of the lasing cavity. The amount of
astigmatism varies with diode lasers of different types as well as diode lasers of the same type. This condition can be corrected by placing optical components outside the laser cavity, such as an anamorphic prism pair.

The diode head of the PicoQuant diode laser used in the studies reported in this document was actively mode-locked and driven by an electrical short pulse generator which supplied high repetition rate current pulses (80 MHz) with a FWHM of 500 ps. The FWHM of the output optical pulses was < 100 ps. For pulse formation, a current pulse is introduced at the p-n junction, simultaneously forming the electron-hole pairs. Recombination of the electron hole pairs after the current pulse produces the pulse of light with a wavelength dictated by the band gap energy of the semiconductor.

2.3.2.2. Detectors.

For TCSPC measurements, a detector is needed that has a low timing dependence on wavelength, a low timing jitter, low intensity after pulsing, high amplification, low noise, and a wide spectral range. Ultimately, it is these characteristics of the detector which dictate the overall timing response of the TCSPC instrument. There are three general classes of detectors available for TCSPC experiments. The classes include photomultiplier tubes, microchannel plate photomultipliers, and avalanche photodiodes.
2.3.2.2.1. Photomultiplier Tubes.

A photomultiplier tube (PMT) consists of a photocathode and a series of dynodes which serve as the built-in gain medium. The photocathode is a thin film of metal on the inside of a window. Incident photons strike the photocathode surface and cause electrons to be released from the surface of the photocathode. The electrons released from the photocathode are accelerated through the dynode chain. At each dynode, the electrons striking the dynode cause secondary electrons to be released. The photocathode is generally kept at a high negative potential, typically -1000 to -2000 V, in order to reduce the overall noise by preventing spontaneous release of electrons. The dynodes are also held at negative potentials, but these potentials increase towards zero along the dynode chain. The photocathode to first dynode potential difference causes an ejected electron to be accelerated toward the first dynode. Upon collision with the first dynode the photoelectron causes 5 to 20 additional electrons to be ejected, depending on the voltage difference to this dynode. This process continues down the dynode chain until a current pulse arrives at the anode. The size of this pulse depends on the overall voltage applied to the PMT. Higher voltages increase the number of electrons generated from each dynode, hence higher amplification. If the voltage of the PMT is too high, electrons can be spontaneously ejected from the photocathode or any of the dynodes, decreasing the signal-to-noise ratio by increasing the noise. Typical gain of a PMT is on the order of $10^6$.  

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
For quantitative measurements, the anode current must be proportional to the light intensity. A nonlinear response can result from excessive current being drawn from the photocathode. Under high intensity illumination, the potential on the photocathode can be decreased because its current-carrying capacity is limited. This decreases the potential difference between the photocathode and the first dynode, which also decreases the overall gain. In addition, excessive photocurrents can damage light-sensitive photocathodes, resulting in a loss of gain and excessive dark current. The dark current from the PMT is the current in the absence of incident light, which can be reduced by cooling the photocathode.

2.3.2.2.2. Microchannel Plate Photomultipliers.

Microchannel plate photomultipliers (MCP) work on the same premise as do PMTs, except instead of dynodes, they use thin plates of glass with many microscopic channels through them. The surfaces of each plate between the channels are coated with a thin conducting layer, and a voltage (~1000 V) is placed across the thickness of the plate. Each channel, which has a diameter of 12-25 μm, is lined with a secondary emitting surface and functions as an individual electron multiplier which releases secondary electrons upon contact with the incident electron. The photoelectron generated from the photocathode travels a short distance, typically 3 mm, to the first MCP, enters a channel, and strikes the wall of the channel generating secondary electrons. These secondary electrons are accelerated further down the channel, colliding with the walls and causing the
release of more electrons. The electrons then traverse and exit the column where they spread out and enter a number of adjacent channels in the next MCP. MCP photomultipliers typically have two or three plates. After the cascade passes through the last plate, it is collected at the anode as a pulse of current.

2.3.2.2.3. Single Photon Avalanche Diodes.

Single photon avalanche diodes (SPAD) are semiconductor based so they function on the same premise as do diode lasers (Section 2.3.2.1.1.2.), except that SPADs are run under reverse bias and above the breakdown voltage. Operation above the breakdown voltage is called Geiger operation. At this bias, the detector current remains zero until a carrier, such as a fluorescence photon, reaches the active layer of the p-n junction and triggers the cascade of electrons, in which many electron-hole pairs are formed. The amount of electron-hole pairs that are formed represent the gain of the photodetector, which is determined by the energy of the incident photon and the efficiency of the detector at the incident photon's wavelength. Photons with higher energies traverse farther into the active layer of the semiconductor, therefore, more electron-hole pairs are formed. When the first electron-hole pair is formed within the semiconductor material, the leading edge of the avalanche current marks the photon arrival time. Once the avalanche is triggered, an output pulse is generated from the detector and sent to the counting electronics. Once the output pulse is formed, the SPAD voltage is decreased below the breakdown voltage for electron-hole pairs to recombine. After this
occurs, the SPAD bias is then restored to the operating value. Therefore, the
dead time of the detector is determined by the recovery time and typically sets an
upper limit on the dynamic range of the detector to photocurrent rates of \(~500\)
Kcps.

The SPAD, like the other photodetectors, can be triggered by photons and
also by carriers due to thermal effects inside the semiconductor. These processes
cause a self-triggering of the cascade of the electron-hole pairs within the
semiconductor material which is called the dark rate of the detector, \(R\). The
statistical fluctuations of these events, which compete with photons in triggering
the detector, reduce the detector sensitivity.

One way to compare the sensitivity of photodetectors is in the
determination of the Noise Equivalent Power, \(NEP\), since it takes into account the
quantum efficiency, \(QE\), and the dark counting rate, \(R\), of the photodetector. The
NEP is defined as the signal power required to attain a unity signal-to-noise ratio
within a 1 s integration time and is determined by the expression:\(^{29}\)

\[
NEP = \left( \frac{h\nu}{QE} \right) \sqrt{2R} . \tag{2.27}
\]

In Geiger mode, the \(QE\) is a product of the absorption efficiency of the
semiconductor and the triggering probability. The \(QE\) is the probability for a
photon to be absorbed in the semiconductor and to be collected by the avalanching
process. The triggering probability is the probability for a carrier to trigger the
avalanche when crossing the junction, which increases with the excess bias above the breakdown voltage. The SPAD has an operating optimum bias when the $NEP$ is at a minimum.

2.3.2.2.4. Timing and Jitter.

All photodetectors have some degree of time jitter, or variations in the arrival time of the anode pulse relative to the primary photoelectron event. First, the electron must pass out of different depths of the semiconductor material to arrive at the vacuum interface with a variable amount of kinetic energy in a variable direction. The electron can be generated from any portion of the illuminated photocathode. Different electrons have different trajectories and require different lengths of time to arrive at the anode. The second source of time jitter is the individual electron multiplier. Some electron cascade will pass through the multiplier faster than others.

The transit time of a PMT is the time interval between the arrival of a photon at the photocathode and the arrival time of the amplified pulse at the anode. Since electrons ejected in the multiplication process will have a range of velocities and may also travel different paths, there will be a spread in the transit times, which for PMTs, are on the order of $2 \text{ ns}$.

The spread in the transit time depends primarily upon the energy of the primary photoelectron and the point on the surface of the photocathode from which it was emitted, and also the wavelength of the incident photon. The coating
of the photocathode is wavelength sensitive, therefore the choice the detector with
the appropriate wavelength range is important to reduce the transit time spread.

As compared to ordinary PMT with dynodes, the transit time for the MCP
is much less due to the shorter distance the photoelectrons have to travel from the
photocathode to the anode; therefore, MCPs can time events much more
accurately than dynode-based tubes. For a TCSPC device, this translates into a
much narrower instrument response function.

With respect to conventional PMTs, SPADs have extended sensitivity in
the near-infrared region, with quantum efficiencies as high as 30% at 800 nm.
Also, the timing response for a SPAD has a resolution on the ps time scale, along
with small dead times and transit time spreads, due to the decreased distance the
photoelectron needs to traverse to initiate a response as compared to the other
detectors.

2.3.2.3. TCSPC Electronics.

2.3.2.3.1. Discriminators.

The output of the fluorescence detector consists of a broad distribution of
pulse heights, some pulses generated by dark noise, some generated by single
photon events, and some by multiple photon events. It is important to send these
pulses through a discriminator in order to improve the signal-to-noise ratio and to
furnish the TAC with constant amplitude pulses that are independent of the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
photodetector pulse shapes. Discriminators provide a timing definition which is independent of pulse height and also discriminate against low-amplitude noise.

There are two types of discriminators: leading edge (LED) and constant fraction discriminators (CFD). The CFD is more suited to single photon timing studies than the LED, since with the LED photodetector pulses of different amplitudes will not cross the discriminator level at the same time, introducing error into the measurement.

Figure 2.5 demonstrates the use of a LED. A broad distribution of pulse heights from the photodetector pulses can give rise to errors when timing is performed via a LED. As can be seen from the figure, pulses A and B are emitted at the same time after excitation (t₀) but have different amplitudes and are seen to cross the discriminator level at different times. At discriminator level 1 and 2, it can be seen from the figure that pulse A would appear to arrive faster than does pulse B at these discriminator levels, even though they were initiated at the same time. Also note the relative time errors Δ₁ and Δ₂. Different discriminator thresholds introduce more error than others depending upon the amplitudes of the generated pulses.

In order to eliminate the timing errors in single photon counting experiments, discrimination employing a constant fraction approach is employed. With constant fraction discrimination, the pulses are timed from a point on the leading edge that is a fixed fraction of the pulse height. For pulses of similar shape but different amplitudes, the timing point is constant. Figure 2.6a illustrates
Figure 2.5. Timing errors associated with leading edge discriminators. Both pulses are initiated at $t_0$; however, their arrival time is different due to the threshold level and the amplitude of the pulse.
Figure 2.6. Schematic diagram of the function of a constant fraction discriminator. The timing is from a position on the rising edge that is set to a fraction of the input pulse height. In (a) is shown the input pulse that is inverted and delayed by time $\delta$, while in (b) is shown the undelayed pulse attenuated to a maximum amplitude ($-fV_s$). In (c) is shown the zero crossover pulse, where the timing is initiated.
the way in which constant fraction discrimination is achieved. Suppose the input pulse has amplitude $V_\text{a}$ and is to be timed at an amplitude $fV_\text{a}$ on the leading edge. In the discriminator, the input pulse is split into two paths. The first path delays the pulse by a factor $\delta$ and inverts it, while in the other path, the undelayed pulse is attenuated to a maximum $fV_\text{a}$ (Figure 2.6b). Both signals are then added to form the zero crossing signal (Figure 2.6c). Only pulses with amplitudes greater than a given threshold, set on the front panel, are timed.

The discriminator level for the excitation pulses is usually set to reject dark noise caused by signal pulses. The setting of the discriminator level for the fluorescence pulses is much more critical. Setting the discriminator level too low allows for the accumulation of background events in the fluorescence decay; whereas, setting the discriminator level too high allows for multiphoton events to be counted. Fluorescence photodetector pulses have a wide amplitude distribution, therefore, there is no clear dividing line between pulses arising from dark noise and pulses arising from fluorescence photons. Increasing the discriminator threshold seems to increase the signal-to-noise ratio. Consequently, the chosen discriminator level will be a compromise between acceptance of relatively more single photon pulses and rejection of dark noise.

2.3.2.3.2. Time-to-Amplitude Converter.

The TAC functions to determine the time interval between the excitation pulse and the subsequent fluorescence photon arriving at the detector. Upon
receipt of a "start" pulse, and after a certain fixed delay, a timing capacitor is charged linearly from a constant current source. The charge on the capacitor is discontinued upon arrival of a "stop" pulse and an output pulse is generated with an amplitude proportional to the time between the "start" and "stop" pulses. If no "stop" pulse is received after a time called the TAC range, charging is automatically stopped. Again, a fixed time elapses before the capacitor is reset, at which time the instrument is ready to accept another "start" pulse.

2.3.2.3.3. Multichannel Analyzer.

The basic multichannel analyzer (MCA) consists of an analog-to-digital converter (ADC), a memory comprised of channels for storing data and data input and output facilities. A standard instrument generally incorporates lower and upper discriminator levels and two modes of data collection - pulse height analysis mode for the display of fluorescence decay profiles and multichannel scaling mode which bins the data into certain time increments. The data is usually displayed on a computer terminal or on an oscilloscope.

For pulse height analysis experiments, between 200 and 600 channels are sufficient for a decay curve; however, increasing the number of channels in the decay and subsequent reduction in the time per channel improves the timing resolution of the instrument. A drawback, if considered one, is the increase in the data accumulation time for increased number of channels.
2.4. Instrumental Response and Convolution.

The instrument response function of a TCSPC instrument is determined by numerous factors, including the excitation pulse width, the timing jitter associated with the detector, the timing electronics (particularly the discriminator), and the optical components. These factors cause the measured excitation pulse to be broader than the pure optical component.

If the impulse response of the $i^{th}$ component in the system has a FWHM of $\Delta t_i$ and the FWHM of the excitation pulse is $\Delta t_o$, then the measured instrumental FWHM $\Delta t_m$ will be given approximately by,\textsuperscript{13,14}

$$\Delta t_m \approx \left[ \Delta t_o^2 + \sum_i (\Delta t_i^2) \right]^{1/2}.$$ \hspace{1cm} (2.28)

The effect of having an instrumental response function is that the measured fluorescence decay form departs from the true fluorescence response function as described by Equation 2.3. The measured fluorescence decay $F(t)$ can be analyzed, however, because $F(t)$ can be expressed by the convolution of the instrument response function, $P(t)$, and the theoretical fluorescence response function, $i(t)$, by,\textsuperscript{13}

$$F(t) = \int_0^t P(t')i(t-t')dt'$$ \hspace{1cm} (2.29)
for pulsed excitation where \( t' \) defines the variable time delays or channel numbers of the infinitesimally small time widths \( dt' \) or channel widths of which \( P(t) \) is composed. By measuring \( P(t) \) experimentally over \( i \) channels, the convolved form of \( F(t) \) can be obtained from Equation 2.29 assuming a functional form for \( i(t) \). The expression explaining the convolution is given by:\textsuperscript{13}

\[
F(i) = P(i) \otimes \frac{1}{\tau} \exp(-i/\tau) \tag{2.30}
\]

and

\[
F_r(i) = B + A \cdot F(i + \Delta) \tag{2.31}
\]

for comparing with the data where \( i \) is an integer denoting the data channels, \( B \) is the background, \( A \) is a scaling factor, \( \Delta \) is the shift parameter and \( \tau \) is the fluorescence lifetime measured in channels.

2.5. Nonlinear Least Squares Analysis.

The standard statistical procedure used to assess the goodness-of-fit is a nonlinear least squares analysis known as the chi-squared test, \( \chi^2 \). Equation 2.31 needs to be fitted to a fluorescence decay data (convolved decay of fluorescence response function and instrumental response function). The \( \chi^2 \) value is a measure of the error between the actual data and the fitted function. Errors may include nonlinearity in the TAC along with presence of high background levels due to
scattered photons within the actual decay. The $\chi^2$ value determined is a function of the parameters given in Equations 2.30 and 2.31, namely,

$$\chi^2 = \chi^2(A, B, \tau, \Delta).$$  \hspace{1cm} (2.32)

The least squares method aims to determine the best-fit parameters $A$, $B$, $\tau$, and $\Delta$ that will yield the lowest possible value for $\chi^2$. By definition,

$$\chi^2 = \frac{1}{\sigma(i)} \sum \frac{(Y(i) - F_r(i))^2}{\sigma(i)^2} = \sum W(i)^2$$ \hspace{1cm} (2.33)

where $Y(i)$ is the fluorescence datum value, $F_r(i)$ is the fitting function value, $\sigma(i)$ is the statistical uncertainty of the datum value $Y(i)$, and $W(i)$ is the weighted residual.

For any value of $i$, the numerator in Equation 2.33 is the actual deviation between the datum value $Y(i)$ and the corresponding fitting function $F_r(i)$. The denominator represents the noise, which is determined by the deviations from statistical considerations. Rewritten, Equation 2.33 becomes,

$$\chi^2 = \sum \frac{\text{actual deviation}}{\text{expected deviation}}^2.$$ \hspace{1cm} (2.34)
If the fitting function is appropriate, the actual deviation should be equal to the expected deviation. The weighted residual is then unity for each term of the sum, and

\[ \chi^2 = \text{number of data points (N)}. \quad (2.35) \]

However, this is not quite right due to the fact this makes the weighted residual and \( \chi^2 \) values statistical quantities with associated distribution functions and that it does not take into account the number of fitted parameters. A more accurate but less precise expression for Equation 2.35 is,

\[ \chi^2 \approx (N - \nu) \quad (2.36) \]

where \( \nu \) is the number of fitted parameters. Therefore, increasing the number of fitted parameters would decrease the observed \( \chi^2 \) for a given value of \( N \). An expression to normalize \( \chi^2 \) so it would be independent of \( (N - \nu) \) is,

\[ \chi^2_N = \frac{\chi^2}{(N - \nu)} \approx 1 \quad (2.37) \]
for a good fit. For single photon counting experiments, the expected deviation, $\sigma(i)$, which characterizes the random noise, can be estimated from the data function using Equation 2.33, thus:

$$
\sigma(i) = \left[Y(i)\right]^{1/2}
$$

Weighted residual values are important for many reasons. Weighted residual values can show where the misfit occurred in the fitting of the data. Also, their normalization compensates for the varying data precision within the data set and from data set to data set. The deviations are expressed in terms of the standard deviations of the associated data noise. Moreover, the relationship between the weighted residuals and $\chi^2$ are straightforward.

2.6. References.


CHAPTER 3

Error Analysis of Simple Algorithms for Determining Fluorescence Lifetimes in Ultradilute Solutions

3.1. Introduction.

The determination of the fluorescence lifetime from an exponential decay process has been accomplished by a variety of methods, with the nonlinear least squares algorithm, accompanied by convolution or deconvolution of the instrument response function with the goodness of fit determined by the value of $\chi^2$, being one common method. The difficulty associated with this approach is the extensive amount of computational time associated with the determination, which can be a severe limitation when large amounts of data must be processed.

For the case where $n = 1$ (single exponential decay), there are several simple algorithms for determining both the preexponential and the exponential factors which describe a decay process. One method is the maximum likelihood estimator (MLE). In this algorithm, the lifetime can be calculated via the relationship:

$$1 + (e^{T/\tau_f} - 1)^{-1} - m(e^{mT/\tau_f} - 1)^{-1} = N_t^{-1/2} \sum_{i=1}^{m} iN_i$$

(3.1)

where $m$ is the total number of time channels in the decay spectrum, $T$ is the time width in each channel (ps), $N_t$ is the total number of photocounts in the calculation, and $N_i$ represents the number of photocounts in the $i^{th}$ time channel. The left-
hand side of Equation 3.1 is not dependent upon the data and is only a function of
\( \tau_s \), while the right-hand side is determined from the experimental data. The
difetime can be abstracted from the data with the use of graphical, tabulation, or
reiterative techniques. This algorithm has been used to calculate fluorescence
lifetimes of single molecules with high accuracy and precision.\(^6\),\(^7\) The relative
standard deviation, \( \frac{\sigma_{\tau_f}}{\tau_f} \), for MLE lifetime determinations can be evaluated from
the following expression;\(^8\)

\[
\frac{\sigma_{\tau_f}}{\tau_f} = N_t^{-1/2} \frac{1 - e^{-T/\tau_f}}{[(1 - e^{-T/\tau_f})^2 - (T/\tau_f)^2 e^{-T/\tau_f})]^{1/2}}
\]

(3.2)

In the case where \( T \ll \tau_s \), the relative standard deviation can be calculated from;

\[
\frac{\sigma_{\tau_f}}{\tau_f} = N_t^{-1/2}
\]

(3.3)

Another simple algorithm that can be used to extract the decay parameters
of a single exponential process is the rapid lifetime determination method (RLD).\(^9\)
This procedure is a variation of the maximum likelihood estimator which involves
binning the data into two contiguous areas of equal time widths, with the
fluorescence lifetime calculated via the expression;
\[ \tau_f = \frac{-\Delta t}{\ln(D_1 / D_0)} \]  

(3.4)

where \( D_0 \) and \( D_1 \) represent two areas under the decay profile of time width \( \Delta t \) and are evaluated by summing the number of photocounts in each time bin within the boundaries defining \( D_0 \) and \( D_1 \). Figure 3.1 shows a schematic of the RLD calculation. The relative standard deviation, \( \frac{\sigma_{\tau_f}}{\tau_f} \), for the RLD method may be calculated via the following expression:

\[ \frac{\sigma_{\tau_f}}{\tau_f} = \frac{-\Delta t}{[\ln(D_1 / D_0)]^2} \left( \frac{\sigma_{D_0}^2}{D_0^2} + \frac{\sigma_{D_1}^2}{D_1^2} \right)^{1/2} \]  

(3.5)

where \( \sigma_{D_0} \) and \( \sigma_{D_1} \) are the standard deviations in \( D_0 \) and \( D_1 \), respectively. In photon counting experiments, \( \sigma_{D_0} \) and \( \sigma_{D_1} \) can be determined by taking the square root of the total number of counts in each time interval since the probability of observing any specific number of counts is given by the Poisson probability function, with a mean \( \mu \) and a variance \( \sigma^2 = \mu \).

Tellingheusen and Wilkerson have evaluated the performance of the MLE for decay profiles constructed of equal width time bins with the use of Monte Carlo simulations consisting of approximately 10-25 photocounts which were free from background photocounts.\(^8\) The major results from this study indicated that
Figure 3.1. Decay profile for IR-125 showing the RLD method for lifetime determination. The decay profile is divided into two equal widths, $\Delta t$, and the counts over $\Delta t$ are summed. The lifetime is then calculated via Equation 3.4. The solid lines represent the boundaries for the time intervals.
maximum likelihood estimators of $\tau_f$ showed extreme bias when the reduced time, $\Gamma = T_f/\tau_f$, where $T_f$ is the total time interval of the calculation within the fluorescence decay profile, was less than 4 and the number of fluorescence counts comprising the decay profile ranged between 10 and 20. This bias was small when $\Gamma > 6$. For small reduced times, the reciprocal of $\tau_f(k)$ was concluded to be a better estimator due to the small biases observed in the simulations for a finite $T_f$. In addition, small reduced times were shown to give large relative errors in both the $\tau_f$ and $k$ values. When the data was binned and $\tau_f$ was calculated by the RLD method, the Monte Carlo simulation results denoted a slight increase in the standard deviation when compared to the minimal binning case, and also little was gained using unequal bin widths.

Near-IR fluorescence has been shown to be an attractive alternative to visible fluorescence due to the intrinsically smaller backgrounds associated with this region of the electromagnetic spectrum. The use of near-IR radiation and detection reduces the fluorescence background due to the fact that few substances intrinsically fluoresce in this region. Since the simple algorithms investigated make no distinction between multiexponential and single exponential decays, minimal impurity contribution is necessary in the case of ultradilute analyses, since it has been shown that increased background biases the calculation. In addition, the scattering contribution, in the form of Raman and/or Rayleigh photons, is dramatically reduced in the near-IR as compared to the visible based upon the $\lambda^4$ dependence of the Raman cross-section. The result is larger fluorescence
observation windows void of solvent Raman bands in the near-IR. When scattered photons, which have early arrival times, are included into the calculation, a bias can be introduced in the lifetime determination, especially when the relative numbers of these photons are high with respect to the number of fluorescence photons.

The dyes typically used in the near-IR applications are the tricarbocyanine dyes, which possess heteroaromatic fragments linked by a polymethine chain. Difficulties associated with near-IR fluorescence in many applications are the lack of sufficient labels for tagging various classes of compounds, the intrinsically short upper state lifetime of the near-IR dyes which places instrumental constraints on the system, and the poor photophysical properties associated with these dyes in aqueous solvents. The photophysics can be improved through the addition of organized media into the aqueous solvent or the use of nonaqueous solvents.

In this chapter, we will discuss the use of these simple algorithms for fluorescence lifetime determination of several near-IR dyes with nanosecond and subnanosecond lifetimes in the limit of ultradilute concentrations ($10^{-11}$ to $10^{-12}$ M) and short integration times (ranging from 1-10 s to allow low numbers of photocounts in the decay profile) in static solutions. In particular, the effect of the background contribution resulting from scattered photons will be investigated. The experiments involve the fluorescence lifetime determination via the MLE and RLD algorithms for three near-IR fluorescent dyes, IR-125, IR-132, and
dithiatricarbocyanine iodide (DTTCI), whose structures are shown in Figure 3.2, under ultradilute conditions.

3.2. Experimental.

3.2.1. Instrumentation.

The near-IR TCSPC was constructed in house and is shown in Figure 3.3. The excitation source of the near-IR TCSPC device consisted of a mode-locked Ti:sapphire laser pumped by the all-lines output of an argon ion laser (Mira 900-F and Innova 310, respectively; Coherent Lasers, Palo Alto, CA). The laser generated nearly transform-limited pulses, with a temporal width of 120 fs (FWHM) and a bandwidth of approximately 11 nm at a repetition rate of 76 MHz. The Ti:sapphire laser was set at an operating wavelength of 785 nm via an internal birefringent filter and was vertically polarized at the flow cell. The mode structure of the laser was predominantly TEM$\text{oo}$, which allowed for tight focusing of the beam. The near-IR light was focused into a square-bore capillary tube, which served as the observation cell, with the use of a laser singlet diode lens (Melles Griot, Irvine, CA), with a $1/e^2$ beam waist of approximately 10 $\mu$m. The fluorescence was collected with the use of a 40x, 0.85 N.A. epi-fluorescence microscope objective (Nikon, Natick, MA) and imaged onto a slit serving as a spatial filter to reduce the amount of scattered photons generated at the air/glass interface of the cell from reaching the photodetector. The slit width was set at 0.4 mm. The effective sampling volume, assuming a cylindrical probe volume, was
Figure 3.2. Structures of the near-IR commercial dyes, DTTCI, IR-132, and IR-125.
Figure 3.3. Diagram of our near-IR TCSPC instrument. M, Mirror; L, Lens; C, Capillary; BD, Beam dump; MO, Microscope objective; SPAD, Single photon avalanche diode detector; CFD, Constant fraction discriminator; TAC, Time-to-amplitude converter; ADC, Analog-to-digital converter.
calculated to be 3.14 pL. The fluorescence was further isolated from the scattered photons with the use of a bandpass filter (Omega Optical, Brattleboro, VT) with a center wavelength of 850 nm and a half-bandwidth of 30 nm. The collected fluorescence was then focused onto the photodetector with a 6.3× microscope objective. The photodetector was a single photon avalanche photodiode (SPAD, EG&G Optoelectronics Canada, Vaudreuil, Canada) mounted on a thermoelectric cooler. The detector was operated at 30 V below its breakdown voltage and possessed a dark count rate of ~100 counts per second. The pulses generated by the SPAD were amplified by a 2 GHz amplifier (Philips Scientific, Mahwah, NJ) and conditioned with the use of a constant-fraction discriminator (CFD, Tennelec TC 754, Oak Ridge, TN). The CFD pulses from the SPAD were sent into the gate and stop inputs of a time-to-amplitude converter (TAC, Tennelec TC 863). The stop pulse for the TAC was generated by an intracavity photodiode monitoring the pulse train from the Ti:sapphire laser and conditioned by the CFD. The fluorescence decay profiles were collected into 4096 time bins with the use of a PCAII A/D board and software (Tennelec Nucleus) on a PC486 computer. Calibration of the time bins in the pulse height analyzer yielded a value of 2.88 ps per channel. The instrument response function of this system was measured to be 164 ps (FWHM).

All data analysis software was written in Turbo Pascal. Unless otherwise stated, the mean fluorescence lifetimes, <τγ>, were calculated with the use of Γ = 10 × <τγ> for MLE and Γ = 5 × <τγ> for RLD. Lifetimes calculated with the use
of MLE and a reduced time of $\Gamma = 10 \times <\tau_f>$ result in small biases when calculating $<\tau_f>$ directly. Calculation of $<\tau_f>$ via the RLD method has been previously shown to yield small relative standard deviations in the determinations. The lifetimes calculated by the MLE algorithm were determined by the use of the experimental data to evaluate the right-hand-side of Equation 3.1.

Monte Carlo simulations were written to model the experimental data by convolving the experimental instrument response function with the calculated decay using the true decay parameter, $\tau_f$. The correct number of background and fluorescence counts were Poisson distributed into the appropriate time bins within the convolved function.

In order to effectively sample the same molecules in the ultradilute experiments, the flow was interrupted during the measurement. The laser beam impinging on the cell was blocked and the flow started in order to sweep fresh sample into the detection zone. The flow was then stopped and the solution was allowed to reach quiescence, after which the excitation beam was unblocked and data acquisition allowed to commence. Under these conditions, only random diffusion and thermal convection caused by local heating due to molecular absorption of light resulted in fresh molecules entering the detection zone during the measurement. The number of molecules sampled during a typical measurement could be estimated from the size of the probe volume and the dye's concentration.
The true fluorescence lifetimes, $\tau_f$, for the near-IR dyes in ethanol were evaluated with the use of a reiterative, nonlinear least-squares algorithm, with the goodness-of-fit determined by the value of $\chi^2$. Typical decay profiles for DTTCI, IR-132, and IR-125 along with the instrument response function, are shown in Figure 3.4. The concentration of the dye was adjusted so that the background was less than 1% of the total counting rate. In order to reduce anisotropies due to rotational diffusion, a Glan-Thompson polarizing prism was placed in the optical train during these measurements and set at the magic angle (54.7º). The $\tau_f$ values calculated in this fashion were found to be: IR-125, $0.57 \pm 0.01$ ns ($\chi^2 = 1.25$); IR-132, $0.76 \pm 0.01$ ns ($\chi^2 = 1.30$); DTTCI = $1.12 \pm 0.01$ ns ($\chi^2 = 1.33$). Previous studies using phase-resolved fluorescence indicated a lifetime for DTTCI in ethanol of $1.33 \pm 0.02$ ns ($\chi^2 = 21$).27 The difference in our lifetime and that observed previously may arise from varying amounts of water present in the ethanol solvent since the lifetimes of a number of near-IR dyes have been shown to be sensitive to the presence of trace amounts of water.22

3.2.2. Reagents and Chemicals.

The near-IR dyes were obtained from Kodak Chemicals (Rochester, NY) and used as received. Spectroscopic-grade ethanol (Mallinckrodt, Paris, NY) was used for all solutions. Stock solutions of the dyes (1 µM) were made monthly and stored in the dark in a refrigerator at 10°C. Over this period of time,
Figure 3.4. Decay profiles for instrumental response, DTTCl, IR-132, and IR-125. [DYE] $\sim 5 \times 10^{-10}$ M; $\lambda_{\text{max}} = 785$ nm, P = 10 mW.
no degradation in the stock dye solutions was apparent, as determined by
monitoring the emission intensity of the dye solutions. The dilute dye solutions
were prepared daily from serial dilutions of the stock dye solutions.

3.3. Results and Discussion.

In Figure 3.5 are shown $<\tau_f>$ values for three near-IR dyes determined by
the MLE and RLD algorithms as a function of the background-to-fluorescence
(B/F). In addition, the lifetimes calculated with the use of the MLE method for
Monte Carlo simulations are presented, which were constructed with the
appropriate background and fluorescence counts to match the experimental
conditions. Fair agreement between the experimental data and simulation results
was observed. In these determinations, the calculations were initiated at $t = 0$,
which was assumed to occur at the channel within the decay profile with the
maximum number of counts. Figure 3.5 shows that decreases in $<\tau_f>$ were
observed with respect to $\tau_f$ as the B/F ratio was increased. The lifetimes at the
high B/F ratios (50%) were consistently lower for the MLE method when
compared to the RLD procedure. At the 50% B/F ratio, the relative errors were
found to be ~18% and ~27% for IR-125, ~12% and ~16% for IR-132, and ~8.7%
and ~14% for DTTCI via the RLD and MLE, respectively. The relative standard
deviation (RSD) was calculated from;

$$\text{RSD} = \frac{\tau_f - <\tau_f>}{\tau_f} \times 100 \quad (3.6)$$
Figure 3.5. Calculated lifetimes as a function of the background-to-fluorescence ratio for the near-IR dyes DTTCI (diamonds), IR-132 (squares), and IR-125 (circles) via the MLE (closed symbols) and the RLD (open symbols). Monte Carlo simulation results as calculated by the MLE are designated by (×).
where $\tau_f$ is the “true” fluorescence lifetime as calculated by nonlinear least squares analysis at high concentrations and long integration times and $<\tau_f>$ is the fluorescence lifetime calculated via the MLE of RLD algorithms. These relative errors were found to consistently increase as the B/F ratio was increased. The larger relative errors at high B/F ratios result from increased contributions of scattered photons in the calculation. Since the arrival time within the decay profile of these photons was at early times within the decay profiles, inclusion of these photons biased $<\tau>$ to lower values at high B/F ratios where their relative contribution in the calculation was high. The larger relative errors for the MLE determination indicated a greater sensitivity to the presence of background photons. The errors were also found to be greater for IR-125, which has the shortest lifetime, with the longer-lifetime dye, DTTCI, demonstrating smaller relative errors.

A plot of the number of occurrences versus $<\tau>$ for these dyes was found to yield a normal distribution for both the MLE and RLD. The standard deviations associated with the distributions were found to be in good agreement with those predicted by Equations 3.2 and 3.5. As can be seen through inspection of Equations 3.3 and 3.5, lower dye concentrations result in fewer fluorescence counts and larger relative standard deviations. The relative standard deviations were found to be approximately 0.8 - 1.0% for the MLE and RLD methods at a B/F ratio of 1% and increased to approximately 2.0 - 2.7% at a B/F ratio of 50%.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Background photons from the solvent originate from two sources, Raman and Rayleigh scattered and/or fluorescence photons from impurities present in the solvent. Ultrasensitive fluorescence experiments using visible excitation and detection have shown that the detection efficiency is limited primarily by the presence of impurity components in the solvent when implementing time-gated detection. In ultrasensitive near-IR experiments, the detection efficiency was significantly improved due to the smaller background level arising from these impurity components. In the present applications, the background photons from solvent impurities can cause significant errors in the calculated lifetime, especially at low dye concentrations (high B/F ratios) since the algorithms do not differentiate between mono- and multiexponential decay processes. In the multiexponential case, the resulting lifetime would be a weighted average of the lifetimes of the dye studied and the fluorescence impurities associated with the solvent. MLE analysis of the response function for the solvent blank (ethanol) provided a value of 0.11 ns. Moreover, an autocorrelation analysis, which is sensitive to correlated bursts of photons from fluorescence components traveling through the detection zone, for the ethanol blank yielded no observable nonrandom correlation. These results indicate the absence of long-lived fluorescence components in the solvent, and thus the dominant background source consists primarily of Raman and Rayleigh scattered photons.

In order to increase the accuracy of the lifetime calculation at high B/F ratios, the calculation was initiated at a channel later in the decay profile to reduce
the relative contribution of scattered photons into the calculation. Figure 3.6 shows \( \langle \tau_f \rangle \) calculated by the MLE and RLD methods as a function of the start channel for IR-125 and DTTCI. The lifetimes calculated by the MLE procedure demonstrated a gradual increase until a channel shift of approximately 35 channels (100 ps) for IR-125 and 30 channels (86 ps) for DTTCI was reached, after which fairly constant lifetime values in good agreement with \( \tau_f \) were obtained. Figure 3.7 shows the decay profiles of IR-125 and DTTCI under ultradilute conditions along with the start channels for the lifetime calculation. Notice that shifting the initial time channel to times later in the decay aids in effectively removing scattered photons from the calculation.

Since the experimental data does not differentiate between background and fluorescence photons, Monte Carlo simulations were performed to determine the relative contribution of background photons in the calculation at time shifts yielding lifetimes in agreements with \( \tau_f \). The simulation results indicated that, at B/F ratio of 50%, the relative contribution of background photons in the lifetime determination at a time shift of 100 ps was approximately 15% for IR-125 and 10% for DTTCI. When the time shift was 86 ps, the relative contribution from background photons was 21% for IR-125 and 14% for DTTCI. The larger relative background contributions for IR-125 at these time shifts compared to DTTCI arise from the shorter lifetime associated with IR-125. The results also indicated that, for MLE determinations, the contribution of the background photons must be significantly reduced in order to achieve lifetime values with high
Figure 3.6. Calculated fluorescence lifetimes for the dyes IR-125 (circles) and DTTCI (squares) as a function of the start channel in the calculation at a B/F ratio of 50% via the MLE (open symbols) and RLD (closed symbols).
Figure 3.7. Decay profiles for IR-125 and DTTCI at [DYE] = 5 × 10^{-12} M and 1 s integration period. The dashed lines represent time shifts employed for calculation of the fluorescence lifetime, 35 channels for IR-125 and 30 channels for DTTCI.
accuracy. In addition, dyes with longer lifetimes require smaller time shifts due to the smaller relative contribution of background photons included in the calculation.

When a time shift is employed, the total number of counts (background and fluorescence counts) included in the calculation is reduced with a corresponding increase in the relative standard deviation (loss in precision) when one is shifting the start channel to later times within the decay profile. The relative standard deviation at a B/F ratio of 50% was found to increase from 2.1% at a zero time shift to approximately 5.6% for a time shift of 100 ps for IR-125. For DTTCI, the relative standard deviation was 1.9% for a zero time shift and increased to 4.0% at a time shift of 86 ps.

Shifting the start channel in the calculation to later times in the decay profile can also result in biases due to the exclusion of fluorescence photons with early arrival times. In order to evaluate these biases, Monte Carlo simulations were performed to construct decay profiles and use the MLE method to evaluate $\langle \tau \rangle$ for dyes with $\tau_f$ values of 1.12 ns, 0.76 ns, and 0.57 ns. The decay profiles were constructed with 0 background counts and 10,000 fluorescence counts. The results of these simulations revealed that the relative error in $\langle \tau \rangle$ was 4.6% for $\tau_f = 1.12$ ns at a time shift of 250 ps, with smaller time shifts resulting in smaller relative errors. For $\tau_f = 0.76$ ns, the relative error was determined to be 10.1% for a time shift of 130 ps (45 channels), and when $\tau_f = 0.57$ ns, the relative error was 10.2% for a time shift of 100 ps (35 channels). These results indicated that early arriving photons make a significant contribution into the calculation for species with
shorter lifetimes whereas, for longer-lifetimes, the relative contribution to the calculation is less. Elimination of these photons introduces biases into the MLE calculation for short $\tau_f$ values, while for longer lifetimes, larger time shifts can be tolerated without introducing significant biases. The lifetimes calculated as a function of channel shift (see Figure 3.4) were corrected for this bias. Lifetimes calculated by the RLD method at a B/F ratio of 50% were found to be insensitive to the time shift and yielded consistently lower $<\tau_f>$ values as compared to $\tau_f$ (see Figure 3.4). Monte Carlo simulation results with the use of background-free decay profiles constructed from 10,000 fluorescence photons also demonstrated the insensitivity of $<\tau_f>$ to the start channel for $\tau_f$ values ranging from 0.57 to 1.12 ns. A shift in the calculation to later time channels within the decay profile does not increase the accuracy of the determination with the use of the RLD method, but does result in a loss of precision due to decreases in the number of photocounts included in the calculation (see Equation 3.5). Therefore, the RLD methods should nominally be used with zero time shifts and high dye concentrations (low B/F ratios) in order to obtain fairly accurate results with good precision.

In Table 3.1, $<\tau>$ values calculated with the use of the MLE method for these near-IR dyes at a concentration of $5 \times 10^{-12}$ M and integration times of 1 and 10 s are presented. At this concentration, the approximate number of molecules sampled ($N_m$) was estimated by the use of:

$$N_m = CN_A V_p$$  \hspace{1cm} (3.7)
Table 3.1. Fluorescence lifetimes for IR-125, IR-132, and DTTCI in ethanol as calculated by the MLE algorithm. [DYE] = 5 \times 10^{-12} \text{ M.}

<table>
<thead>
<tr>
<th></th>
<th>Integration Time (s)</th>
<th>$\tau_f (\text{ns})^a$</th>
<th>Counts$^b$</th>
<th>$\sigma_a (\text{ns})^c$</th>
<th>$\sigma_a (\text{ns})^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR-125$^e$</td>
<td>1</td>
<td>0.58</td>
<td>2250</td>
<td>$\pm 0.04$</td>
<td>$\pm 0.02$</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.57</td>
<td>21775</td>
<td>$\pm 0.02$</td>
<td>$\pm 0.007$</td>
</tr>
<tr>
<td>IR-132$^e$</td>
<td>1</td>
<td>0.75</td>
<td>2625</td>
<td>$\pm 0.04$</td>
<td>$\pm 0.02$</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.76</td>
<td>25150</td>
<td>$\pm 0.01$</td>
<td>$\pm 0.006$</td>
</tr>
<tr>
<td>DTTCI$^f$</td>
<td>1</td>
<td>1.12</td>
<td>3440</td>
<td>$\pm 0.03$</td>
<td>$\pm 0.02$</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.12</td>
<td>31235</td>
<td>$\pm 0.01$</td>
<td>$\pm 0.005$</td>
</tr>
</tbody>
</table>

$^a$ Represents the average of six replicate sets.

$^b$ Photocounts included in lifetime determination.

$^c$ Standard deviations calculated from six replicate measurements.

$^d$ Standard deviations calculated via Equation 3.3.

$^e$ Lifetime values calculated with a time shift of 125 ps.

$^f$ Lifetime values calculated with a time shift of 100 ps.
where $C$ is the dye concentration, $N_A$ is Avogadro's number, and $V_p$ is the probe volume. At this dye concentration and a 3.14 pL probe volume, $N_m = 10$. The effective number of molecules sampled is independent of the integration time since the volumetric flow rate was stopped during the measurement. In addition, the B/F ratio at this concentration is independent of the integration time. It was noticed that, during data acquisition, the steady-state count rate decreased due to photobleaching of the dye molecules in the excitation beam. In the case of DTTCI, significant reductions in the counting rate were observed during data acquisition when compared to IR-125 and IR-132, indicating a poorer photochemical stability.

If no new molecules are swept into the detection zone during data acquisition, the count rate should approach that of the solvent blank. Since the counting rate remained above the background level in all cases, new molecules were assumed to have been swept into the detection zone through random diffusion and/or thermal gradients. The number of sampled molecules calculated in Table 3.1 should therefore be considered provisional. For IR-125 and IR-132, time shifts of 125 ps were sufficient to give $<\tau_f>$ values in good agreement with $\tau_f$ whereas, a time shift of 100 ps was necessary for DTTCI to give $<\tau_f>$ in good agreement to $\tau_f$. The time shifts required for good agreement with $<\tau_f>$ were similar for both integration times for all dyes. At this dye concentration and appropriate time shift, the lifetime values calculated were in excellent agreement with $\tau_f$ for the dyes studied. For IR-125, $<\tau_f>$ was determined to be 0.58 ns for a 1 s integration time and 0.57 ns for a 10 s integration time. For DTTCI, similar lifetime values were obtained for
the 1 and 10 s integration times. Also, these results showed that longer integration times do not necessarily result in better accuracy. The accuracy in the MLE method was determined, in part, by the background in the form of scattered photons which were included in the calculation and was set by the B/F ratio (i.e., dye concentration) and the time shift necessary to reduce the number of these background photons in the determination to an acceptable level. The precision in the measurements was found to improve with increasing integration time due to the fact that more photocounts were being accumulated in the decay profile. For IR-125, the RSDs were found to be 6.9% for a 1 s integration time and deceased to 3.5% for a 10 s integration time. For DTTCI, the RSDs were 2.7% and 1.0% at 1 and 10 s integration times, respectively.

3.4. Conclusions.

Fluorescence lifetimes associated with single exponential decays can be calculated with little computational effort and a high level of accuracy and precision with the use of the MLE and RLD algorithms. When the background-to-fluorescence ratio is small, both algorithms yield similar levels of accuracy and precision for dyes with nanosecond and subnanosecond lifetimes. In the limit of ultradilute dye concentrations (high B/F ratio), where the background in the form of scattering makes a relatively large contribution into the decay profile, the MLE method was shown to be superior to the RLD method under appropriate calculation conditions. The accuracy was found to be better for the MLE.
algorithm when compared to the RLD method with the use of a time shift in the
decay profile in order to reduce the amount of scattered photons included in the
calculation. For ultradilute solutions at short integration times, the lifetimes
calculated via the MLE method were found to agree favorably with those obtained
in the high concentration experiments and evaluated with the use of a nonlinear
least-squares method. The negligible contribution of fluorescence impurities
arising from the solvent blank with the use of near-IR excitation and detection
allows for the determination of fluorescence lifetimes in complex matrices under
dilute conditions.

In summary, the ability to measure fluorescence lifetimes in static solutions
via two simple algorithms, the MLE and RLD, with high accuracy and precision in
the limits of low concentrations and short integration times was demonstrated.
Since the detection scheme involved in our DNA sequencing application involves
fluorescence lifetime discrimination of labeled oligonucleotides separated by
capillary gel electrophoresis, methods of calculating the fluorescence lifetimes of
the labeled oligonucleotides during the separation quickly and accurately is
essential. The results from these static experiments have shown the ability to
determine fluorescence lifetimes at low concentrations and short integration times,
which mimics the conditions for components separated by capillary
electrophoresis.
3.5. References.


CHAPTER 4

On-Line Fluorescence Lifetime Determinations in Capillary Electrophoresis

4.1. Introduction.

Several research groups have demonstrated the ability to make on-line fluorescence lifetime determinations using both time\textsuperscript{1-4} and frequency\textsuperscript{5-8} domain measurements in high-performance liquid chromatography (HPLC). The advantages of determining lifetimes during an analytical separation include peak identification, analysis of coeluting components, and multiplexing applications. Much of the on-line lifetime applications performed thus far involved the determination of coeluting peaks during the HPLC separation of polyaromatic hydrocarbons (PAHs). A photophysical characteristic associated with PAHs that simplifies lifetime determinations in HPLC applications is their long lifetimes, ranging from 10-50 ns, which relaxes the instrumental criteria for making the measurement. McGown and co-workers have utilized phase-resolved methods and heterogeneity analysis during the HPLC separation to determine if more than two components were present in the detection region (coelution of peaks).\textsuperscript{5-8} By monitoring the phase and demodulation lifetimes, the presence of multiple components and their fractional contribution to the decay was determined. Lytle and co-workers have used time-resolved fluorescence techniques for the analysis of PAHs.\textsuperscript{3} In their method, a nitrogen laser was used as the excitation source with the emission monitored at two different times within the decay so a "ratiogram" could be constructed.
An advantage of using time-resolved techniques over phase-resolved techniques is that time-filtering can be simultaneously employed, which can improve the signal-to-noise (SNR) during the separation by discriminating against interferences with short lifetimes or scattering photons.\textsuperscript{1-2,9} Time-filtering has been used in capillary electrophoresis (CE) applications with a nanosecond, diode-pumped Nd-YLF laser serving as the excitation source for the fluorescence.\textsuperscript{10,11} The results indicated an improvement in the signal-to-noise ratio by discriminating against short-lived interferences present in the biological sample. The authors demonstrated the time-filtering capabilities of their system; however, measurements of the fluorescence lifetimes of the separated components were not acquired.

In applications requiring the monitoring and identification of multiple dyes during an analytical separation, lifetimes can improve the efficiency in the identification process when compared to spectral wavelength discrimination. An example of multifluor analysis is DNA sequencing, where the identity of a terminal nucleotide base during the separation can be accomplished via spectral emission wavelengths associated with the different fluorescent probes.\textsuperscript{12-15} Identification based on differences in emission wavelengths can produce errors in the base calling, due to broad, overlapping emission profiles which can result in cross-talk between detection channels. Lifetimes can be measured with high precision under appropriate conditions and produce little cross-talk, yielding definitive
identification of the particular chromophore during the analytical separation when the dyes have distinct lifetime values.

On-line fluorescence lifetime determinations in CE applications represents new challenges not typically encountered in HPLC separations. These challenges include short residence times of the chromophore within the detection zone and low loading masses. Both of these conditions limit the number of photocounts that can be accumulated within the decay profile, producing poor photon statistics and inaccurate lifetime values with poor precision, especially in highly scattering media, such as gel matrices. Several groups have demonstrated the ability to determine lifetimes of single molecular events in solution using pulsed laser excitation and time-gated detection. In these studies, decay profiles were constructed from 20-200 photocounts and the lifetimes calculated via simple algorithms to reduce the amounts of computational time associated with the determination.

Near-IR fluorescence can be an appealing alternative to visible fluorescence when attempting to perform on-line measurements under ultradilute conditions and complex sample matrices. The advantages of near-IR fluorescence monitoring include smaller contributions from scattered photons due to the smaller Raman cross-section in the near-IR and fewer fluorescence interferences. However, a major disadvantage of near-IR fluorescence is the intrinsically short upper-state lifetime associated with the chromophores used in these applications and the poor photophysical properties associated with these dyes in predominately aqueous solvents. Due to the dye’s short lifetimes, which can range between 200-
1,000 ps, certain instrumental criteria must be met in order to perform this type of analysis with time domain methods. These criteria include subnanosecond pulse widths delivered from the excitation source and a detector with a small transit time spread.

For on-line analysis of fluorescence lifetimes, it is advantageous to have algorithms to calculate $\tau_f$ simply and precisely under conditions where the photon statistics are poor. Several simple algorithms have been developed to determine nanosecond and subnanosecond fluorescence lifetimes. Two examples include the maximum likelihood estimator (MLE) and the rapid lifetime determination (RLD) methods. We have shown that highly accurate and precise lifetimes of near-IR fluorescent dyes with subnanosecond lifetimes can be calculated via the MLE and RLD using time-correlated single photon counting with a passively modeocked Ti:sapphire laser and a single-photon avalanche diode detector at low concentrations and short integration times in static solutions.

The difficulty associated with the poor photophysics of many near-IR dyes in aqueous solvents can be circumvented to a certain degree by modifying the application to use an organic solvent. For example, we have improved the sensitivity and resolution of several near-IR dyes separated by free solution CE in buffers composed predominately of methanol. The detection limits for some model near-IR dyes separated by CE were found to be in the 100-400 molecule range in a running buffer composed of 95/5 methanol/water; whereas, the detection limit increased to 120,000 molecules for a running buffer consisting of
60:40 methanol/water. The increase in the detection limit with a higher water content running buffer was ascribed to quenching effects exhibited by the aqueous solvent on the chromophore.\textsuperscript{21}

In this chapter, experiments were performed to investigate the on-line measurement of fluorescence lifetimes for several near-IR chromophores separated by free solution capillary electrophoresis using time-correlated single photon counting.\textsuperscript{29} The instrument used in this study was a passively mode-locked Ti:sapphire laser for excitation and a single photon avalanche diode detector, with the instrument response function having a FWHM of 165 ps,\textsuperscript{21,27} appropriate for making subnanosecond lifetimes. The chromophores are the tricarbocyanine dyes that show absorption and emission properties in the near-IR and possess fluorescence lifetimes in predominately methanol solvents which range from 500-1,000 ps. To improve the photophysical characteristics of the near-IR dyes during the CE, running buffers composed of high methanol concentrations were used in the separation. Also, fluorescence lifetime measurements of DNA sequencing fragments labeled with a near-IR label on the 5'-end of a primer and separated by CE are presented.

4.2. Experimental.

4.2.1. Instrumentation.

The instrument used is similar to that described previously in Chapter 3 with the following exceptions (see Chapter 3, Figure 3.3). The fluorescence
emission from the capillary tube was collected by a 60×, N.A. = 0.85 microscope objective (Nikon, Natick, MA) with the collected radiation imaged onto a spatial filter with a slit width set to 1.2 mm. The decay profiles were constructed by a pulse height analyzer (PHA) board and software (PCA II, Tennelec Nucleus, Oak Ridge, TN). The output from the time-to-amplitude converter was digitized by an analog-to-digital converter and placed into the appropriate time bin within the decay profile by the PHA hardware and software. The normal intensity electropherogram was constructed by monitoring the fluorescence counting rate during the separation using a multichannel scaler resident in the PC.

4.2.2. Free Solution Capillary Electrophoresis.

The electrophoresis was performed in a capillary (Polymicro, Phoenix, AZ) with an internal diameter of 75 μm. The optical window was produced using a low-temperature flame to remove the polyimide coating. In free solution CE, the distance from the injection end to the detector was set at 45 cm, with a total length of the capillary set at 50 cm. The ends of the capillary were adjusted to the same height to prevent siphoning of solvent into the capillary. The high voltage was supplied by a Spellman high-voltage power supply (CZ1000R, Plainview, NY) and operated in a normal mode (anode at injection end) for free solution CE and reverse polarity for capillary gel electrophoresis. The high-voltage end of the capillary was placed in a protective interlock box constructed in-house. The running buffers used for free solution CE consisted of 95/5 methanol/triply distilled
water. The carrier buffer also contained 20 mM borate with a pH=9.4. The carrier buffers were not purged with an inert gas to remove O\textsubscript{2}, since our previous work has shown that O\textsubscript{2} has a negligible effect on the photophysics of many tricarbocyanine dyes.\textsuperscript{30} All near-IR fluorescent dyes for free solution CE were obtained from Kodak (Rochester, NY) and used as received. The structures of the dyes are shown in Figures 3.2 and 4.1. Stock dye solutions were prepared in 100% methanol at 0.1 mM and stored in the dark in a refrigerator at 10°C. The solutions for CE analysis were prepared daily from serial dilutions of the stock dye at the appropriate concentrations in the carrier buffer. The column was conditioned daily by flushing the capillary with 1 N NaOH for 30 min and then rinsing with triply distilled water for 30 min. The column was then allowed to equilibrate with the running buffer for 30 min at 5 kV for 30 min. All samples were electrokinetically injected onto the column. Photocounts in the intensity electropherogram were typically integrated for 0.4 s and were not subjected to any type of filtering algorithm.

4.2.3. CE Lifetime Determinations.

The fluorescence decay profiles were collected over the electrophoretic peak with data integration commencing when the fluorescence counting rate exceeded the average background rate by ~25% and accumulation ceased when the counting rate dropped below this level. The integration time was determined therefore by the time width of the respective electrophoretic bands. The
Figure 4.1. Chemical structures of Hexamethylindotricarbocyanine iodide (HITCI), IR-140, and IR-144.
fluorescence lifetimes were calculated via the MLE and RLD algorithms, with the expressions for the fluorescence lifetime calculation given by Equations 3.1 and 3.3 and the standard deviations calculated by Equations 3.2 and 3.5, respectively.

4.2.4. Preparation of Gel Columns and DNA Sequencing Fragments.

The gel columns (Polymicro, Phoenix, AZ) were prepared by standard techniques. The total length of the column was 45 cm, with the distance from injection to detection being 35 cm. The wall of the capillary was conditioned with the following solutions in the order given, 1 N NaOH (10 min), triply-distilled water (10 min), 1 N HCl (10 min) and finally triply-distilled water (10 min). The wall of the capillary was derivatized with a 50/50 [3-(methacryloxy)propyl]trimethylsiloxane/methanol (Aldrich Chemical) solution overnight followed by drying in an oven at 110 °C. The unpolymerized polyacrylamide gel solution (3% T/ 3% C, Sigma Chemicals) was introduced into the column via aspiration. This gel solution contained 8 M urea as a denaturing agent, 1X TBE (TRIS-borate, EDTA, pH = 8.3) and riboflavin, which served as the photoinitiator for polymerization. After filling the column with the gel solution, the capillary was capped at each end, placed in an ice bath, and exposed to UV light for 12 hr. Following polymerization, the column ends were clipped and pre-run at 5 kV for a period of 30 min prior to performing the sequencing run.

The sequencing ladder was prepared from the M13mp18 template using standard Sanger dideoxy termination protocols with only the C-terminated
fragments generated and the Sequenase Version 2 DNA sequencing kit (United States Biochemical, Cleveland, OH). Two picomoles of a near-IR dye-labeled M13 universal sequencing primer (Li-Cor, Lincoln, NE) was annealed to a M13 template at 65°C for 2 min. The construct was allowed to cool slowly (30 min) to room temperature (25°C). After cooling, 0.2 μmol of DTT, 0.1 μmol MnCl₂, 2 μL of 1:5 diluted dNTP labeling mix, 0.004 units of pyrophosphatase, and 0.4 units of the sequenase enzyme were added to the labeled template. A 3.5 μL aliquot of this mixture was placed in a microcentrifuge tube containing 3.2 pmol of ddCTP. Additional volumes of dNTPs were added to achieve an overall ratio of dNTP:ddCTP of 1200:1. The mixture was incubated at 37°C for 30 min after which 4.0 μL of a stop solution (95% formamide) was added to the reaction vessel. Prior to injection onto the gel column, the extension mixture was heated to 85°C and cooled quickly. The sample was electrokinetically injected onto the column at 11.25 kV for 2 min. The separation was performed using a field strength of 250 V/cm.

4.3. Results and Discussion.

4.3.1. Comparison of MLE and RLD Lifetime Algorithms in Free Solution CE.

In Figure 4.2 is shown an intensity electropherogram for the CE separation of the near-IR fluorescent dyes, DTTCI (cationic) and IR-125 (anionic) along with the associated decay profiles with the boundaries for the lifetime calculation within the decay shown by the dashed lines. The concentration of the dyes injected onto
Figure 4.2. Free solution capillary electropherogram and the associated decay profiles for DTTCI and IR-125. [DYE] = 40.0 pM. The running buffer consisted of 95/5 methanol/borate, pH=9.4. The dyes were electrokinetically injected onto the column at 5 kV for 5 s and the separation performed at 491 V/cm. $\lambda_{\text{max}} = 785$ nm, $P = 10$ mW.
the column in this case was 40.0 pM and represents approximately 491 (DTTCI)
and 142 (IR-125) zmol of material. The mass injected onto column was
determined from the following expression,\(^{32}\)

\[
Q = \frac{L_d A C t_{inj} V_{inj}}{t_{mig} V_{ele}} \times \frac{1}{1000 \text{ cm}^3}
\] (4.1)

where \(Q\) is the amount injected (moles), \(L_d\) is the length of the capillary to the
detector (cm), \(A\) is the area of the capillary (area of a cylinder = \(\pi r^2\), cm\(^2\)), \(t_{inj}\) is the
injection time (s), \(V_{inj}\) is the injection voltage (V), \(t_{mig}\) is the migration time (s), and
\(V_{ele}\) is the electrophoresis voltage (V). The integration time for the construction
of these decay profiles was determined by the residence time of the electrophoretic
band within the detection zone and were found to be approximately 2 s for DTTCI
and 8 s for IR-125 at this concentration ([40 pM]). As can be seen from these
decay profiles, DTTCI exhibits a longer lifetime (smaller slope in the semi-log plot)
compared to IR-125, consistent with the experimentally determined lifetimes of
these dyes at high concentrations in static solutions using standard nonlinear least
squares fitting routines (\(\tau_f = 935\) ps for DTTCI and \(\tau_f = 471\) ps for IR-125).

Table 4.1 shows the fluorescence lifetime values calculated via the MLE
and RLD methods using Equations 3.1 and 3.3 at several different concentrations
of DTTCI and IR-125 separated by free solution CE along with the standard
deviations calculated from replicate measurements (\(\sigma_{exp}\)) and also using Equations
Table 4.1. Fluorescence lifetime determinations in capillary electrophoresis using the MLE and RLD methods for the fluorescent dyes DTTCI and IR-125. The lifetimes were calculated over a $5 \times \tau_f$ interval. The lifetimes for these dyes obtained under static conditions and high concentrations were 935 ps ($\pm$ 10 ps) for DTTCI and 471 ps ($\pm$ 2 ps) for IR-125.

<table>
<thead>
<tr>
<th></th>
<th>Conc. (pM)</th>
<th>Inj. moles (zmol)</th>
<th>$\tau_f$(ps)$^a$</th>
<th>$\sigma_{exp}$(ps)$^b$</th>
<th>$\sigma_{calc}$(ps)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MLE</td>
<td>RLD</td>
<td>MLE</td>
</tr>
<tr>
<td>DTTCI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>400.0</td>
<td>4910</td>
<td>936</td>
<td>922</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>40.0</td>
<td>491</td>
<td>924</td>
<td>939</td>
<td>18</td>
<td>26</td>
</tr>
<tr>
<td>4.0$^d$</td>
<td>49.1</td>
<td>943</td>
<td>910</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>IR-125</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>400.0</td>
<td>1420</td>
<td>481</td>
<td>482</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>40.0</td>
<td>142</td>
<td>485</td>
<td>487</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>4.0$^d$</td>
<td>14.2</td>
<td>483</td>
<td>454</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>0.4$^d$</td>
<td>1.42</td>
<td>482</td>
<td>423</td>
<td>14</td>
<td>23</td>
</tr>
</tbody>
</table>

$^a$ Represents the average of five to eight replicate sets.

$^b$ Standard deviations calculated from six replicate measurements.

$^c$ Standard deviations calculated via Equations 3.2 and 3.5 for the MLE and RLD, respectively.

$^d$ Lifetime values calculated with a time shift of 86 ps.
3.2 and 3.5 ($\sigma_{\text{calc}}$). The time period over which the calculations were performed at any particular dye concentration was chosen in order to diminish the number of background and scattered counts included into the calculation. The scattered photons, resulting from Rayleigh and Raman scattering, can bias the fluorescence lifetimes to shorter values due to the fact that these photons are coincident with the laser pulse. Reduction in the contribution of these photons into the calculation can be accomplished by shifting the start channel of the calculation to a later time bin within the decay profile due to the temporal characteristics of these photons. A random distribution of background photons in all time bins within the decay profile was also observed. These background photons arose from the dark counts associated with the photodetector and stray light from the capillary tube walls impinging onto the face of the photodetector. The presence of these photons in the latter time channels of the decay profile biased the calculated lifetime to longer values, especially in the lower concentration conditions where the contribution of these photons were relatively large. In order to reduce the contribution of these photons, an average number of background photons was calculated from an area of the decay profile in which the probability of finding fluorescence photons was minimal and subtracted from each channel within the decay profile.

In order to access the relative contributions of the scattered and background photons into the calculations at various fluorescence photon levels, Monte Carlo simulations were performed with the appropriate lifetime and numbers of scattered, background, and fluorescence photons to model
experimental conditions. When decay profiles were constructed with no background or scattered photons and 10,000 fluorescence counts, it was discovered that shifting the start time in the calculation by 28 ps resulted in a lifetime value that was 6% longer than the expected lifetime for input values of 471 ps and approximately 3% longer for lifetimes of 950 ps when the MLE method was used. This bias was found to be cumulative up to approximately four-28 ps time shift intervals. When the RLD method was used, no bias was found in the simulation results for a time shift up to 112 ps. The simulation results also indicated that the presence of scattered photons introduced no significant bias when the background-to-fluorescence (B/F) ratio was less than 10% for both algorithms. When the B/F ratio was increased to over 20%, significant biases were observed. Shifting the start time in the calculation 86 ps from the channel with the maximum number of photocounts significantly reduced the scattered photon contribution into the calculation and improved the accuracy. The presence of the background photons into the calculation could be corrected for by subtracting a constant count number from each time bin over the entire decay profile. This constant was determined by averaging the number of counts in each time bin from the end channel in the calculation to the last time bin in the decay profile containing counts.

As can be seen from Table 4.1, the lifetimes calculated via the MLE algorithm at all dye concentrations agreed favorably to the accepted lifetime values for DTTCI and IR-125. At an injection concentration of 400.0 pM, the calculated
fluorescence lifetimes were determined to be 936 ps for DTTCl and 481 ps for IR-125, while at an injection concentration of 0.4 pM, the lifetime for IR-125 was 482 ps. The slightly longer calculated lifetime for IR-125 was attributed to the inability to effectively remove all background photocounts in the latter time bins within the decay profile. For IR-125 at a concentration of 0.4 pM, only 1.42 zmol of material was injected onto the column. Since approximately 10% of the material is effectively sampled due to the size of the separation column (i.d. = 75 μm), the focused laser beam (ω₀ = 5 μm) and slit width (1.2 mm), the decay profile for IR-125 at this dye concentration was constructed from approximately 85 molecules. This decay profile consisted of approximately 4,750 counts and was constructed using an integration time of 1 s in this case. Since the steady-state counting rate in the absence of any dye was determined to be 2,700 counts/s, the net fluorescence counts comprising the decay profile consisted of 2,050 photocounts, with a B/F photocount ratio of 132%. Due to the high B/F ratio, a time shift was implemented. When the time shift was inserted (86 ps), the counts included into the calculation were 2,780. Our simulation results indicated that under these experimental conditions, the time shift reduced the B/F ratio to approximately 8.5%, which subsequently reduced the number of scattered photons into the calculation to approximately 234.

In the case of the RLD method, a consistently lower lifetime was calculated for both dyes at the lower concentrations, even when the appropriate time shift was used. At a concentration of 400 pM, the lifetime determined via the RLD
algorithm for DTTCI was found to be 922 ps, while for IR-125, the lifetime was 482 ps. When the injected concentration was reduced to 4.0 pM, the lifetimes calculated were 910 and 454 ps for DTTCI and IR-125, respectively. These results are consistent with our previous research on static solutions, which indicated that the MLE algorithm yielded better accuracy in ultradilute conditions, even when a time shift was used.

The high accuracy obtained in the determinations, especially at the low dye concentrations, results partly from the use of near-IR fluorescence monitoring. Since few compounds show intrinsic fluorescence in the near-IR, few interferences are included into the decay profile in the form of background photons. This is particularly important when using the MLE and RLD algorithms, since they cannot differentiate between a single and/or multiexponential decays. In the presence of these interferences, the calculated lifetime represents a weighted average of the components comprising the decay, reducing the accuracy in the determination. In addition, the lower Raman scattering cross-sections in the near-IR when compared to the visible reduces the contribution of scattered photons into the decay profile as well.

Also included in Table 4.1 are the standard deviations using both the MLE and RLD algorithms. The calculated standard deviations were evaluated using equations 3.2 and 3.5, while the experimental standard deviations were obtained from 5-8 replicate measurements. In general, the MLE method produced smaller calculated and experimental standard deviations, consistent with our previous
ultrasensitive lifetime measurements in static solutions. For DTTCI at an injection concentration of 400.0 pM the experimental relative standard deviations (RSD, see Chapter 3, Equation 3.6) were found to be 0.7% and 1.5% for the MLE and RLD methods, respectively, while for IR-125 at this concentration, the RSDs were 0.8% and 1.5% for the MLE and RLD algorithms, respectively. At injection concentrations of 4.0 pM, the experimental RSDs were 2.4% and 3.6% for the MLE and RLD methods for DTTCI and for IR-125, these deviations were 1.9% and 2.4% for the MLE and RLD procedures, respectively. The reduced precision at the lower dye concentration results from the lower number of photons included into the calculation and also, the increased relative contribution of the scattered and background photons into the determination.

Inspection of the standard deviations for the MLE and RLD methods indicated consistently higher experimental standard deviations than the calculated standard deviations. The calculated standard deviation represents variability in the determination associated only with photon statistics (i.e., number of counts in the decay profile), while the experimental standard deviation has contributions from both the photon statistics and experimental conditions, such as instrument stability, dye microenvironment, and scattered and background photon contributions to the decay. In the case of DTTCI, the dye's photophysics represents a significant contribution to the uncertainty of the measurement. Due to the presence of the two charged alkyl sulfonate groups on IR-125, the dyes photophysics are less dependent on the nature of the solvent, whereas for the singly charged cationic
dye, DTTCI, its photophysics are more sensitive to its immediate environment. Therefore, the variability in the lifetimes for DTTCI from run-to-run may result primarily from small changes in the microenvironment resulting from a mixed methanol/aqueous running buffer producing changes in the dye’s lifetime. In the case of IR-125, the relatively short lifetime of the dye makes it difficult to effectively minimize the relative contribution of scattered photons in the calculation without sacrificing a large number of fluorescence photons, ultimately reducing the precision in the experiment.

4.3.2. Component Identification via Fluorescence Lifetime Discrimination.

In order to determine the ability to identify unknown peaks in an electropherogram using lifetime matching, a capillary electrophoretic separation of six near-IR dyes was performed with the lifetimes acquired for each peak in the electropherogram. The results are shown in Figure 4.3. As can be seen, four of the near-IR dyes elute early and are most likely cationic in nature, while the two late eluting peaks are probably anionic. Decay profiles were constructed over a time width of each band with the lifetimes calculated via the MLE algorithm. The identity of each peak was subsequently determined from the calculated lifetime and the known lifetime of the components, as determined by high concentration, static measurements via a nonlinear least squares analysis. The assignment of the peaks is given in Figure 4.3 along with the calculated lifetime values, with the decay profiles for each near-IR dye shown in Figure 4.4. For confirmation of the
Figure 4.3. Capillary electropherogram of six near-IR fluorescent dyes with peak identification via fluorescence lifetime matching. The lifetimes were calculated using the MLE method with the calculation commencing at the channel with the maximum number of counts. [DYE] = 100 pM. The mixture was electrokinetically injected onto the column at 5 kV for 5 s and a separation field strength of 367 V/cm.
Figure 4.4. Decay profiles for the six near-IR dyes in Figure 4.2. The integration times ranged from 2 s for DTTCI to 10 s for IR-125. The solid lines represent the slope of the respective decay profile.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
migration order, each dye was individually injected onto the CE column and the mobility calculated. The migration order predicted from lifetime identification agreed favorably with that found from the mobilities of the components. The advantage of lifetime matching for peak identification versus single component injections is that the identification process can be performed in a single CE run; whereas, in electrophoretic mobility matching, multiple CE runs are required.

4.3.3. Effect of Neighboring Components on Lifetime Determination.

To investigate the effects of neighboring components and background on the lifetime calculation using the MLE algorithm, the integration time for the construction of the decay profile was incrementally changed during the electrophoretic separation. Figure 4.5a shows an expanded view of an electropherogram consisting of three cationic dyes, HITCI ($\tau_f = 491$ ps), IR-140 ($\tau_f = 732$ ps) and IR-132 ($\tau_f = 697$ ps) along with the integration times used in the determination. In order to allow increasing levels of background and scattered photons into the calculation, the integration time was increased in the electropherogram of IR-140 alone. Figure 4.6 shows a series of decay profiles that were constructed from the electropherogram of IR-140 by itself using various integration times. As can be seen from the data in Figure 4.6, the decay profile collected over a 2 s integration time showed the absence of a prompt peak arising from scattered photons. When the integration time was 25 s, there was evidence of a prompt peak in the early time bins (nonlinear semi-log plot) and the background

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 4.5. CE separation of three cationic near-IR dyes (a) and lifetimes (b) as a function of the integration time. [DYE] = 40 pM. The integration times and the area they cover is shown in (a). The lifetimes were calculated using the MLE method with a 0 (circles) and 30 (squares) channel time shift. The electropherogram consisted of either IR-140 only (closed symbols) and HITCI, IR-140, and IR-132 (open symbols). The separation parameters were the same as listed in Figure 4.3.
Figure 4.6. Decay profiles for IR-140 only at various integration times corresponding to $t_1$, $t_2$, and $t_3$ from Figure 4.5.
level has increased, as seen by the increasing number of photocounts in time channels below channel number 400 (prior to excitation) and above channel number 1800 (minimal fluorescence photons).

Figure 4.5b shows the calculated lifetime values with and without introducing a time shift (86 ps) into the calculation for IR-140. Increasing the integration time for accumulation of counts into the decay profile for the electropherogram of IR-140 alone indicated that a calculated lifetime was independent of the integration time when a time shift of 86 ps was introduced. In the absence of a time-shift, a noticeable decrease in the fluorescence lifetime was observed. Since the background was composed primarily of scattered photons which have a temporal response coincident to the laser pulse, inclusion of these photons into the calculation would introduce a bias into the determination and lower the calculated fluorescence lifetime value. When photons from neighboring components were included into the lifetime calculation, a significant reduction in the lifetime for IR-140 was observed using a 30 channel (86 ps) time shift. The calculated lifetime of IR-140 decreased from 728 ps to 664 ps when the calculation included photons from neighboring components. This resulted from the fact that the calculated lifetime values represented a weighted average of the three components comprising the decay. The reduced lifetime arises from the fact that the two neighboring components have shorter lifetimes than IR-140. When not incorporating a time shift into the determination in the presence of neighboring constituents, the lifetime does decrease but not to the same degree as was seen for
IR-140 by itself. This results from the fact that more long-lived fluorescence photons were included into the decay with longer integration times than was the case for IR-140 by itself.

4.3.4. Lifetime Determination of Near-IR Dye-Labeled DNA Fragments Separated via Capillary Gel Electrophoresis.

In order to investigate the feasibility of acquiring lifetimes during the capillary gel electrophoretic separation of sequencing ladders, C-terminated fragments produced from standard Sanger chain-terminating protocols and labeled with a near-IR fluorophore on the primer were electrophoresed with lifetimes determined for the various components within the electropherogram. The challenge in performing lifetime measurements in CGE as compared to free solution CE is that the gel matrix in CGE produces a larger scattering background and introduces impurity components (fluorescence) into the determination as compared to CE. The electropherogram of these C-terminated fragments are shown in Figure 4.7a. In Figure 4.7, an expanded view of a section of the electropherogram is shown along with the integration periods that were used in order to construct the decay profiles. The integration time was set to 3-5 s. In Figure 4.7c, the decay profiles for the gel only (prompt peak, no dye-labeled oligonucleotide in the detection zone) and the near-IR dye-labeled oligonucleotide are shown. When the normalized prompt peaks generated from the gel column was compared to the free solution column, no residual fluorescence was observed in the gel case indicating little fluorescence interference contribution from the gel.
Figure 4.7. Capillary gel electropherogram of C-terminated DNA fragments (a) and an expanded view (b) showing the integration times over which the decay profiles were accumulated. In (c) is shown the decay profile for dye-labeled IRD41 labeled C-terminated fragment.
matrix and other additives when using near-IR excitation. As can be seen from this figure, the choice of the start time in the calculation (86 ps) eliminated a large fraction of the scattered photons produced from the gel matrix. Using this time shift, concentration, and injection conditions, approximately 20000 counts were included in the calculation. The average lifetime determined by the MLE algorithm was found to be 581 ps with a standard deviation of ±9 ps (RSD = 1.9%). Using Equation 3.3 and the average number of counts included into the calculation produced a standard deviation of approximately ±4 ps. The high precision obtained indicates that minimal fluorescent and scattering interferences contribute to the decay in CGE, a result of near-IR fluorescence monitoring.

4.4. Conclusions.

We have demonstrated the ability to accurately determine the fluorescence lifetimes of components separated via capillary electrophoresis with high precision in the zmol regime using time-correlated single photon counting. A comparison of two simple algorithms (MLE and RLD) for calculating lifetimes were compared and the data indicated that the MLE produced higher accuracy and precision in ultradilute conditions. Decay profiles for IR-125 were constructed from 85 molecules and a lifetime determined with high accuracy and precision using the MLE method during CE. The favorable accuracy and precision was aided by the use of near-IR fluorescence detection, which minimizes background contributions from scattered and impurity fluorescence photons. The identity of unknown
components in CE were definitively identified via lifetime matching in a single electrophoretic run. In addition, the lifetime of C-terminated oligonucleotides separated via CGE were calculated with high precision. This result indicates that lifetime discrimination can be a viable approach to base-calling in sequencing applications. If definitive identification of the terminal base could be accomplished with lifetime differences for a series of dyes at 3σ, then our data indicates that only a 27 ps difference would be required.

One disadvantage of near-IR lifetime monitoring is the short upper state lifetimes associated with these dyes, especially in predominately aqueous media. This requires the inclusion of large percentages of organic solvents into the running buffer, such as methanol, which not only increases the fluorescence lifetime, but also improves the photophysical characteristics of the dye (i.e., photon statistics, quantum yield). Even in organic solvents, these dyes display lifetimes in the several hundred picosecond range, requiring a time-correlated single photon counting instrument with a timing response in the 100-200 ps regime. In this study, the use of a passively mode-locked Ti:sapphire laser and a single photon avalanche diode detector produced an instrument with the desired characteristics. The avalanche diode is a convenient device for the present application due to its favorable timing response, high sensitivity, low dark count rate, low cost, and long lifetime. While the Ti:sapphire laser is a solid state instrument, it requires pumping by an Ar ion laser, making it difficult to operate and cost-prohibitive for the development of a time-correlated instrument for CE applications. However,
pulsed diode lasers can be constructed at a fraction of the cost of the Ti:sapphire laser system and can achieve light levels and pulse widths appropriate for this application. The use of a pulsed diode laser and avalanche photodiode can be used to construct a low cost and rugged time-correlated single photon counting device for lifetime measurements in many different analytical applications, including CE.

4.5. References.


CHAPTER 5

An All Solid-State Near-IR Time-Correlated Single Photon Counting Instrument for Dynamic Lifetime Measurements in DNA Sequencing Applications

5.1. Introduction.

Tremendous efforts are currently being invested into developing new technologies for sequencing the human genome. The major thrusts of the technology development are directed towards increasing the speed of acquiring sequencing data, multiplexing instruments to improve throughput, and improving the accuracy in base-calling during the separation process. The general protocol for identifying the four constituent bases (adenine, A, cytosine, C, guanine, G, and thymine, T) is to attach one of four unique fluorescent probes to each base and use spectral discrimination for identification. While this has been a fairly robust method and is the technique commonly incorporated into many commercial automated DNA sequencers, the protocol does present itself with potential difficulties, including the need for multiple excitation sources and/or detection channels, electrophoretic mobility differences between the four fluorescent labels and cross-talk between detection channels due to spectral overlap which can produce errors in identifying the constituent bases during the sequence analysis. In high throughput instruments, where many sequencing lanes must be run in parallel, the necessity for multiple detection channels to process the fluorescence from each label can make the sequencing device instrumentally intensive.
We have recently suggested an approach to base-calling in DNA sequencing applications using fluorescence lifetime discrimination of dyes with similar absorption and emission maxima along with similar electrophoretic mobilities, but possess unique fluorescence lifetimes. Several advantages are associated with lifetime discrimination, including; (1) the calculated lifetime is immune to concentration differences; (2) the fluorescence lifetime can be determined with higher precision than fluorescence intensities and; (3) only one excitation source is required to efficiently excite the fluorescent probes and only one detection channel is needed to process the fluorescence for appropriately selected dyes. One of the potential difficulties arising from the use of lifetime discrimination in base-calling applications for DNA sequencing is the poor photon statistics produced from the need for making a dynamic measurement (the chromophore is resident in the excitation beam for 1-5 s) and the low mass loading levels associated with capillary electrophoresis. This common fractionating method used for DNA sequencing results in a typical loading range of 0.01 - 1.0 attomole of material for each electrophoretic band.

We have recently demonstrated that a simple computational method, known as the maximum likelihood estimator (MLE), in conjunction with near-IR fluorescence can be used for lifetime determinations of C-terminated DNA fragments with high accuracy and precision. The difficulty in obtaining an accurate and precise lifetime value is compounded by the presence of the gel matrix in which the DNA is sieved which can result in a large amount of scattering.
and impurity fluorescence. However, using near-IR fluorescence, where matrix interferences are significantly reduced compared to visible excitation, significantly improves the accuracy and precision in the measurement. In these experiments, a passively mode-locked Ti:sapphire laser pumped by the all-lines output of an Ar ion laser and conventional time-correlated single photon counting electronics were used to analyze the time-resolved fluorescence of DNA fragments labeled with a single dye and separated via capillary gel electrophoresis.

Another potential difficulty associated with lifetime measurements in DNA sequencing applications is the need for sophisticated and complex instrumentation. The use of mode-locked lasers and the extensive electronics required for TCSPC can make the measurement difficult, especially for those not well trained in laser operations. While others have shown that pulsed diode lasers can be used for TCSPC, these devices did not possess the ability to make dynamic measurements of chromophores with subnanosecond lifetimes with sufficient sensitivity to determine lifetimes for small amounts of fluorescing material.

In this chapter, we wish to discuss the development of a simple TCSPC apparatus which uses all solid-state components and basically is turn-key in operation. The excitation source consists of a pulsed GaAlAs diode laser with a single photon avalanche diode (SPAD) serving as the photodetector. In addition, the system utilizes a PC-board in which all of the electronics needed for making a TCSPC measurement are situated. The use of this instrument will be demonstrated in DNA sequencing applications by identifying bases in a two-dye
labeled experiment with the oligonucleotides separated via capillary gel electrophoresis (CGE).

5.2. Instrumentation.

5.2.1. Pulsed Diode-Based Near-IR TCSPC System.

A block diagram of the near-IR laser-induced fluorescence system for capillary electrophoresis utilizing the pulsed diode laser source (PicoQuant GmbH, model DL-4040, Berlin Germany) and TCSPC board (PicoQuant GmbH, model SPC-300, Berlin Germany) is shown in Figure 5.1. The laser source was an actively pulsed solid-state GaAlAs diode with a repetition rate of 80 MHz, and an average power of 5.0 mW at a lasing wavelength of 780 nm. The laser beam was converted from an elliptical to circular shape using external cavity correction optics. The diode head was driven by an electrical short pulse generator which supplied high repetition rate picosecond current pulses. The laser driver consisted of an RF pulse generator, fast switching stage, coax line driver and a pulse shaper stage (see Figure 5.2a). The driver delivered 3.6 W (50 Ω) current pulses at 80 MHz to the diode head with a FWHM of 500 ps.

The laser was focused onto a capillary tube to an approximately 14 μm (1/e²) beam waist using a diode laser singlet lens (Melles Griot, Irvine, CA). The emission was collected in a conventional 90⁰ format with a 40X high NA microscope objective (Nikon, Natick, MA, NA=0.85) and the emission spatially filtered with a slit (width = 0.4 mm). The fluorescence was further isolated from
Figure 5.1. Diagram of our diode-based near-IR TCSPC instrument. M, Mirror; L, Lens; C, Capillary; BD, Beam dump; MO, Microscope objective; SPAD, Single photon avalanche diode detector; CFD, Constant fraction discriminator; TAC, Time-to-amplitude converter; ADC, Analog-to-digital converter.
Figure 5.2. Block diagram of the electronics for the pulsed diode laser (a) and the TCSPC board (b). CFD, Constant fraction discriminator; TAC, Time-to-amplitude converter; ADC, Analog-to-digital converter.
scattering photons using an 8 cavity interference bandpass filter (CWL = 850 nm, HBW = 30 nm, Omega Optical, Brattleborough, VT). The filtered fluorescence was then focused onto the photodetector by a 10× microscope objective producing an image of approximately 20 μm on the face of the photodetector. The photodetector was a single photon avalanche diode (SPAD, EG&G Electrooptics Canada, Vaudreuil, Canada) stationed on a thermoelectric cooler with a photoactive area of $1.77 \times 10^{-4}$ cm$^2$ (i.d. = 150 μm) and dark count rates of approximately 100 cps. The pulses from the photodetector were amplified (Phillips Scientific, Mahwah, NJ) 20-fold and sent to the time-correlated single photon counting board. The board, which plugs directly into the PC-bus, consisted of a constant fraction discriminator, time-to-amplitude converter, analog-to-digital converter, and multichannel analyzer with 128 parallel channels (see Figure 5.2b).\textsuperscript{12,13} The electronics have a dead time of <260 ns, allowing efficient processing of single photon events at counting rates exceeding 2 million cps. The timing jitter in the electronics was determined to be < 20 ps. The controlling software was written in LabView (National Instruments, Austin, TX). The electronics allowed collection of 128 decay profiles with a timing resolution of 9.77 ps per channel. The instrument response function (IRF), determined from the gel only, was found to be 275 ps (FWHM). In the present experiments, each decay profile was collected for 2 s during the electrophoresis, allowing a total data acquisition time of 256 s after which time, the data was dumped to memory producing a down time of approximately 25 s. The duty cycle, which is defined as
the ratio of the down time to the collection time, was 10%.

The intensity electropherograms were acquired using a PC-resident time/counter board (Computer Boards, INC, model CIO-CTR05, Mansfield, MA). Counts were accumulated for 1 s intervals of time during the electrophoresis to construct the intensity electropherograms.

5.2.2. Capillary Gel Electrophoresis.

The gel columns, which consisted of a 5% crosslinked denaturing polyacrylamide gel (75 µm i.d., 375 µm o.d., Polymicro, Phoenix, AZ) were prepared using published procedures.14 The column was cut to a total length of 60 cm, with the distance from injection to detection being 53 cm. The voltage to the capillary column was supplied by a Spellman high voltage power supply (CZ1000R, Plainview, NY). In all cases, the electrophoresis was performed using a field strength of 250 V/cm. DNA samples were inserted onto the gel column using an electrokinetic mode, in which the capillary tube was inserted into a DNA sample, a 15 kV voltage applied for 90 s, and then placing the capillary tube back into the running buffer subsequent to performing the electrophoresis. The running buffer for the electrophoresis consisted of a TRIS buffer, with borate and EDTA (pH = 8.3) containing 7.3 M urea as the denaturant.
5.2.3. Preparation of DNA Sequencing Ladders.

The DNA sequencing ladders were prepared using standard Sanger sequencing methods.\textsuperscript{15,16} The DNA primers were labeled with one of two near-IR dye-labeled primers (Li-COR, Lincoln, NE). The structures of the dye along with their absorption/emission maxima and fluorescence lifetimes are shown in Figure 5.3. All the chemical properties shown in Figure 5.3 were determined in an unpolymerized gel matrix consisting of 5\% acrylamide, TRIS/borate buffer with EDTA and urea. IRD40 was used to label DNA fragments terminated in A and IRD41 was used for labeling of the C-terminated DNA fragments in the sequencing experiments.

5.3. Results and Discussion.

Due to the similar absorption and emission maxima of the labeling fluorescent dyes used in this experiment, dideoxynucleotide base identification using spectral discrimination would be difficult in DNA sequencing experiments. However, the dyes selected show distinct fluorescence lifetimes ($\Delta \tau = 69$ ps), which should permit facile identification using lifetime discrimination for an instrument which possessed an instrument response function adequate for measuring subnanosecond lifetimes since, in the present case, these dyes show lifetimes which range from 580-650 ps. Performing a static experiment (non-capillary gel electrophoresis) over long integration times and high dye concentrations to minimize scattering photon contributions into the decay and
Figure 5.3. Chemical structures and properties of the near-IR dye-labeled primers.

IRD40
Abs $\lambda_{\text{max}} = 783$ nm
Em $\lambda_{\text{max}} = 805$ nm
Lifetime = 650 ps
$\mu_{\text{ep}} = 1.83 \times 10^{-4}$ cm$^2$/Vs

IRD41
Abs $\lambda_{\text{max}} = 787$ nm
Em $\lambda_{\text{max}} = 807$ nm
Lifetime = 581 ps
$\mu_{\text{ep}} = 2.02 \times 10^{-4}$ cm$^2$/Vs
using non-linear least squares methods to determine $\tau_f$ values of 650 ps for IRD40/DNA primer and 581 ps for IRD41/DNA primer were found, with both adequately described by a monoexponential function ($\chi^2 \sim 1.03$). In this case, the lifetimes of these probes were determined in an unpolymerized gel matrix with urea (DNA sequencing conditions), since the photophysics of these type of dyes have been shown to depend dramatically upon the solvent system.$^{17}$

Figure 5.4a shows the intensity electropherogram for the dye-labeled DNA primers only electrophoresed in the capillary gel column. In this experiment, each electrophoretic band contained $\approx 6.2 \times 10^{-21}$ moles (3900 molecules) of dye-labeled DNA, which was calculated from the apparent mobility of the dye, primers, injection conditions and the dye concentration. As can be seen in this figure, two bands are present, even though both dyes are attached via a linker to the same sized DNA primer. From charge considerations only (IRD41 is neutral and IRD40 is anionic), one would expect that the IRD40-labeled primer would migrate faster than the IRD41-labeled primer. However, the point of covalent attachment of the dye to the primer linkage also affects the frictional factor which influences the mobility, resulting in a faster migration rate for the IRD41 dye/ primer, irrespective of charge considerations only.$^{14}$ The electrophoretic mobility of the IRD40 labeled primer was determined to be $1.83 \times 10^{-4}$ cm²/Vs and for the IRD41 labeled primer, the electrophoretic mobility was calculated to be $2.02 \times 10^{-4}$ cm²/Vs. In Figure 5.4b is shown the decay profiles for the gel matrix and the fluorescently labeled primers integrated over the points indicated in Figure 5.4a.
Figure 5.4. Capillary gel electropherogram of dye-labeled DNA primers (a) and time-resolved decay profiles (b) of the gel matrix, IRD40 and IRD41 dye-labeled primers. The capital letters in (a) represent time intervals over which the decay profiles were collected. [DYE] = 1 pM.
In order to calculate the fluorescence lifetimes for these DNA bands on-line during the electrophoresis, maximum likelihood estimators were used, which is given by Equation 3.2, where in this case, $T$, the time width of each bin, was 9.77 ps and $m$, the time interval over which the lifetime was calculated, 10 ns. The lifetimes shown in Table 5.1 were calculated from the right-hand-side of Equation 3.2. The relative precision in the measurement using this relationship is simply given by $N_i^{(1/2)}$ when $\tau_f >> T$ as is the case for the present conditions. In order to minimize the amount of scattering photons included into the calculation for the lifetimes, the determination was carried out over a time interval that was shifted by 97 ps (see solid line in Figure 5.4b) from the channel containing the maximum number of photocounts with 440 channels (4.3 ns) included into the calculation. The calculated lifetimes for the points indicated in Figure 5.4a are shown in Table 5.1 along with the total number of counts included into the calculation as well as the standard deviation in the measurement, which was determined from $N_i^{(1/2)}$ multiplied by the observed lifetime. As can be seen from these results, the apparent lifetimes for points A and J, where only the gel matrix contributes to the decay, was found to be approximately 412-422 ps, but with significantly fewer photocounts included in the calculation when compared to the case where dye-labeled DNA was present. When dye/DNA was resident within the detection volume, the lifetime values varied, with the value determined by the identity of the dye. At point B, a value of 585 ps was determined, which corresponds to the value calculated in the static case for IRD41. At point D, the lifetime value was
Table 5.1. Calculated fluorescence lifetimes for the near-IR dye-labeled DNA sequencing primers. The letters indicate the points during the electrophoresis in which the decay profiles were constructed (see Figure 5.4a). Each decay profile was collected over a 2 s interval.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Lifetime (ps)</th>
<th>Number Photocounts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Standard Deviation&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>422</td>
<td>11,060 ± 4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>585</td>
<td>73,945 ± 2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>582</td>
<td>71,336 ± 2</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>595</td>
<td>68,654 ± 2</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>645</td>
<td>46,844 ± 3</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>660</td>
<td>58,784 ± 3</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>662</td>
<td>53,727 ± 3</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>666</td>
<td>31,731 ± 4</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>661</td>
<td>23,270 ± 4</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>412</td>
<td>12,152 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Includes only those counts used in the lifetime calculation.

<sup>b</sup> Calculated by Equation 3.3.
seen to be greater than that expected for IRD41 alone. This resulted from the fact that the algorithm used to calculate the lifetime cannot differentiate between a mono- and multiexponential decay, and the calculated lifetime represents a weighted average of the various components comprising the decay. At this point during the electrophoresis, both IRD40 and IRD41 were resident within the detection zone. At points F-I, the lifetime values were similar to that calculated for IRD40 in the static experiment, since only this dye was expected to be present in the detection zone.

In Figure 5.5 is shown the intensity electropherogram for A- and C-terminated DNA sequencing fragments separated by capillary gel electrophoresis. In this particular experiment, IRD41 was used to label the DNA fragments that were terminated in a C and IRD40 was used for labeling the A-terminated DNA fragments. Peaks were still observed after 9,000 s with fluorescence signals on the order of 3,000-5,000 counts per peak. Since only two dyes were available, the T's and G's were not identified. In Figure 5.6 is shown the decay profiles for the gel matrix, IRD41 and IRD40 dye-labeled DNA fragments represented by (*) in Figure 5.5. The number of photocounts in this case was substantially less than that found in the case of the primers only. Therefore, the scattering photons make a larger contribution into the decay, which can potentially introduce bias into the calculated lifetime. Over the time interval in which the lifetime was calculated, 17,373 counts were included in the determination for the IRD41 DNA fragment and 26,096 counts for the IRD40 DNA fragment.
Figure 5.5. Steady-state fluorescence intensity electropherogram of A- and C-terminated DNA fragments separated by capillary gel electrophoresis. The sequencing ladder was injected for 90 s at 15 kV with the separation carried out at the same field strength.
Figure 5.6. Time-resolved decay profiles for the gel matrix and two bands taken from Figure 5.5, which are indicated with an asterisk.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
In Figure 5.7 is shown an expanded view of the intensity electropherogram shown in Figure 5.5 along with the identity of the terminal base and some of the calculated lifetimes, while in Figure 5.8 is shown a three dimensional plot of intensity (cps) vs. time (ps) vs. time (s). Over the entire electropherogram, the average lifetime value determined for the IRD40 A-terminated DNA fragments was found to be 669 ps, with a standard deviation of ± 42 ps, while for the IRD41 C-terminated DNA fragments, the average lifetime was 528 ps and a standard deviation of ± 68 ps. Both of these values are similar to the lifetime values calculated in the static case. However, the standard deviations in these measurements were larger than expected based upon the average number of counts included into the determination. For the A-fragments the average number of photocounts in the lifetime calculation was found to be 19,928 and for the C-fragments, 10,781 counts, which would result in standard deviations of ± 5 ps and ± 6 ps, respectively, if the precision in the measurement was determined primarily by photon statistics. The large standard deviation in these experiments results from the fact that the TCSPC electronics sequentially collect the decay profiles over 2 s intervals during the electrophoresis. In some cases, the data accumulation time for construction of the decay profile may not be aligned (in time) with the time interval over which the electrophoretic band is resident within the laser beam. This would result in a decay profile that was constructed on the rising or falling edge of the electrophoretic band and inclusion of a disproportionately large amount of scattering photons, biasing the determination to lower lifetime values.
**Figure 5.7.** Expanded view of the intensity electropherogram shown in Figure 5.5 with bases identified via lifetime discrimination.
Figure 5.8. Time-resolved decay profiles for a series of 2 s intervals as collected by the TCSPC board. The series shown here is labeled 3-D in Figure 5.7.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
(see Figure 5.9). In addition, if two bands were present in the detection zone during decay profile construction, the calculated lifetime would represented a weighted average of both dyes. These situations could be corrected by including a threshold condition into the hardware and/or software, in which the decay profiles were collected only over time intervals in which the steady-state counting rate exceeded the average background rate by some predetermined value.

5.4. Conclusions.

We have demonstrated the use of a simple, all solid-state TCSPC device to dynamically measure fluorescence lifetimes in DNA sequencing applications. The major components of the instrument consisted of a pulsed-diode laser, which produced picosecond pulses at 80 MHz, a single photon avalanche diode and counting electronics resident in a PC. The TCSPC device demonstrated a timing resolution of 275 ps, allowing measurements of chromophores with subnanosecond lifetimes using simple algorithms. In addition, the device was sensitive enough to acquire lifetimes with reasonably high precision during short integration times and low concentrations of chromophores within the sampling zone. The instrument is also easy to operate and economical and will find applications in other areas of biology and chemistry, where time-resolved fluorescence has found many applications for studying various phenomena.
Figure 5.9. Expanded view of Figure 5.5 showing the inability of the counting board to effectively integrate over only one peak in the electropherogram. The data collection time for each decay profile was set for 2 s. The vertical solid lines show the boundaries for accumulation of counts into the decay profile.
5.5. References.


CHAPTER 6

Lifetime Determinations for Generated Gaussian Peaks as a Function of Electrophoretic Resolution

6.1. Introduction.

The use of the simple algorithms as described in Chapters 3-5 permit the calculation of fluorescence lifetimes at very low photocount levels with high accuracy and precision and minimal computational effort.\(^1\) The major disadvantage associated with these algorithms is their inability to distinguish between single and multiexponential decays; therefore, only a single lifetime can be calculated from any given decay profile. The resulting lifetime is consequently a weighted average of the various components comprising the decay.

Since the proposed detection scheme for DNA base-calling applications outlined in Chapter 1 involves the fluorescence lifetime discrimination of four near-IR dye-labeled dideoxynucleotide terminated DNA fragments possessing unique fluorescence lifetimes, overlap of the electrophoretic peaks can introduce base-calling errors when poor electrophoretic resolution results and these simple algorithms are implemented. As can be seen from Figure 5.9, the decay profile associated with the time interval between 3577-3579 s would be composed of two separate electrophoretic peaks. In theory, if both peaks were A-terminated IRD40-labeled DNA fragments, the lifetime associated with this decay, barring any interference from the background, would be 660 ps as determined by the MLE or RLD methods. Likewise, if both peaks were C-terminated IRD41-labeled
fragments, the lifetime calculated from the decay would be 581 ps for the MLE or RLD. However, if one peak was a C-terminated fragment and the other was an A-terminated fragment, the resulting calculated lifetime would be a weighted average of the two components comprising the peak with a calculated value which would range between 581 ps and 660 ps depending on the fractional contribution of each component. With our DNA base-calling scheme, four separate tags having four distinct lifetimes are required and, since the fractionation step does not completely separate the individual components, an understanding of the importance of the effects of overlapping peaks is needed, along with the ability to determine the fluorescence lifetimes at short integration times and in the limit of low photocounts.

For our high-throughput DNA sequencing methodology, the detection scheme needs to be able to handle large amounts of data. In this application, computer software is being written to process the data, which will collect the electropherogram [intensity vs. time (ms)] data and simultaneously construct decay profiles over specified time intervals. The ability to calculate fluorescence lifetimes over very short time intervals from Gaussian-shaped electrophoretic peaks is of utmost importance. In order to aid in the base-calling accuracy in DNA sequencing applications and demonstrate the ability to calculate fluorescence lifetimes from very short counting intervals, Monte Carlo simulations were developed to assess the performance of the MLE and RLD algorithms in
determining the decay parameters from neighboring Gaussian peaks of varying resolution.

Monte Carlo simulations derive their name from the use of random numbers to model experiments using probability functions. These probability distributions are generated using analytical expressions describing the physical event. Thus, Monte Carlo simulations provide the ability to compare experimental results with results generated from modeled systems.

In this chapter, Monte Carlo simulations of generated Gaussian peaks were performed and lifetime values determined from decay profiles constructed from time periods along the electropherogram. The performance of the MLE and RLD algorithms of generated peaks which simulated actual DNA sequencing data, in which the electrophoretic peaks contained relatively low numbers of photocounts, was studied. In this chapter, Monte Carlo simulations were used to study the effect of electrophoretic resolution between peaks on the calculated lifetime using both the MLE and RLD algorithms. In addition, accuracy and precision were analyzed for the calculated lifetimes during the electrophoresis as a function of integration time.

6.2. Development of Monte Carlo Simulations.

The computer program for the Monte Carlo simulations was written in Turbo Pascal (See Appendix). A flow chart of the program is shown in Figure 6.1. In general, the program constructs an electropherogram in which the time
**Figure 6.1.** Flowchart of the computer program for Monte Carlo simulations.
scale on the x-axis is divided into 0.2 s intervals. Gaussian peaks were then constructed from fluorescence counts input by the user and then superimposed onto a background. The decay profiles were then formed from time slices along the electrophoresis time scale with the correct number of background counts and fluorescence counts. From the convolved decay profile, the fluorescence lifetimes were then calculated via the MLE and RLD algorithms as explained by Equations 3.1 and 3.4, respectively.

6.2.1. Input Parameters and Basic Equations.

In order to construct the Gaussian peaks with the appropriate resolution between them, there are many input parameters needed, such as the length of the capillary from injection to detection ($L_d$, cm), electric field strength of the separation ($E$, V/cm), electrophoretic mobility of each component ($\mu_{ep}$, cm$^2$/Vs), time width at half height for each peak ($w_{1/2}$, s), total number of fluorescence counts in each peak ($N_f$), fluorescence lifetimes for the components ($\tau_f$, ps) and average number of background counts per electrophoresis time channel ($N_b$). The numbers input for this application were obtained from results of actual DNA sequencing experiments performed in our lab.$^{3,4}$

The following expression was used to calculate the migration time, $t_{mg}$:

$$t_{mg} = \frac{L_d}{E\mu_{ep}}$$  \hspace{1cm} (6.1)
where $\mu_{ep}$ is the electrophoretic mobility.

The resolution ($res$) between two neighboring peaks was calculated from the relation:\textsuperscript{6}

$$res = \frac{t_{mig}[2] - t_{mig}[1]}{\left(\frac{1}{w_{1/2}[2]} + \frac{1}{w_{1/2}[1]}\right)}.$$ \hspace{1cm} (6.2)

The expression for calculating the standard deviation, $\sigma_G$, for each of the electrophoretic peaks was:\textsuperscript{7}

$$\sigma_G = \frac{w_{1/2}}{2.354}.$$ \hspace{1cm} (6.3)

\textbf{6.2.2. Insertion of Background Counts into Simulated Electropherogram.}

For construction of the electropherogram, the background counts were inserted into each of the 2000 electrophoretic channels. Since the background counts were distributed by Poisson statistics, the standard deviation, $\sigma_b$, was calculated from the square root of the mean of the average background. The probability function, $P_b$, for the distribution of the background counts into the time channel was calculated from:\textsuperscript{6}

$$P_b = \frac{e^{-((T1-N_b)^2/2(\sigma_b)^2)}}{\sigma_b \sqrt{2\pi}}.$$ \hspace{1cm} (6.4)
where $TI$ was a randomly selected count number. In this case, $P_b$ was calculated using a Gaussian function since the mean count rate, $N_b$, was $>20$, in which case Poisson distributions can be adequately described as a Gaussian function. After $P_b$ was calculated, a test function was generated, $T2$, which was a random number between 0 and 1. If the condition $P_b > T2$ was true, the background count number generated for that channel ($TI$) was accepted. This process was repeated until the 2000 time channels had the appropriate background counts.

### 6.2.3. Creation of Gaussian Peaks.

The mean position of each electrophoretic peak within the 2000 time channels was determined from the migration time for each component. The electrophoretic peak shape in these simulations was assumed to be Gaussian. For each peak, the fluorescent photon distribution was determined over 400 channels in 0.2 s intervals centered at $t_{mg}$. The expression used for determining the distribution of the fluorescence counts within the Gaussian peaks, $P_G$, was:

$$P_G = \frac{e^{-((0.2 \cdot x)^2 / (2(\sigma_G)^2))}}{\sigma_G \sqrt{2\pi}}$$  \hspace{1cm} (6.5)

where $x$ was a randomly selected channel between 0 and 400. A random number was generated between 0 and 1 ($TI$), and if the condition $P_g[x] > TI$ was true, a
fluorescent count was inserted into that time channel (x). This process was repeated until all the fluorescence photocounts, \( N_f \), were placed into the appropriate time bin. After the fluorescence photocounts were placed in the appropriate time bins, the background was added to each channel to complete the electropherogram.

6.2.4. Construction of the Decay Profile.

The next step was to determine the interval over which the decay profile was to be constructed. The start channel and stop channel in the electropherogram were selected as input parameters by the operator. From the simulated electropherogram, the program was able to calculate the contributions of fluorescence counts from each peak into the simulated decay along with the total background contribution. The percent composition of each component, \( P_{k\alpha} \), in the decay profile was calculated from:

\[
P_{k\alpha} = \frac{\sum_{n=s}^{f} N_{p\alpha}(n)}{\sum_{n=s}^{f} N_T(n)} \quad (6.6)
\]

where \( N_{p\alpha}(n) \) was the number of fluorescence counts in the decay profile from only one component in channel \( n \), \( N_T(n) \) is the total number of fluorescence counts in channel \( n \), \( s \) is the start channel, and \( f \) is the end channel.
6.2.5. Model Decay Profile.

The model decay profile, before convolving with the instrument response function, was calculated from;

\[ I_{f(t)} = \sum_{i=1}^{n} P_{k_\gamma(i)} e^{-t/\tau_{f_i}} \]  

(6.7)

where \( \tau_{f_i} \) is the fluorescence lifetime of each component comprising the decay with the sum was carried out over the total number of components, \( i \), and \( t \) represents the time bin within the decay. The variable ‘\( t \)’ was selected over 4096 time channels with each channel representing 2.88 ps. These values were normalized to produce a probability distribution, \( P_{f(t)} \), using;

\[ P_{f(t)} = \frac{I_{f(t)}}{I_{f(max)}} \]  

(6.8)

where \( I_{f(t)} \) is the fluorescence intensity in each channel \( t \) and \( I_{f(max)} \) is the maximum fluorescence intensity in a time channel.
6.2.6. Prompt Function with Correct Number of Background Counts.

Using an experimental instrument response function, the probability of placing a background photocount within the response function, \( P_{d(i)} \), was determined from:

\[
P_{d(i)} = \frac{c_i}{T_{mx}}
\]

(6.9)

where \( c_i \) corresponds to the counts in time channel \( i \) and \( T_{mx} \) is the maximum number of counts from any channel \( i \) within the experimental response function. A channel number was randomly selected, \( TI \), with a value between 1 and 4096. A second random number was then generated (\( T2 \)) with a value between 0 and 1. If the condition \( P_{d(i)} [TI] > T2 \) was true, a background photon was placed in time channel \( TI \). This procedure was repeated until the appropriate number of background counts were placed within the simulated decay.

6.2.7. Convolving Background Prompt Function and Fluorescence Decay Profile.

For construction of the simulated decay profile, the instrumental response function, given in the form of a probability \( (P_{d(i)}) \) and the fluorescence decay probabilities \( (P_{f(i)}) \) were convolved to form a single function through the use of a fast Fourier transform. After production of the convolved decay profile, the
probability distribution \( P_{\tau_0} \) for each time channel was then determined and a new decay profile was formed with the correct number of fluorescence photocounts. This was accomplished by selecting a time channel \( t \) and then generating a test function, \( T/I \). If the condition, \( P_{\tau_0} > T/I \) was true, then the photocount was inserted into time channel \( t \). This process was repeated until the pre-selected number of fluorescence photocounts had been placed into the convolved decay profile. The final decay was constructed by adding the background counts from the prompt function with the correct number of background counts to the fluorescence counts over 4096 time channels. The fluorescence lifetimes were then calculated using Equations 3.1 and 3.4 for the MLE and RLD, respectively, at a 45 channel (130 ps) time shift from the channel with the maximum number of counts and over an interval of \( 5 \times \tau_f \).

6.3. Results and Discussion.

In Figures 6.2 and 6.3 are shown the Monte Carlo simulation results for determination of fluorescence lifetime values as calculated by the MLE and RLD methods, respectively, for two generated Gaussian peaks. The input parameters for the construction of the two peaks and also the decay profiles were as follows: 

\[ \mu_{ep}[1] = 6.6667 \times 10^{-5} \text{ cm}^2/\text{Vs}; \quad w_{1/2}[1] = 5.00 \text{ s}; \quad N_f[1] = 75000 \text{ counts}; \quad \tau_f[1] = 750 \text{ ps}; \quad \mu_{ep}[2] = 6.6511 \times 10^{-5} \text{ cm}^2/\text{Vs}; \quad w_{1/2}[2] = 8.00 \text{ s}; \quad N_f[2] = 65000 \text{ counts}; \quad \tau_f[2] = 850 \text{ ps} \text{ and } N_b = 2200 \text{ counts per channel.} \] 

The two peaks in this case had a resolution of 0.53 (Equation 6.2).
Figure 6.2. Simulated electropherogram containing two generated Gaussian peaks (resolution = 0.53) with the lifetimes in 1 channel (0.2 s) intervals calculated via the MLE method. The dashed line represented the weighted lifetime as calculated by the fractional composition of each peak. The vertical lines represented the standard deviations calculated from three replicate measurements. The horizontal dotted lines represented the expected lifetimes for peaks 1 and 2.
Figure 6.3. Simulated electropherogram containing two generated Gaussian peaks (resolution = 0.53) with the lifetimes in 1 channel (0.2 s) intervals calculated via the RLD method. The dashed line represented the weighted lifetime as calculated by the fractional composition of each peak. The vertical lines represented the standard deviations calculated from three replicate measurements. The horizontal dotted lines represented the expected lifetimes for peaks 1 and 2.
As can be seen from Figures 6.2 and 6.3, the MLE and RLD methods were able to determine lifetimes which agreed favorably with the true lifetime of component 1 comprising peak 1 when the calculation was centered on peak 1. However, the calculated lifetime deviated from the true lifetime of component 2 comprising peak 2 when the calculation was centered on peak 2. From the simulation results for channel 15000, it was determined that the fractional contribution from component 1 was 0.937 (~2810 counts) and 0.063 (~191 counts) from component 2, resulting in a calculated lifetime which agreed to the true lifetime of component 1 due to the small contribution of component 2 into the calculation. For channel 15035, centered on peak 2, the lifetime values determined for the MLE and RLD methods were less than the expected value. The expected lifetime for this time interval was 850 ps (component 2) since the fractional contribution for this time channel from component 1 was small (0.00943 component 1; and 0.991 component 2). The lifetime values were lower than what was expected due to the fact the decay for this channel was constructed from fewer fluorescence photocounts and high number of background counts.

Since the resolution of the two peaks was not baseline resolved, time channels between the center of both peaks were comprised of contributions from each component. In this case, the observed lifetime calculated by the MLE and RLD methods would be a weighted average of the components within the given time period. For example, the lifetimes that were calculated at channel 15019, where there is significant contribution from both components within the decay
profile, were 795 ± 23 ps and 814 ± 21 ps for the MLE and RLD, respectively. This time period had a fractional contribution from component 1 of 0.361 (~583 counts) and component 2 had a fractional contribution of 0.639 (~1030 counts) superimposed on a background of ~2110 counts. Thus, the weighted average lifetime was calculated to be 813 ps, which agreed with the determined value. Therefore, peak identification via lifetime discrimination of the two components would be difficult via the use of these simple algorithms when selecting time channels along the electrophoresis axis which contains sizable contributions from both components.

For these simulations, the preferred results would be a lifetime value of 750 ps for all channels which contain predominately fluorescence counts associated with component 1 (peak 1) and 850 ps for all channels which contain fluorescence signal from component 2 (peak 2). If peak overlap occurs, then the lifetime algorithm should be able to extract a biexponential decay giving values of 750 and 850 ps and also the fractional contribution of each within the decay. The major drawback with the MLE and RLD algorithms is the ability to determine only a single lifetime from a multiexponential decay.

At channel 15000, the background contribution in the decay was found to be ~1990 counts, yielding a background-to-fluorescence ratio (B/F) of 0.66 in this case. For the lifetime determinations, the start channel within the decay profile was shifted 130 ps in order to aid in removing background due to scattered photons which biases the observed lifetimes to lower values.¹ However, the use
of a time shift also biases the calculated lifetimes by exclusion of early arriving fluorescence photocounts. Therefore, corrections were made within the calculation to offset this bias. When the time shift was implemented, the number of background counts in the determination at channel 15000 was found to be ~252 counts and the fluorescence signal was ~2480, reducing the B/F ratio to 0.102. The calculated lifetimes were $761 \pm 9$ ps and $739 \pm 17$ ps for the MLE and RLD algorithms, respectively, which agreed favorably with the true lifetime of component 1. The expected standard deviations, as calculated from Equations 3.3 and 3.5, were $\pm 14$ ps and $\pm 20$ ps for the MLE and RLD methods, respectively.

In comparison, for channel 15035, the number of counts included in the calculation was ~1260 fluorescence counts and ~213 background counts, giving a B/F ratio of 0.169. The calculated lifetimes were $825 \pm 6$ ps and $816 \pm 16$ ps for the MLE and RLD, respectively. The lower lifetimes were a direct result of the high B/F ratio while the precision in the measurements was a function of the number of counts within the calculation. The expected standard deviations calculated from Equations 3.3 (MLE) and 3.5 (RLD) were $\pm 22$ ps and $\pm 28$ ps, respectively.

In Figure 6.4 are shown the calculated fluorescence lifetimes via the MLE and RLD over 10 channel (2 s) integration periods, 14995-15005, 15014-15024, and 15030-15040, for the same Gaussian peaks shown in Figure 6.2. Longer integration times allow the ability to accumulate more photocounts within the decay, which improves the precision in the measurement (see Equations 3.2, 3.3,
Figure 6.4. Simulated electropherogram containing two generated Gaussian peaks (resolution = 0.53) with the lifetimes in 10 channel (2 s) intervals ($t_1,t_2,t_3$) calculated via the MLE (circles) and RLD (squares) methods. The dashed line represented the weighted lifetime as calculated from the fractional composition of each peak. The error bars represented the standard deviations calculated from three replicate measurements (larger error bars - RLD). The horizontal lines represented the expected lifetimes for peak 1 and 2.
and 3.5). For example, the lifetime calculated via the MLE method at a 0.2 s integration period was 761 ± 9 ps for channel 15000 in the electrophoresis compared to 753 ± 1 ps for a 10 channel integration (channels 14995-15005). For the RLD, the calculated fluorescence lifetime for channel 15000 was 739 ± 17 ps for a one channel integration and 753 ± 12 ps for a 10 channel (2 s) integration (channels 14995-15005). The number of fluorescence counts in this time period was found to be ~29100 counts with a total background of ~20900 counts. The fractional contribution of two components for this time period was 0.939 (~27300) and 0.0610 (~1770) for components 1 and 2, respectively, giving a weighted average lifetime of 756 ps. The number of photocounts that were found in the calculation with a 140 ps time shift was ~21400 fluorescence counts and ~1430 background counts, with a B/F ratio of 0.0668, which was about half of what was seen for the 1 channel integration at channel 15000. The decrease in the number of background counts along with the increased number of fluorescence photocounts included in the calculation increases the precision in the measurements as well. The expected standard deviations were ± 5 ps and ± 6 ps for the MLE and RLD, respectively.

From Figure 6.4, it can be seen that for both the MLE and RLD methods, the calculated fluorescence lifetime for 10 channel integrations over peak 2, centered at channel number 15035, gave values lower than the true lifetime. Calculation of τr for a 10 channel integration from channels 15030-15040 yielded lifetime values for the MLE of 815 ± 5 ps and 816 ± 12 ps for the RLD. The
precision shown for this 10 channel interval were better than the precision calculated from a single channel interval (channel 15035) for both methods, however the accuracy in the observed lifetimes did not change.

For the 10 channel time interval from 15014-15024, the fractional contribution of component 1 was 0.361 (~5760 counts) and the contribution of component 2 was 0.639 (~10107 counts), thus, the weighted lifetime was calculated to be 813 ps and the resulting decay was biexponential. The decay profile constructed from this interval was composed of ~12700 fluorescence and ~1430 background photocounts (B/F = 0.113). The calculated lifetimes for this period were 800 ± 15 ps and 805 ± 11 ps for the MLE and RLD, respectively, which agreed favorably with the weighted lifetime. Although the accuracy of the calculated lifetime was consistent with the weighted average, it did not agree with either the true lifetime for component 1 or 2.

The decay profiles constructed from the photocounts in the 10 channel integration periods of 14995-15005 (t1), 15014-15024 (t2) and 15030-15040 (t3) are shown in Figure 6.5. Note that the t1 integration decay profile has the larger slope in the semi-log plot, indicating the shortest lifetime, whereas the integration time of t3 channels had the smallest slope in the semi-log plot, indicating the longest lifetime. The decay constructed from t2 was a biexponential decay due to the fact that both peaks contributed significantly to the total number of counts within this time period.
Figure 6.5. Decay profiles from times $t_1$, $t_2$, and $t_3$ from Figure 6.4. The diagonal solid lines represent the slope as determined by the calculated lifetime. The horizontal solid line represents the start channel within the calculation. Time period $t_2$ was fitted to a biexponential decay.
In order to further study the effect of resolution on the fluorescence lifetime calculation, two Gaussian peaks were generated which possessed resolutions of 0.23 and 1.0 with lifetimes calculated over 0.2 s integration times via the MLE. The input parameters were the same as in Figure 6.2, except that $\mu_2 = 6.6600 \times 10^5 \text{ cm}^2 / \text{Vs}$ for a resolution of 0.23 and $\mu_2 = 6.6600 \times 10^5 \text{ cm}^2 / \text{Vs}$ for a resolution of 1.0. Figures 6.6 and 6.7 show the results for these simulations. For Figure 6.6, the calculated lifetime values by the MLE method for Gaussian peak 1, centered at channel 15000, and Gaussian peak 2, centered at channel 15015, were consistent with the weighted average lifetime value but did not agree with the actual lifetime values associated with each individual component. However, it should be pointed out that while this algorithm produced only an average value for a decay composed of multiple components, nonlinear least squares could be used to determine the individual lifetimes of each component comprising the decay. Therefore, while the electrophoresis may provide only marginal separation (low resolution between neighboring peaks), lifetime analysis via nonlinear least squares would help determine the identity of each component.

From Figure 6.7, where both peaks are baseline resolved, the calculated lifetimes more favorably agreed with the expected lifetimes for components 1 and 2. The lifetime calculated at the channel centered on peak 1 (channel 15000) and peak 2 (channel 15065) were determined to be $749 \pm 8 \text{ ps}$ and $822 \pm 37 \text{ ps}$, respectively which agreed within one standard deviation of the input lifetimes of the individual peaks ($\tau_1 = 750$ and $\tau_2 = 850 \text{ ps}$). Also, longer integration times can
Figure 6.6. Simulation of two generated Gaussian peaks with a resolution of 0.23. The lifetimes were calculated over one channel (0.2 s) intervals. The error bars represented the standard deviation in the measurement. $\tau_1$ represented the fluorescence lifetime of peak 1 while $\tau_2$ represented the fluorescence lifetime of peak 2.
Figure 6.7. Simulation of two generated Gaussian peaks with a resolution of 1.0. The lifetimes were calculated over one channel (0.2 s) intervals. The error bars represented the standard deviation in the measurement. $\tau_1$ represents the fluorescence lifetime of peak 1 while $\tau_2$ represented the fluorescence lifetime of peak 2.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
be used to increase the precision in the measurements without a significant loss in accuracy. For example, the calculated lifetime over the time period 14995-15005 was \(746 \pm 5\) ps, while the lifetime calculated over the time period 15063-15068 was \(821 \pm 23\) ps. In comparison, calculated lifetimes for similar intervals centered on each peak from Figure 6.2 gave values of \(786 \pm 5\) ps (channels 14995-15005) and \(796 \pm 8\) ps (channels 15013-15018).

6.4. Conclusions.

The fluorescence lifetimes associated with single exponential decays can be calculated with little computational effort by the use of simple algorithms, such as the MLE and RLD. These algorithms will be used as a basis for on-line data analysis for DNA base-calling applications via fluorescence lifetime discrimination in which the dye-labeled DNA fragments are separated via CGE. In DNA separations via CGE, small amounts of material are loaded onto the column, which permit only short integration times and therefore, low photocount levels used to construct the decay profiles. The results indicated that for two neighboring peaks with a resolution of 0.23, these simple algorithms are not adequate for lifetime discrimination since the calculated lifetime is a weighted average of the components comprising the decay. Methods such as nonlinear least squares analysis would be needed to identify the individual components along with the fractional composition of each. When two Gaussian peaks were constructed with a resolution of 0.53, the peak identification was possible by the MLE when the
lifetimes were calculated over intervals within the peak that did not contain a significant fraction of fluorescence counts from the other component. When the two peaks were baseline resolved, peak identification was easily accomplished via the MLE method since there was no overlap between peaks. In addition, baseline resolution allowed the use of longer integration times, thus improving the precision in the measurement without sacrificing accuracy.

6.5. References.


CHAPTER 7

Conclusions and Future Work

The focus of this work was to demonstrate the viability of using time-correlated single photon counting detection for the analysis of near-IR dye-labeled DNA fragments separated via CGE and the determination of the fluorescence lifetimes of the collected decay profiles via two simple algorithms, the MLE and RLD methods. The parameters used to determine the fluorescence lifetimes in the limit of low photocounts were also studied in detail by Monte Carlo simulations.

In Chapter 1, the background literature on the Human Genome Initiative and the current technologies were outlined, along with an explanation of how our research group will construct a high-throughput DNA sequencing system. The use of a temporal discrimination technique for base-calling was explained, along with the advantages over spectral discrimination. The advantages of near-IR fluorescence excitation and detection as compared to visible detection were also discussed, with the major advantage being the fact that relatively few compounds intrinsically fluoresce in this region of the electromagnetic spectrum.

In Chapter 2, a thorough explanation of TCSPC was given. This chapter included a description of the instrumentation necessary for making TCSPC measurements along with an explanation of nonlinear least squares analysis for determination of the fluorescence lifetime. This data analysis method offers the advantage of being able to distinguish between mono- and multiexponential decay profiles, but it suffers from the large amounts of computational time needed for the
analysis. Therefore, this method is not an optimal choice for data analysis when large amounts of data need to processed, which is the case for high-throughput DNA sequencing applications.

In Chapter 3, two simple computational algorithms were introduced for fluorescence lifetime determination, the MLE and RLD method. These algorithms have been shown to give favorable lifetime values in the limit of low photocounts. The performance of these algorithms were evaluated for three commercially available near-IR dyes under ultradilute conditions and short integration times. The results indicated that both algorithms were able to give favorable results for the fluorescence lifetimes of dyes under ultradilute conditions when a time-shift was implemented to reduce the contribution of background photons from scattering. Monte Carlo simulations were also performed under similar time shifts to show the effects of a time shift on the determined lifetime value.

In Chapter 4, the ability of determining the fluorescence lifetimes of components separated via CE was shown. The calculated fluorescence lifetime via the MLE method for IR-125 (482 ± 14 ps) in predominately methanol was shown to agree favorably with the "true" fluorescence lifetime (473 ps) for a 1.42 zmol mass injection. Additionally, fluorescence lifetime matching was used to identify six near-IR fluorophores separated by CE. The advantage of this technique is the ability to determine the identity of the individual components in a single electrophoretic run. Lastly, it was shown that the fluorescence lifetimes of near-IR dye-labeled DNA fragments could be obtained with high accuracy and
precision. The MLE calculated results indicated a fluorescence lifetime of 581 ± 9 ps for 35 individual peaks along the electropherogram, which agrees with the “true” lifetime, 581 ps, of the near-IR label. This means that if definitive identification of the terminal base could be accomplished with lifetime differences for a series of dyes at 3σ, then only a 27 ps difference of the fluorescence lifetimes is needed.

In Chapter 5, the use of a pulsed-diode laser was demonstrated as an excitation source and a PC board containing the CFD, TAC, and MCA was utilized for the counting electronics. The performance of this system for DNA sequencing applications was determined. IRD40 labeled A-terminated fragments (τf = 660 ps) and IRD41 labeled C-terminated fragments (τf = 581 ps) were separated via CGE and the decay profiles (2 s intervals) were collected during the entire electrophoresis. The results indicated that fluorescence lifetimes could be determined using this instrument; however, the precision in the measurements was not as good as seen in Chapter 4, mainly due to the time interval for the decay profile collection.

In Chapter 6, Monte Carlo simulations were performed to study the effect of overlapping peaks and ultrashort integration times on the calculated fluorescence lifetime via the MLE and RLD algorithms as a function of electrophoretic resolution. A computer program was written in order to generate Gaussian peaks at various resolutions superimposed onto a background level and time slices along the electropherogram (in 0.2 s intervals) were chosen to construct
decay profiles, from which fluorescence lifetimes were subsequently determined. It was found that for peaks with low resolution (<0.20) that the use of these simple algorithms were not adequate for lifetime discrimination. A method such as nonlinear least squares analysis is needed which can determine lifetimes from multiexponential decays. If the resolution was 0.5, identification of the components of the peaks could be determined via lifetime discrimination if the appropriate time intervals were used to construct the decay profile. For peaks with a resolution of 1.0, the calculated lifetimes agreed favorably with the expected results, and also, longer integration times could be used to increase the precision in the measurements.

Future work will involve a four-dye experiment using our heavy-atom modified dyes attached to amine modified DNA primers or dideoxynucleotides, subsequent separation via CGE and base-calling using lifetime discrimination using the MLE algorithm. The accuracy in the base-calling from this experiment will be compared with results from visible fluorescence DNA sequencing experiments.
APPENDIX

Computer Program for Lifetime Determination of Generated Gaussian Peaks at Various Resolutions
PROGRAM SIMULATION1;
{THIS PROGRAM FUNCTIONS TO SIMULATE GAUSSIAN ELECTROPHORETIC PEAKS AT VARYING FLUORESCENCE COUNTS, BACKGROUND COUNTS, ELECTROPHORETIC MOBILITIES, ETC... FOR THE DETERMINATION OF THE FLUORESCENCE LIFETIME VIA THE MAXIMUM LIKELIHOOD ESTIMATOR AND RAPID LIFETIME DETERMINATION METHOD.}

Uses
Crt, {Unit found in TURBO.TPL}
Graph; {Unit found in GRAPH.tpu for 5.5}

CONST
WIDTH : REAL = 50.0;
PI : REAL = 3.141592354;
NN : INTEGER = 32;
CHO : INTEGER = 1;
MARKER : INTEGER = 10;
FLAG1 : LONGINT = 0;
FLAG2 : INTEGER = 0;
MARKER1 : INTEGER = 0;
FLAG3 : INTEGER = 0;
FLAG4 : LONGINT = 0;
CHECK10 : CHAR = 'N';
SKIP : LONGINT = 0;
DUMMY : INTEGER = 0;
MAX_PROMPT : REAL = 0.0;
TEMP1 : INTEGER = 0;
TEMP2 : REAL = 0.0;
PROMPT_MAX1 : INTEGER = 0;

TYPE
SMARRAY = ARRAY[1..4] OF REAL;
REALARRAYNN2 = ARRAY[1..4096] OF SINGLE;
TITLE = STRING [33];

VAR
SAVE_DATA : INTEGER; {USER INPUT OPTION TO SAVE DATA}
RLD_RANGE : INTEGER; {USER INPUT (LIFETIMES) FOR CALCULATION VIA RLD}
MLM_RANGE : INTEGER; {USER INPUT (LIFETIMES) FOR CALCULATION VIA MLM}
HIGH_CHANMLM : INTEGER; {UPPER CHANNEL LIMIT IN DECAY FOR MLM CALCULATION}
HIGH_CHANRLD : INTEGER; {UPPER CHANNEL LIMIT IN DECAY FOR RLD CALCULATION}
IND100, IND200 : INTEGER; {SET OF INTEGERS USED TO DETERMINE DECAY CHANNEL WITH MAXIMUM COUNTS}
FWHM_PROMPT : INTEGER; {FULL WIDTH AT HALF MAXIMUM FOR PROMPT FUNCTION}
HALF_CHAN1, HALF_CHAN2 : INTEGER; {USED TO CALCULATE FWHM BY DIVIDING PROMPT INTO TWO HALVES}
HALF_MAX : INTEGER; {CALCULATES NUMBER OF COUNTS BY DIVIDING MAX NUMBER OF COUNTS IN PROMPT FUNCTION BY 2}
DIFFER : INTEGER; {DIFFERENCE IN CHANNEL NUMBERS BETWEEN PROMPT AND CONVOLVED DECAY PROFILES}
TAU_EST10 : REAL; {TEST FOR CALCULATED LIFETIME}
TAU_EXP : REAL; {EXPECTED LIFETIME OF DECAY}
DELTARDL : INTEGER; {TIME WIDTH OF D0 AND D1 FOR RLD CALCULATION}
SUM_RD0 : REAL; {TOTAL COUNTS IN D0 FOR RLD CALCULATION}
SUM_RD1 : REAL; {TOTAL COUNTS IN D1 FOR RLD CALCULATION}
TOT_SUM: REAL; {COUNTS IN D1/COUNTS IN D0 FOR RLD CALCULATION}
TAU_ESTRDL : REAL; {NUMBER OF TIME CHANNELS FOR LIFETIME DETERMINATION (1 CHANNEL = 2.88 PS)}
TAU_TRDL : REAL; {LIFETIME CALCULATED BY RLD}
BACK_GR : INTEGER; {BACKGROUND}
CHANNEL_NUM : REAL; {NUMBER OF TIME CHANNELS IN CONVOLVED DECAY PROFILE}
TOTAL_COUNTS : LONGINT; {TOTAL SIGNAL COUNTS IN CONVOLVED DECAY PROFILE FOR MLE CALC}
DELAY1 : INTEGER; {CHANNEL DELAY FROM PROMPT MAX TO BEGIN LIFETIME CALC}
MT1 : INTEGER; {MIGRATION TIME OF PEAK 1 IN CHANNELS (0.2 SEC/CHANNEL}
MT2 : INTEGER; {MIGRATION TIME OF PEAK 2 IN CHANNELS (0.2 SEC/CHANNEL}
MTIME1 : INTEGER; {MIGRATION TIME OF PEAK 1 IN CHANNELS ADJUSTED BY 13500 CHANNELS}
MTIME2 : INTEGER; {MIGRATION TIME OF PEAK 2 IN CHANNELS ADJUSTED BY 13500 CHANNELS}
MAX_PROMPT1 : REAL; {HIGHEST NUMBER OF COUNTS IN
CONVOLVED DECAY}
MP1 : REAL; {HIGHEST NUMBER OF COUNTS IN CONVOLVED DECAY
PROFILE}
TAU_TIME : REAL; {LIFETIME OF CONVOLVED DECAY PROFILE FOR
MLM}
TAU_EST : REAL; {NUMBER OF TIME CHANNELS FOR MLE
CALCULATION}
SUM_TIME : REAL; {CHANNEL NUMBER * NUMBER OF COUNTS IN
CHANNEL FOR MLM CAL}
LOW_CHAN : INTEGER; {PROMPT MAX + DELAY1}
BACK : INTEGER; {BACKGROUND}
CHAN_PROMPT : INTEGER; {USED TO ALIGN FLUORESCENCE DECAY
WITH BACKGROUND DECAY FOR CONVOLVED DECAY PROFILE}
TYP1 : BOOLEAN; {USED TO ALIGN FLUOR AND BACK. DECAY FOR
CONVOLVED DECAY}
NORM : REAL; {FOR PLOTTING}
ISIGN : INTEGER; {FOR FFT OR INVERSE FFT}
PROB : REAL; {INVOLVED WITH CONSTRUCTION OF CONVOLVED
DECAY}
TEST1 : INTEGER; {INVOLVED WITH CONSTRUCTION OF
CONVOLVED DECAY}
PROMPT_MAX : INTEGER; {DECAY TIME CHANNEL WITH MAXIMUM
COUNTS}
CHAN_TIME : REAL; {TIME WIDTH OF EACH DECAY CHANNEL, 2.88
PS}
CHAN_NUM : INTEGER; {TOTAL NUMBER OF TIME CHAANELS IN
DECAY, 4096}
COMPTS : INTEGER; {NUMBER OF COMPONENTS TO SIMULATE}
IND10, IND11, IND12, IND13 : INTEGER; {INTEGERS IN FOR..TO LOO}
NUM : LONGINT; \{THE NUMBER OF DATA POINTS TO FFT. MUST BE 4096 FOR FFT OF SINGLE FUNCTION AND 2048 FOR AUTOCORRELATION OR FFT OF TWO FUNCTIONS.\}
BAC : ARRAY[1..4000] OF INTEGER; \{ARRAY FOR BACKGROUND COUNTS\}
NDATA, DATA, DECAY : REALARRAYNN2; \{DATA ARRAYS\}
ANS, DATA_1, DATA_2, DATA_3, DATA_PROB : \^REALARRAYNN2; \{DATA ARRAYS\}
BIG, PER, SCAL, SMALL : REAL; \{PLOT\}
N, I, J, NLIM : INTEGER; \{PLOT\}
GD, GM : INTEGER; \{PLOT\}
Z, Z1 : REAL; \{GAUSSIAN FUNCTION FOR PEAK CONSTRUCTION FOR ELECTROPHOROGRAM\}
BAC1 : INTEGER; \{BACKGROUND\}
SIG_COUNT : SMARRAY; \{FLUORESCENCE COUNTS IN ELECTROPHOROGRAM PEAKS\}
TOT_FLU : REAL; \{TOTAL FLUORESCENCE COUNTS TO BE PUT IN CONVOLVED DECAY\}
FLU_CNT1 : REAL; \{FLUORESCENCE COUNTS FROM PEAK 1 IN CONVOLVED DECAY\}
FLU_CNT3 : REAL; \{FLUORESCENCE COUNTS FROM PEAK 2 IN CONVOLVED DECAY\}
PER_FLU1 : REAL; \{PERCENT FLUORESCENCE FROM PEAK 1 IN CONVOLVED DECAY\}
PER_FLU2 : REAL; \{PERCENT FLUORESCENCE FROM PEAK 2 IN CONVOLVED DECAY\}
TWIDTH : SMARRAY; \{ARRAY TO INPUT PEAK WIDTHS AT HALF HEIGHT\}
THEO_PLATE : SMARRAY; \{THEORETICAL PLATES\}
ELEC_MOB : SMARRAY; \{ARRAY TO INPUT ELECTROPHORETIC MOBILITIES\}
MIG_TIME : SMARRAY; \{MIGRATION TIME, IN SECONDS\}
SD : SMARRAY; \{STANDARD DEVIATION\}
RAN1 : REAL; \{RANDOM NUMBER\}
BAC_STDEV : REAL; \{BACKGROUND STANDARD DEVIATION\}
LEN_CAP : REAL; \{TOTAL CAPILLARY LENGTH\}
LEN_CAP1 : REAL; \{CAPILLARY LENGTH TO DETECTOR\}
FLD_STGH : REAL; \{FIELD STRENGTH\}
TOT_BACK : REAL; \{TOTAL BACKGROUND\}
AVG_BACK : INTEGER; \{AVERAGE BACKGROUND / CHANNEL\}
SIGMA : INTEGER; \{STANDARD DEVIATION FOR BACKGROUND\}
S_CHAN : INTEGER; \{START TIME (S) FROM ELECTROPHOROGRAM TO BUILD DECAY\}
S_CHAN1 : INTEGER; \{START TIME (CH) FROM ELECTROPHOROGRAM TO BUILD DECAY\}
E_CHAN : INTEGER; {END TIME (S) FROM ELECTROPHEROGRAM TO
BUILD DECAY}
E_CHAN1 : INTEGER; {END TIME (CH) FROM ELECTROPHEROGRAM TO
BUILD DECAY}
REPEAT_CALC : INTEGER; {USER INPUT TO REPEAT CALCULATION}
MOLES : INTEGER; {WHETHER YOU WANT TO ENTER FLU. COUNTS OR MOLECULES}
NO MOLE : SMARRAY; {NUMBER OF MOLECULES TO ENTER}
NOPHPERCH : SMARRAY; {NUMBER OF MOLECULES/CHANNEL FOR GAUSSIAN PEAK}
NOPHPERCH1 : REAL; {TOTAL NUMBER OF MOLECULES/CHANNEL FOR GAUSSIAN PEAK}
PROB_MOL : REAL; {PROBABILITY OF A MOLECULE IN CHANNEL 1}
PROB_SD : REAL; {PROBABILITY OF BACKGROUND COUNTS}

{*******************************************************************************}

PROCEDURE READDATA(VAR DATA10 : REALARRAYNN2);

VAR
INDEX9 : INTEGER;
INDEX10 : INTEGER;
INDEX2 : INTEGER;
INDEX5 : INTEGER;
FILE : TEXT;
DATASTRING : STRING [100];
DATA1 : STRING [9];
CODE : INTEGER;
DUMMYSTRING : STRING [100];
NEXT_CHAR : STRING [1];
BEGIN
ASSIGN (FILE, SFILE);
{$I-}
RESET (FILE);
{I+};
IF IORESULT <> 0 THEN
BEGIN
WRITELN ('FILE NOT FOUND');
WRITELN ('HIT ENTER TO CONTINUE');
READLN;
WRITELN ('FLAG1,' ',IORESULT');
HALT(1);
END;
FOR INDEX9 := 1 TO 6 DO
BEGIN
READLN(RFILE, DUMMYSTRING);
END;
FOR INDX2 := 1 TO CHAN_NUM DO
BEGIN
READLN (RFILE, DATASTRING);
DATA1 := COPY (DATASTRING,6,14);
NEX_CHAR := COPY (DATA1,1,1);
WHILE NEX_CHAR = ' ' DO
BEGIN
DELETE (DATA1, 1, 1);
NEX_CHAR := COPY (DATA1, 1, 1);
END;
VAL (DATA1, DATA10[INDX2], CODE);
IF CODE <> 0 THEN
BEGIN
WRITELN (CODE);
HALT(1);
END;
END;
CLOSE (RFILE);
END;

***********

PROCEDURE EWRITE;

VAR
CH : CHAR;
BEGIN
GOTOXY (1,25);
CLREOL;
FILENAME := ' ';
WRITELN ('UNABLE TO WRITE DATA TO DISK, PRESS <RETURN> TO CONTINUE');
READLN;
END;

***********
PROCEDURE WRITEDATA (VAR DATA5 : REALARRAYNN2;
    NN5 : INTEGER);

TYPE
    RC = RECORD
        DATA_ARRAY : ARRAY [1..16] OF CHAR; {WRITE UP TO A 10 DIGIT
         NUMBER
         TERMINATED IN A CR LF}
    END;

VAR
    RFILE : FILE OF RC;
    RRECORD : RC;
    Q : STRING [9];
    R : STRING [4];
    IX : INTEGER;
    IY : REAL;
    INDX2 : INTEGER;
    INDX5 : INTEGER;
    STRINGDATA : STRING [16];
    INDX : INTEGER;

BEGIN
    ASSIGN (RFILE, FFILE_NAME);
    {$I-}
    REWRITE (RFILE);
    {$I+}
    IF IORESULT <> 0 THEN
    BEGIN
        EWRITE;
        EXIT;
    END;
    WITH RRECORD DO
    BEGIN
        FOR INDX2 := 1 TO (NN5) DO {MUST HAVE NO MORE THAN 4096
         DATA POINTS}
        BEGIN
            IY := DATA5[INDX2];
            IX := INDX2;
            STR (IX:9:3,Q);
            STR (IX:4,R);
            STRINGDATA := R + ',' + Q + CHR(13) + CHR(10);
            FOR INDX5 := 1 TO 16 DO
            BEGIN
                DATA_ARRAY[INDX5] := STRINGDATA[INDX5];
            END;
        END;
    END;
PROCEDURE FOUR1 (VAR DATA: REALARRAYNN2;
    NNN, ISIGN: INTEGER);
    {REPLACES DATA BY ITS DISCRETE TRANSFORM, ISIGN = 1,
    THEN FFT, IF ISIGN = -1 THEN INFFT.}
VAR
    NN2 : INTEGER;
    II,JJ,N,MMAX,M,J,ISTEP,I : INTEGER;
    WTEMP,WR,WPR,WPI,WI,THETA : REAL;
    TEMPR, TEMPI, WRS, WIS : DOUBLE;
BEGIN
    N := 2*NNN;
    J := 1;
    FOR II := 1 TO NNN DO {BIT REVERSAL PART OF PROGRAM}
        BEGIN
            I := 2*II-1;
            IF J > I THEN
                BEGIN
                    TEMPR := DATA[J];
                    TEMPI := DATA[J+1];
                    DATA[J] := DATA[I];
                    DATA[J+1] := DATA[I+1];
                    DATA[I] := TEMPR;
                    DATA[I+1] := TEMPI;
                END;
            M := N DIV 2;
            WHILE (M >= 2) AND (J > M) DO
                BEGIN
                    J := J-M;
                END;
            IF IORESULT <> 0 THEN
                BEGIN
                    EWRITE;
                    HALT(1);
                END;
        END;
END;
\[ M := M \div 2; \]
\[ \text{END;} \]
\[ J := J+M; \]
\[ \text{END;} \]
\[ \text{MMAX} := 2; \]
\[ \text{WHILE } N > \text{MMAX DO} \quad \{ \text{START OF THE DANIELSON-LANCZOS CALC.} \} \]
\[ \begin{align*}
\text{BEGIN} & \quad \{ \text{OUTER LOOP EXECUTED LOG NN TIMES} \}
\text{ISTEP} := 2^{\text{MMAX}}; \\
\text{THETA} := \frac{6.28318530717959}{(I\text{SIGN} \cdot \text{MMAX})}; \\
\text{WPR} := -2.0 \cdot \text{SQR} (\sin(0.5 \cdot \text{THETA})); \\
\text{WPI} := \sin (\text{THETA}); \\
\text{WR} := 1.0; \\
\text{WI} := 0.0; \\
\text{FOR II} := 1 \text{ TO MMAX DIV 2 DO} \quad \{ \text{INNER LOOP EXECUTED LOG MMAX TIMES} \}
\begin{align*}
\text{BEGIN} & \quad \{ \text{INNER LOOP EXECUTED LOG MMAX TIMES} \}
\text{M} := 2^{\text{II}} - 1; \\
\text{WRS} := \text{WR}; \\
\text{WIS} := \text{WI}; \\
\text{FOR JJ} := 0 \text{ TO } (N-M) \text{ DIV ISTEP DO} \\
\begin{align*}
\text{BEGIN} & \quad \{ \text{INNER LOOP EXECUTED LOG MMAX TIMES} \}
I := M+JJ \cdot \text{ISTEP}; \\
J := I+\text{MMAX}; \\
\text{TEMPR} := \text{WRS} \cdot \text{DATA}[J] - \text{WIS} \cdot \text{DATA}[J+1]; \\
\text{TEMPI} := \text{WRS} \cdot \text{DATA}[J+1] + \text{WIS} \cdot \text{DATA}[J]; \\
\text{DATA}[J] := \text{DATA}[I] - \text{TEMPR}; \\
\text{DATA}[J+1] := \text{DATA}[I+1] - \text{TEMPI}; \\
\text{DATA}[I] := \text{DATA}[J] + \text{TEMPR}; \\
\text{DATA}[I+1] := \text{DATA}[J+1] + \text{TEMPI}; \\
\end{align*}
\end{align*}
\end{align*}
\end{align*}
\text{END;} \\
\text{WTEMP} := \text{WR}; \\
\text{WR} := \text{WR} \cdot \text{WPR} - \text{WI} \cdot \text{WPI} + \text{WR}; \\
\text{WI} := \text{WI} \cdot \text{WPR} + \text{WTEMP} \cdot \text{WPI} + \text{WI}; \\
\text{END;} \\
\text{MMAX} := \text{ISTEP}; \\
\text{END;} \\
\text{END;} \]

************

PROCEDURE TWOFFT (VAR DATA3,DATA5 : REALARRAYNN2; 
VAR FFT1,FFT2: REALARRAYNN2; 
N: INTEGER);
VAR
NN3,NN4,NN,JJ,J : INTEGER;
REP,REM,AIP,AIM: REAL;
BEGIN
NN := N+N;
NN4 := NN+2;
NN3 := NN+3;
FOR J := 1 TO N DO
BEGIN
JJ := J+J;
FFT1[JJ-1] := DATA3[J]; \{ PACKS TWO ARRAYS INTO ONE \}
FFT1[JJ] := DATA5[J];
END;
FOUR1(FFT1,N,1);
FFT2[1] := FFT1[2];
FFT1[2] := 0.0;
FFT2[2] := 0.0;
FOR JJ := 1 TO N DIV 2 DO
BEGIN
J := 2*JJ+1;
REP := 0.5*(FFT1[J]+FFT1[NN4-J]);
REM := 0.5*(FFT1[J]-FFT1[NN4-J]);
AIP := 0.5*(FFT1[J+1]+FFT1[NN3-J]);
AIM := 0.5*(FFT1[J+1]-FFT1[NN3-J]);
FFT1[J] := REP;
FFT1[J+1] := AIM;
FFT1[NN4-J] := REP;
FFT1[NN3-J] := -AIM;
FFT2[J] := AIP;
FFT2[J+1] := -REM;
FFT2[NN4-J] := AIP;
FFT2[NN3-J]:= REM;
END;
END;

**********
PROCEDURE REALFT(VAR DATA: REALARRAYNN2;
N1, ISIGN: INTEGER);
VAR
BEGIN
THETA := 6.28318530717959/(2.0*N1);
C1 := 0.5;
IF ISIGN = 1 THEN
BEGIN
   C2 := -0.5;
   FOUR1(DATA,N1,+1); {CALCULATE THE FFT}
END
ELSE
BEGIN
   C2 := 0.5;
   THETA := -THETA;
END;
WPR := -2.0*SQR(SIN(0.5*THETA));
WPI := SIN(THETA);
WR := 1.0+WPR;
WI := WPI;
FOR I := 2 TO N1 DIV 2 DO
BEGIN
   I1 := I+I-1;
   I2 := I1+1;
   I3 := N1+N1+3-I2;
   I4 := I3+1;
   WRS := WR;
   WIS := WI;
   H1R := C1*(DATA[I1]+DATA[I3]); {SEPARATE 2 TRANSFORMS OUT
OF DATA}
   H1I := C1*(DATA[I2]-DATA[I4]);
   H2R := -C2*(DATA[I2]+DATA[I4]);
   H2I := C2*(DATA[I1]-DATA[I3]);
   DATA[I1] := H1R+WRS*H2R-WIS*H2I;
   DATA[I2] := H1I+WRS*H2I+WIS*H2R;
   DATA[I3] := H1R-WRS*H2R+WIS*H2I;
   DATA[I4] := -H1I+WRS*H2I+WIS*H2R;
   WTEMP := WR;
   WR := WR*WPR-WI*WPI+WR;
   WI := WI*WPR+WTEMP*WPI+WI;
END;
IF ISIGN = 1 THEN
BEGIN
H1R := DATA[1];
DATA[1] := H1R + DATA[2];
DATA[2] := H1R - DATA[2];
END
ELSE
BEGIN
H1R := DATA[1];
DATA[1] := C1 * (H1R + DATA[2]);
DATA[2] := C1 * (H1R - DATA[2]);
FOUR1(DATA, N1, -1);  {INVERSE FFT}
FOR INDX10 := 1 TO (N1*2) DO
BEGIN
DATA[INDX10] := (1/N1) * (DATA[INDX10]);
END;
END;
END;

PROCEDURE PLOT1(DATA5:REALARRAYNN2;  {PLOT FUNCTION}
    N3: INTEGER;
    N5: INTEGER;
    CHECK10: INTEGER;
    HEADER: TITLE);
VAR
    IN1: INTEGER;
    IN2: INTEGER;
    IN3: INTEGER;
    IN4: INTEGER;
    SMALL: REAL;
    BIG: REAL;
    YLIM: INTEGER;
    XLIM: INTEGER;
    SCALX: REAL;
    SCALY: REAL;
    XLIM1: INTEGER;
    YLIM1: INTEGER;
    LOWMODE: INTEGER;
    HIGHMODE: INTEGER;
    ERRORCODE: INTEGER;
    BIGY: STRING [10];
    SMALLY: STRING [10];
BIGX : STRING [4];
LOG : INTEGER;

BEGIN
CLRSCR;
LOG := 2;  {IF LOG = 1 THEN WILL TAKE LOG OF COUNTS TO PLOT}
SMALL := 1.0E+10;
BIG := -1.0E+10;
FOR IN2 := N3 TO N5 DO BEGIN
IF (DATA5[IN2] < SMALL) THEN SMALL := DATA5[IN2];
IF (DATA5[IN2] > BIG) THEN BIG := DATA5[IN2];
END;
SCALX := 590/(N5-N3);
SCALY := 300/(BIG-SMALL);
STR(BIG:10:1,BIGY);
STR(SMALL:10:1,SMALLY);
STR(N5,BIGX);
GD := DETECT;
INITGRAPH(GD,GM,"");
ERRORCODE := GRAPHRESULT;
IF ERRORCODE <> GROK THEN BEGIN
WRITELN('GRAPHICS ERROR', GRAPHERROMSG(ERRORCODE));
WRITELN('PROGRAM ABORTED AT THIS POINT');
READLN;
HALT(1);
END;
GETMODERANGE(EGA64,LOWMODE,HIGHMODE);
SETGRAPHMODE(HIGHMODE);
SETLINESTYLE(SOLIDLN,0,THICKWIDTH);
SETCOLOR(7);
RECTANGLE(20,20,620,330);
SETCOLOR(14);
SETLINESTYLE(SOLIDLN,0,NORMWIDTH);
SETTEXTSTYLE(0,0,1);
TEXTCOLOR(15);
SETTEXTJUSTIFY(0,1);
OUTTEXTXY(10,153,BIGY);
OUTTEXTXY(200,15,READER);
SETTEXTJUSTIFY(2,1);
OUTTEXTXY(620,340,BIGX);

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
FOR IN2 := N3 TO N5 DO
BEGIN
  IF (CHECK10 = 0) THEN
  BEGIN
    IF (DATA5[IN2] <> 0) THEN
    BEGIN
      XLIM := ROUND(((IN2-N3)*SCALX)+25);
      YLIM := ROUND(325-((DATA5[IN2]-SMALL)*SCALY));
      XLIM1 := 325;
      LINE(XLIM, YLIM,XLIM1, YLIM1);
    END;
  END;
  IF (CHECK10 = 1) THEN
  BEGIN
    XLIM := ROUND(((IN2-N3)*SCALX)+25);
    YLIM := ROUND(325-((DATA5[IN2]-SMALL)*SCALY));
    XLIM1 := ROUND(((IN2+1-N3)*SCALX)+25);
    YLIM1 := ROUND(305-((DATA5[IN2+1]-SMALL)*SCALY));
    WRITELN(XLIM,' ',XLIM1);
    LINE(XLIM, YLIMjXLIM 1, YLIM 1);
  END;
  END;
READLN;
CLOSEGRAPH;
END;

***************************************

PROCEDURE CONVLV (VAR DATA:REALARRAYNN2; {CONVOLVES DATA AND PROMPT})
  N:INTEGER;
  VAR RESPNS:REALARRAYNN2;
  M,ISIGN: INTEGER;
  VAR ANS: REALARRAYNN2);
VAR
  NO2, I, II: INTEGER;
  DUM,MAG2: REAL;
  FFT:^REALARRAYNN2;
BEGIN
  NEW(FFT);
  FOR I := 1 TO (M-1) DIV 2 DO
    BEGIN
RESPNS[N+1-I] := RESPNS[M+1-I];
END;
FOR I := (M+3) DIV 2 TO N-((M-1) DIV 2) DO
BEGIN
  RESPNS[I] := 0.0;
END;
TWOFFT(DATA,RESPNS,FFT^,ANS,N);
NO2 := N DIV 2;
FOR I := 1 TO NO2+1 DO
BEGIN
  II := 2*I;
  IF ISIGN = 1 THEN
  BEGIN
    DUM := ANS[II-1];
    ANS[II-1] := (FFT^[II-1]*ANS[II-1]-FFT^[II]*ANS[II])/NO2;
    ANS[II] := (FFT^[II]*DUM+FFT^[II-1]*ANS[II])/NO2;
  END
  ELSE
  BEGIN
    IF ISIGN = -1 THEN
    BEGIN
      IF SQR(ANS[II-1]) + SQR(ANS[II]) = 0.0 THEN
      BEGIN
        WRITELN('PAUSE IN ROUTINE CONVLV');
        WRITELN('DECONVOLVING AT RESPONSE ZERO');
        READLN;
      END;
      DUM := ANS[II-1];
      MAG2 := SQR(ANS[II-1])+SQR(ANS[II]);
      ANS[III] := (FFT^[III]*ANS[III]+FFT^[II]*ANS[II])/(MAG2*NO2);
      ANS[II] := (FFT^[II]*DUM-FFT^[II-1]*ANS[II])/(MAG2*NO2);
    END;
    END;
  END;
END;
ANS[2] := ANS[N+1];
REALFT(ANS,NO2,-1);
DISPOSE(FFT);
END;

FUNCTION RANO (VAR EDUM : INTEGER) : REAL; {RANDOM NUMBER GENERATOR}
VAR
  DUM : REAL;
  J : INTEGER;
BEGIN
  IF IDUM < 0 THEN
    BEGIN
      RANDSEED := -IDUM;
      IDUM := 1;
      FOR J := 1 TO 97 DO DUM := RANDOM;
      FOR J := 1 TO 97 DO RANOV[J] := RANDOM;
      RAN0Y := RANDOM;
    END;
  J := 1 + TRUNC(97.0 * RANOY);
  RAN0Y := RANOV[J];
  RANO := RANOY;
  RANOV[J] := RANDOM;
END;

FUNCTION TWOTOJ (J : INTEGER) : INTEGER;
BEGIN
  IF (J=0) THEN TWOTOJ := 1
  ELSE TWOTOJ := 2 * TWOTOJ(J-1);
END;

PROCEDURE CALC_GAUSS;
VAR
  U1 : REAL;
  U2 : REAL;
  V1 : REAL;
  V2 : REAL;
  S : REAL;
  M : REAL;

FUNCTION TWOTOJ (J : INTEGER) : INTEGER;
BEGIN
  IF (J=0) THEN TWOTOJ := 1
  ELSE TWOTOJ := 2 * TWOTOJ(J-1);
END;

PROCEDURE CALC_GAUSS;
VAR
  U1 : REAL;
  U2 : REAL;
  V1 : REAL;
  V2 : REAL;
  S : REAL;
  M : REAL;
XI : REAL;
X2 : REAL;
BACK1 : REAL;

BEGIN
REPEAT
  U1 := RAN0(IDUM);
  U2 := RAN0(IDUM);
  V1 := 2 * U1 - 1;
  V2 := 2 * U2 - 1;
  S := (V1 * V1) + (V2 * V2);
UNTIL (S <= 1) AND (S <> 0);
  M := SQRT ((-2 * LN(S)) / S);
  XI := V1 * M;
  X2 := V2 * M;
  TEST := RAN0(IDUM);
IF (TEST <= 0.5) THEN BACK1 := (XI)
ELSE BACK1 := (X2);
PROB_GAUSS := ROUND (BACK1 * SQRT(AVER) + AVER);
END;

{*******************************************************************************

BEGIN
NEW(DATA_PROB);
NEW(ANS);
NEW(DATA_1);
NEW(DATA_2);
NEW(DATA_3);
CLRSCR;
RANDOMIZE;
IDUM := -(1+RANDOM(10));
CHAN_NUM := 4096;
CHAN_TIME := 2.8805E-12;
CHAN_ELEC := 2000;
BEGIN
WRITE('ENTER NUMBER OF COMPONENTS IN DATA TO SIMULATE (2 MAX) -> ');
READLN(COMPTS);
WRITE('ENTER THE CHANNEL LENGTH TO DETECTOR (CM) -> ');
READLN(LEN_CAP1);
WRITE('ENTER ELECTRIC FIELD STRENGTH FOR THE SEPARATION (V/CM) -> ');
READLN(FLD_STGH);

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
FOR IND10 := 1 TO COMPTS DO
BEGIN
  ELEC_MOB[IND10] := 0.0;
  TWIDTH[IND10] := 0.0;
  WRITE('ENTER THE ELECTROPHORETIC MOBILITY (CM^2/V S) OF PEAK ','IND10',' -> ');
  READLN(ELEC_MOB[IND10]);
  WRITE('ENTER THE WIDTH AT HALF HEIGHT (S) FOR PEAK ','IND10',' -> ');
  READLN(TWIDTH[IND10]);
END;
FOR IND10 := 1 TO COMPTS DO
BEGIN
  MIG_TIME[IND10] := 0.0;
  MIG_TIME[IND10] := ROUND(LEN_CAP1/(FLD_STGH*ELEC_MOB[IND10]));
  THEO_PLATE[IND10] := 0;
  THEO_PLATE[IND10] := ROUND(5.54 * SQR(MIG_TIME[IND10] / (TWIDTH[IND10])));
END;
MT1 := 0;
MT2 := 0;
MTIME1 := 0;
MTIME2 := 0;
MT1 := ROUND(MIG_TIME[1]) * 5;
IF COMPTS = 2 THEN MT2 := ROUND(MIG_TIME[2]) * 5;
MTIME1 := MT1 - 14500;
IF COMPTS = 2 THEN MTIME2 := MT2 - 14500;
FOR IND10 := 1 TO COMPTS DO
BEGIN
  SD[IND10] := 0.0;
  SD[IND10] := ((TWIDTH[IND10]) / (2.354));
END;
IF COMPTS = 2 THEN
BEGIN
  RES := 0.0;
END;
FOR IND10 := 1 TO COMPTS DO
BEGIN
  WRITELN(' MIGRATION TIME, PEAK ','IND10',' -> ' ,MIG_TIME[IND10]:6:1,' S. ');
  WRITELN(' MIGRATION TIME, PEAK ','IND10',' -> ' ,ROUND(MIG_TIME[IND10] * 5):6,' CHANNELS (0.2 S/CHAN));
WRITE('THEORETICAL PLATES, PEAK 'IND10', 'THEO_PLATE[IND10]:8:1);
WRITE(' THE STANDARD DEVIATION FOR PEAK 'IND10, ' (S) -> 'SD[IND10]:6:4);
END;
IF COMPTS = 2 THEN WRITE(' RESOLUTION BETWEEN PEAK 1 AND 2 -> ',RES:5:4);
WRITE;
WRITE('DO YOU WISH TO ENTER (1) # MOLECULES (2) # FLUORESCENCE COUNTS -> ');
READLN(MOLES);
IF MOLES = 1 THEN
BEGIN
FOR IND10 := 1 TO COMPTS DO
BEGIN
NO_MOLE[IND10] := 0;
NOPHERCH[IND10] := 0;
TAU[IND10] := 0;
WRITE('COMPONENT 'IND10,' LIFETIME (PS) -> ');
READLN(TAU[IND10]);
TAU[IND10] := TAU[IND10] * IE-12;
WRITE('ENTER NUMBER OF MOLECULES TO CONSTRUCT PEAK 'IND10,' -> ');
READLN(NO_MOLE[IND10]);
WRITE('ENTER AVERAGE NUMBER OF PHOTONS PER MOLECULE -> ');
READLN(NOPHERCH[IND10]);
END;
END;
IF MOLES = 2 THEN
BEGIN
FOR IND10 := 1 TO COMPTS DO
BEGIN
SIG_COUNT[IND10] := 0;
TAU[IND10] := 1.0;
WRITE('COMPONENT 'IND10,' LIFETIME (PS) -> ');
READLN(TAU[IND10]);
TAU[IND10] := TAU[IND10] * IE-12;
WRITE('ENTER TOTAL NUMBER OF FLUORESCENCE COUNTS IN PEAK 'IND10,' -> ');
READLN(SIG_COUNT[IND10]);
END;
WRITE('ENTER AVERAGE NUMBER OF BACK COUNTS / CHANNEL (CHANNEL = 0.2 S) -> ');
READLN(BACK);
TOT_BACK := 0;
AVG_BACK := 0;
FOR IND10 := 1 TO 2000 DO
BEGIN
BAC[IND10] := 0;
REPEAT
PROB_SD := 0.0;
TEST := RANO(IDUM) * (2 * BACK);
PROB_SD := (EXP(-(SQR(TEST - BACK) / (2 * SQR(SQRT(BACK)))))) / (SQRT(BACK * 6.283185307));
TEST2 := RAN0(IDUM);
UNTIL PROB_SD > TEST2;
BAC[IND10] := ROUND(TEST);
TOT_BACK := (TOT_BACK + BAC[IND10]);
END;
{AVG_BACK := ROUND(TOT_BACK / 2001);
WRITELNC AVG. BACKGROUND -> 'AVG_BACK:10);}
WRITE('HIT RETURN TO CONTINUE');
READLN;
FOR IND10 := 1 TO 2000 DO
BEGIN
NDATA[IND10] := 0;
DECAY[IND10] := 0;
END;
IF MOLES = 1 THEN
BEGIN
FOR IND12 := -200 TO 200 DO
BEGIN
Z := 0.2 * IND12;
DATA_PROB^[IND12] := 0;
DATA_PROB^[IND12] := (0.39894228 / (SD[1]) * (EXP(-(SQR(Z) / (2 * SQR(SD[1])))))));
END;
FOR IND12 := -200 TO 200 DO
BEGIN
NDATA[IND12] := 0;
DECAY[IND12] := 0;
END;
IF COMPTS = 1 THEN
BEGIN
FOR IND10 := 1 TO ROUND(NO_MOLE[1]) DO
BEGIN
NOPHPERCH1 := 0;
REPEAT
END;
PROB_MOL := 0.0;
TEST := RAN0(IDUM)* (NOPHPERCH[1] * 2);
PROB_MOL := (EXP(-(TEST / NOPHPERCH[1])));
TEST2 := RAN0(IDUM);
UNTIL PROB_MOL > TEST2;
NOPHPERCH1 := ROUND(TEST);
REPEAT
TEST := RAN0(IDUM);
TEST1 := ROUND(TEST * 400);
IF TEST1 = 0 THEN TEST1 := 1;
TEST := RAN0(IDUM);
UNTIL DATA_PROBA[TEST1 - 201] > TEST;
IF DATA_PROBA[TEST1 - 201] > TEST THEN
BEGIN
NDATA[TEST1 + ROUND(MTIME1) - 201] := NDATA[TEST1 + ROUND(MTIME1) - 201] + NOPHPERCH1;
END;
END;
END;
IF COMPTS = 2 THEN
BEGIN
FOR IND10 := 1 TO ROUND(NO_MOLE[2]) DO
BEGIN
NOPHPERCH1 := 0;
REPEAT
PROB_MOL := 0.0;
TEST := RAN0(IDUM) * NOPHPERCH[2] * 2;
PROB_MOL := (EXP(-(TEST / NOPHPERCH[2])));
TEST2 := RAN0(IDUM);
UNTIL PROB_MOL > TEST2;
NOPHPERCH1 := ROUND(TEST);
REPEAT
TEST := RAN0(IDUM);
TEST1 := ROUND(TEST * 400);
IF TEST1 = 0 THEN TEST1 := 1;
TEST := RAN0(IDUM);
UNTIL DATA_PROBA[TEST1 - 201] > TEST;
IF DATA_PROBA[TEST1 - 201] > TEST THEN
BEGIN
DECAY[TEST1 + ROUND(MTIME2) - 201] := DECAY[TEST1 + ROUND(MTIME2) - 201] + NOPHPERCH1;
END;
END;
END;
END;
{BUILDING GAUSSIAN DISTRIBUTION FOR PEAK 1}

IF MOLES = 2 THEN

BEGIN

FOR IND10 := 1 TO COMPTS DO

BEGIN

FOR IND12 := -200 TO 200 DO

BEGIN

NDATA[IND12] := 0;
DECAY[IND12] := 0;
Z := 0.2 * IND12;
DATA_PROB[IND12] := 0;
DATA_PROB[IND12] := (0.39894228 / (SD[IND10]) * (EXP(-(SQR(Z) /
(2 * SQR(SD[IND10])))));
END;

FLAG1 := 0;
IF IND10 = 1 THEN

BEGIN

IF SIG_COUNT[1] > 1 THEN

BEGIN

REPEAT

TEST := RAN0(IDUM);
TEST1 := ROUND(TEST * 400);
IF TEST1 = 0 THEN TEST1 := 1;
TEST := RAN0(IDUM);
IF (DATA_PROB[TEST1 - 201] > TEST) THEN

BEGIN

FLAG1 := FLAG1 + 1;
NDATA[TEST1 + ROUND(MTIME1) - 201] := NDATA[TEST1 +
ROUND(MTIME1) - 201] + 1;
END;

UNTIL (FLAG1 = SIG_COUNT[1]);
END;

END;

IF (IND10 = 2) THEN  {GAUSSIAN DISTRIBUTION, PK 2}

BEGIN

IF SIG_COUNT[2] > 1 THEN

BEGIN

REPEAT

TEST := RAN0(IDUM);
TEST1 := ROUND(TEST * 400);
IF TEST1 = 0 THEN TEST1 := 1;
TEST := RAN0(IDUM);
IF (DATA_PROB[TEST1 - 201] > TEST) THEN

BEGIN

FLAG1 := FLAG1 + 1;

```
DECAY[TEST1 + ROUND(MTIME2) - 201] := DECAY[TEST1 + ROUND(MTIME2) - 201] + 1;
END;
UNTIL FLAG1 = SIG_COUNT[2];
END;
END;
END;
END;
FOR IND13 := 1 TO 2000 DO
BEGIN
IND10 := IND13;
DATA[IND13] := 0;
END;
PLOT1(DATA,1,CHAN_ELEC,0,'GAUSSIAN FUNCTION');
IF (BACK > 1) THEN
BEGIN
WRITE('DO YOU WISH TO SAVE GAUSSIAN FUNCTION (1) YES (2) NO -> ');
READLN(SAVE_DATA);
IF SAVE_DATA = 1 THEN
BEGIN
WRITE('ENTER FILENAME FOR ELECTROPHORESIS SIMULATION -> ');
READLN(FILE_NAME);
WRITEDATA(DATA,CHAN_ELEC);
END;
END;
REPEAT_CALC := 1;
WHILE REPEAT_CALC = 1 DO
BEGIN
CHAN_TIME := 2.8805E-12;
CHAN_NUM := 4096;
WRITE('ENTER START CHANNEL IN ELECTROPHEROGRAM TO BUILD DECAY -> ');
READLN(S_CHAN);
WRITE('ENTER END CHANNEL IN ELECTROPHEROGRAM TO BUILD DECAY -> ');
READLN(E_CHAN);
WRITE('ENTER DELAY FROM PROMPT TO BEGIN CALCULATION -> ');
READLN(DELAY1);
SFILE := 'ME0607A.ASC';
READDATA(DATA);
BAC1 := 0;
FLU_CNT1 := 0;
FLU_CNT3 := 0;
PER_FLU1 := 0;
PER_FLU2 := 0;
TOT_FLU := 0;
S_CHAN1 := 0;
E_CHAN1 := 0;
S_CHAN1 := S_CHAN - 14500;
E_CHAN1 := E_CHAN - 14500;
FOR IND10 := S_CHAN1 TO E_CHAN1 DO
BEGIN
  BAC1 := BAC1 + BAC[IND10];
END;
FOR IND13 := S_CHAN1 TO E_CHAN1 DO
BEGIN
  IND10 := IND13;
  FLU_CNT1 := (NDATA[IND13]) + FLU_CNT1;
  FLU_CNT3 := (DECAY[IND13]) + FLU_CNT3;
END;
IF COMPTS = 1 THEN
BEGIN
  PER_FLU1 := 1;
  TOT_FLU := ROUND(FLU_CNT1);
END;
IF COMPTS = 2 THEN
BEGIN
  TOT_FLU := FLU_CNT1 + FLU_CNT3;
  PER_FLU1 := FLU_CNT1/TOT_FLU;
  PER_FLU2 := FLU_CNT3/TOT_FLU;
END;
WRITEFLUORESCENCE COUNTS, PEAK 1 -> ',FLU_CNT1:10:2);
WRITEFLUORESCENCE COUNTS, PEAK 2 -> ',FLU_CNT3:10:2);
WRITE(TOTAL FLUORESCENCE COUNTS -> ',TOT_FLU:10:2);
WRITE(TOTAL BACKGROUND COUNTS -> ',BAC1:10);
WRITE(FRACTION OF 1',PER_FLU1:10:5);
WRITE(FRACTION OF 2',PER_FLU2:10:5);
DELAY(15000);
IF TOT_FLU = 0 THEN
BEGIN
  TAU_EST := 0.0;
  TAU_TIME := 0.0;
  TAU_ESTRDL := 0.0;
  TAU_TRDL := 0.0;
  SUM_RDO := 0;
  SUM_RD1 := 0;

TOTAL_COUNTS := 0;
BACK_GR := 0;
END;
IF TOT_FLU >= 1 THEN
BEGIN
MAX_PROMPT := 0;
IF (BACK <= 1) THEN PROMPT_MAX := 1;
IF (BACK > 1) THEN
BEGIN
PROMPT_MAX := 0;
FWHM_PROMPT := 0;
HALF_MAX := 0;
FOR IND10 := 1 TO CHAN_NUM DO
BEGIN
DATA_1A[IND10] := 0;
DATA_2A[IND10] := 0;
DATA_3A[IND10] := 0;
IF (DATAJTND10] > MAX_PROMPT) THEN
BEGIN
MAX_PROMPT := DATA[IND10];
PROMPT_MAX := IND10;
END;
END;
HALF_CHAN1 := 0;
HALF_CHAN2 := 0;
HALF_MAX := ROUND(MAX_PROMPT / 2);
IND100 := 0;
IND200 := 0;
REPEAT
IND200 := IND200 + 1;
IND100 := IND100 + 1;
FOR IND10 := 1 TO PROMPT_MAX DO
BEGIN
IF (DATA[IND10] < HALF_MAX+IND100) AND (DATA[IND10] >
HALF_MAX-IND100) THEN HALF_CHAN1 := IND10;
END;
FOR IND10 := PROMPT_MAX TO CHAN_NUM DO
BEGIN
IF (DATA[IND10] < HALF_MAX+IND100) AND (DATA[IND10] >
HALF_MAX-IND100) THEN HALF_CHAN2 := IND10;
END;
UNTIL((HALF_CHAN1<>0) AND (HALF_CHAN2<>0) OR (IND200 =
5000));
FWHM_PROMPT := HALF_CHAN2 - HALF_CHAN1;
PROMPT_MAX := HALF_CHAN1 + (FWHM_PROMPT DIV 2);
FOR IND10 := PROMPT_MAX TO CHAN_NUM DO
  BEGIN
    DATA_3^[IND10] := PER_FLU1 * MAX_PROMPT * (EXP(-((IND10-
PROMPT_MAX) * CHAN_TIME) / TAU[1]));
    IF (COMPTS = 1) THEN DATA_1^[IND10] := DATA_3^[IND10];
    END;
    IF (COMPTS = 2) THEN
      BEGIN
        FOR END10 := PROMPT_MAX TO CHAN_NUM DO
          BEGIN
            DATA_2^[IND10] := PER_FLU2 * MAX_PROMPT * (EXP(-((IND10-
PROMPT_MAX) * CHAN_TIME) / TAU[2]));
            DATA_1^[IND10] := (DATA_2^[IND10] + DATA_3^[IND10]);
          END;
      END;
    END;
    PLOT1(DATA_1[],1,CHAN_NUM,0,'CALCULATED DECAY PROFILE');
    TOTAL_COUNTS := 0;
    FOR IND10 := 1 TO CHAN_NUM DO
      BEGIN
      END;
    FOR IND10 := 1 TO CHAN_NUM DO
      BEGIN
        DATA_2^[IND10] := DATA[IND10];
        DATA[IND10] := 0;
      END;
    FLAG1 := 0;
    FLAG2 := 0;
    IF (BACK > 1) THEN
      BEGIN
        REPEAT
          FLAG2 := FLAG2 + 1;
          TEST := RAN0(IDUM);
          TEST1 := ROUND(TEST * CHAN_NUM);
          IF TEST1 = 0 THEN TEST1 := 1;
          TEST := RAN0(IDUM);
          IF (DATA_PROB^[TEST1] > TEST) THEN
            BEGIN
              FLAG1 := FLAG1 + 1;
              DATA[TEST1] := DATA[TEST1] + 1;
            END;
          UNTIL (FLAG1 = BACK) OR (FLAG2 = 50000);
        FLAG1 := 0;
END;
    PLOT1(DATA, 1, CHAN_NUM, 0, 'PROMPT FUNCTION CORRECT COUNTS');
    IF (FLAG2 = 50000) THEN
        BEGIN
            WRITE ('PROBLEM WITH FLAG2');
            HALT(1);
        END;
    IF (BACK > 1) THEN
        BEGIN
            CHAN_PROMPT := CHAN_NUM;
            TYP1 := ODD(CHAN_NUM);
            FOR IND10 := 1 TO CHAN_NUM DO
                BEGIN
                    ANS^[IND10] := 0.0;
                    DATA_1^[IND10] := DATA_1^[IND10] / MAX_PROMPT;
                    DATA_2^[IND10] := DATA_2^[IND10] / MAX_PROMPT;
                END;
            ISIGN := 1;
            IF (ORD(TYP1) = 0) THEN
                BEGIN
                    CHAN_PROMPT := CHAN_NUM - 1;
                    CONVLV(DATA_1^, CHAN_NUM, DATA_2^, CHAN_PROMPT, ISIGN,
                        ANS^);
                    PROMPT_MAX1 := 0;
                    MAX_PROMPT1 := 0.0;
                    FOR IND11 := 1 TO CHAN_NUM DO
                        BEGIN
                            IF (ANS^[IND11] > MAX_PROMPT1) THEN
                                BEGIN
                                    MAX_PROMPT1 := (ANS^[IND11]);
                                    PROMPT_MAX1 := IND11;
                                END;
                        END;
                    IF (PROMPT_MAX1 > PROMPT_MAX) THEN
                        BEGIN
                            DIFFER := PROMPT_MAX1 - PROMPT_MAX;
                            FOR IND11 := 1 TO (CHAN_NUM - DIFFER) DO
                                BEGIN
                                    ANS^[IND11] := ANS^[IND11 + DIFFER];
                                END;
                            END;
                        IF (PROMPT_MAX1 < PROMPT_MAX) THEN
                            BEGIN
                                DIFFER := PROMPT_MAX - PROMPT_MAX1;
                                FOR IND11 := DIFFER TO (CHAN_NUM - DIFFER) DO
                                    BEGIN
                                    END;
ANS^[IND1-DIFFER] := ANS^[IND1];
END;
END;
FOR IND1 := 1 TO CHAN_NUM-DIFFER DO
BEGIN
DATA_3^[IND1] := 0.0;
DATA_PROBA^[IND1] := ANS^[IND1] / MAX_PROMPT1;
END;
PLOT1(DATA_PROBA^, 1, CHAN_NUM, 0, 'PROBABILITY OF CONVOLV. DATA);
FLAG4 := 0;
REPEAT
TEST := RAN0(IDUM);
TEST1 := ROUND(TEST * (CHAN_NUM-DIFFER));
PROB := DATA_PROBA^[TEST1];
TEST := RAN0(IDUM);
IF (TEST < PROB) THEN
BEGIN
DATA_3^[TEST1] := DATA_3^[TEST1] + 1;
FLAG4 := FLAG4 + 1;
END;
UNTIL (FLAG4 >= TOT_FLU);
END;
FOR IND10 := 1 TO CHAN_NUM - DIFFER DO
BEGIN
DATA_3^[IND10] := DATA_3^[IND10] + DATA^[IND10];
END;
PLOT1(DATA_3^, 1, CHAN_NUM-DIFFER, 0, 'CONVOLVED PROFILE, CORRECT COUNTS');
IF (BACK > 1) THEN
BEGIN
WRITE('DO YOU WISH TO SAVE FFT CONVOLVED DATA (1) YES, (2) NO -> ');
READLN(SAVE_DATA);
IF (SAVE_DATA = 1) THEN
BEGIN
WRITE('ENTER FILE NAME FOR FFT CONVOLVED DATA -> ');
READLN(FILE_NAME);
WRITEDATA(DATA_3^, CHAN_NUM-DIFFER);
END;
END;
PROMPT_MAX := 0;
MAX_PROMPT := 0;
FOR IND10 := 1 TO CHAN_NUM-DIFFER DO
BEGIN

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
IF (DATA_3^[IND10] > MAX_PROMPT) THEN BEGIN
  PROMPT_MAX := IND10;
  MAX_PROMPT := ROUND(DATA_3^[IND10]);
END;
END;
SUM_RDO := 0.00;
SUM_RD1 := 0.00;
SUM_TIME := 0.00;
LOW_CHAN := PROMPT_MAX + DELAY1;
{WRITE(ENTER NUMBER OF LIFETIMES FOR RLD CALC -> );
READLN(RLD_RANGE);
WRITE(ENTER NUMBER OF LIFETIMES FOR MLM CALC -> );
READLN(MLM_RANGE);}
RLD_RANGE := 5;
MLM_RANGE := 5;
IF COMPTS = 1 THEN CHANNEL_NUM := ((TAU[1] * MLM_RANGE) / CHAN_TIME) + PROMPT_MAX + DELAY1;
IF COMPTS = 2 THEN CHANNEL_NUM := (((TAU[1] + TAU[2])/2) * MLM_RANGE) / CHAN_TIME) + PROMPT_MAX + DELAY1;
HIGH_CHANMLM := ROUND(CHANNEL_NUM);
IF COMPTS = 1 THEN CHANNEL_NUM := ((TAU[1] * RLD_RANGE) / CHAN_TIME) + PROMPT_MAX + DELAY1;
IF COMPTS = 2 THEN CHANNEL_NUM := (((TAU[1] + TAU[2])/2) * RLD_RANGE) / CHAN_TIME) + PROMPT_MAX + DELAY1;
HIGH_CHANRLD := ROUND(CHANNEL_NUM);
DELTARDL := (HIGH_CHANRLD - LOW_CHAN) DIV 2;
FOR IND10 := (LOW_CHAN) TO (DELTARDL + LOW_CHAN) DO BEGIN
  SUM_RDO := SUM_RDO + DATA_3^[IND10];
END;
FOR IND10 := (LOW_CHAN + DELTARDL + 1) TO (HIGH_CHANRLD) DO BEGIN
  SUM_RD1 := SUM_RD1 + DATA_3^[IND10];
END;
SUM_TIME := 0.0;
FOR IND10 := LOW_CHAN TO HIGH_CHANMLM DO BEGIN
  SUM_TIME := SUM_TIME + ((IND10-PROMPT_MAX) * DATA_3^[IND10]);
END;
TOTAL_COUNTS := 0;
FOR IND10 := LOW_CHAN TO HIGH_CHANMLM DO BEGIN
TOTAL_COUNTS := TOTAL_COUNTS + ROUND(DATA_3^[IND10]);
END;
BACK_GR := 0;
FOR IND10 := LOW_CHAN TO HIGH_CHANLM DO
BEGIN
  BACK_GR := BACK_GR + ROUND(DATA[IND10]);
END;
TAU_ESTRDL := 0.0;
TOT_SUM := SUM_RD1/SUM_RD0;
IF (TOT_SUM = 0) OR (TOT_SUM = 1) THEN TAU_ESTRDL := 0;
  IF (TOT_SUM > 0) AND (TOT_SUM < 1) THEN TAU_ESTRDL := -(DELTARDL) / (LN(TOT_SUM));
IF COMPTS = 1 THEN TAU_EXP := TAU[1];
TAU_TRDL := 0;
TAU_TRDL := (TAU_ESTRDL * CHAN_TIME) / 1.024;
TAU_EST := 0.0;
IF TOTAL_COUNTS = 0 THEN TAU_EST := 0.0;
  IF TOTAL_COUNTS > 0 THEN TAU_EST := (SUM_TIME / TOTAL_COUNTS) - DELAY1;
TAU_TIME := 0.0;
TAU_TIME := (CHAN_TIME * TAU_EST) * 1.03;
END;
CLRSCR;
WRITELN('ESTIMATED LIFETIME BY MLM (CHANNELS) ->
',TAU_EST:6:4);
WRITELN('LIFETIME IN TIME (PS) -> ',(TAU_TIME*1E12):4:1);
WRITELN;
WRITELN('ESTIMATED LIFETIME BY RDL (CHANNELS) ->
',TAU_ESTRDL:6:4);
WRITELN('LIFETIME IN TIME (PS) -> ',(TAU_TRDL*1E12):4:1);
WRITELN;
CHAN_NUM := HIGH_CHANLM - LOW_CHAN;
FLAG1 := 0;
WRITELN;
WRITELN('THE TOTAL NUMBER OF COUNTS IN D_0 IS ->
',SUM_RD0);
WRITELN('THE TOTAL NUMBER OF COUNTS IN D_1 IS ->
',SUM_RD1);
WRITELN('THE TOTAL NUMBER OF BACKGROUND COUNTS IS ->
',BAC1:6);
WRITELN('THE TOTAL NUMBER OF BACKGROUND COUNTS IN CALCULATION IS ->',BACK_GR:6);
WRITELN('THE TOTAL NUMBER OF SIGNAL COUNTS IN MLM LIFETIME CALC. -> ',TOTAL_COUNTS:6);
WRITELN('THE NUMBER OF DELAYED CHANNELS TO START LIFETIME CALC. -> ',DELAY1);
WRITELN('THE START CHANNEL IN ELECTROPHEROGRAM WAS -> ',(S_CHAN1+14500):5);
WRITELN('THE END CHANNEL IN ELECTROPHEROGRAM WAS -> ',(E_CHAN1+14500):5);
WRITELN('THE FWHM OF THE PROMPT PEAK IS -> ',FWHM_PROMPT:6);
FOR IND10 := 1 TO COMPTS DO BEGIN
  WRITELN('THE ENTERED LIFETIME OF COMPONENT ',IND10:2,' IS -> ',TAU[IND10]);
END;
WRITELN('THE EXPECTED LIFETIME FOR THE DECAY WAS -> ',TAU_EXP);
IF TOT_FLU >= 1 THEN BEGIN
  REPEAT
    TAU_TIME := CHAN_TIME * TAU_EST * 1.03;
    TAU_EST10 := 1 + (1/EXP(CHAN_TIME/TAU_TIME-1)) - 1/((CHAN_NUM)*(EXP((CHAN_NUM)*(CHAN_TIME/TAU_TIME-1))) - 1);
  IF (TAU_EST10 > TAU_EST) THEN TAU_EST := TAU_EST - 0.1;
  IF (TAU_EST10 < TAU_EST) THEN TAU_EST := TAU_EST + 0.1;
  FLAG1 := FLAG1 + 1;
  UNTIL (ABS(TAU_EST - TAU_EST10) <= 0.01) OR (FLAG1 = 100);
END;
WRITELN('HIT RETURN TO CONTINUE');
READLN;
WRITE('DO YOU WISH TO REPEAT CALCULATION (1) YES (2) NO -> ');
READLN(REPEAT_CALC);
END.
DISPOSE(ANS);
DISPOSE(DATA_1);
DISPOSE(DATA_2);
DISPOSE(DATA_3);
DISPOSE(DATA_PROB);
END.
VITA


Upon graduation, he enrolled at Nicholls State University in August, 1986, on a Presidential Scholarship. While attending Nicholls, he was a member and president of the Nicholls Chemical Sciences Society and a member of Phi Kappa Theta fraternity. He graduated with a bachelor of science degree in Biology in May, 1990, and a bachelor of science degree in Chemistry in May, 1992. In August, 1992, he began graduate school at Louisiana State University in the Department of Chemistry. Upon graduation, he plans to work as a Research Scientist for Transgenomics, Inc., of Omaha, Nebraska.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Benjamin L. Legendre Jr.

Major Field: Chemistry

Title of Dissertation: Fluorescence Lifetime Discrimination of Near-Infrared Dye-Labeled Oligonucleotide Fragments Separated by Capillary Gel Electrophoresis for Base-Calling Applications in DNA Sequencing

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination: 10/23/97