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Single-Molecule Imaging With a Custom-Built Fluorescence Microscope

David Allan Ball
University of Tennessee - Knoxville

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Dr. Lloyd M. Davis, Major Professor

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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Dr. Anne Mayhew

Vice Provost and Dean of Graduate Studies

(Original signatures are on file with official student records.)

SINGLE MOLECULE IMAGING
WITH A CUSTOM-BUILT
FLUORESCENCE MICROSCOPE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

David Allan Ball

August 2002

Dedication

This thesis is dedicated in

loving memory

of

Allan D. Ball

June 3, 1952 – January 27, 1999

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Abstract

An apparatus for single-molecule imaging was constructed and used to examine nanomolar solutions of Bodipy Texas Red in water. The illumination source for this microscope is a synchronously pumped dye laser operating at a wavelength of 585 nm. The beam can easily be switched to give total internal reflection or wide field epi-illumination excitation of the sample. The instrument also has the capability of being used for single-molecule detection by confocal epi-illumination and for fluorescence correlation spectroscopy. Movies of single molecules were analyzed using custom software written in Matlab. Molecules stuck to the surface revealed fluctuations in intensity, or blinking, for both forms of illumination. Possible causes for the fluctuations are discussed.

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1 Introduction

In recent years, technological improvements have made single-molecule detection and imaging important tools for the study of biological molecules. In the work presented here, the project was not only to study single dye molecules in solution, but also to build a custom fluorescence microscope for use in this investigation. In the early stages of this work, a commercial Zeiss microscope was modified to enable imaging of single molecules with total-internal reflection (TIR) excitation. As this earlier work is adequately reported in References [1] and [2], this thesis focuses on the later work, in which a custom microscope was built. This microscope is capable of single-molecule imaging by both TIR and wide-field epi-illumination excitation, and also fluorescence correlation spectroscopy, with single-molecule detection by confocal epi-detection.

A basic description of the theory behind single-molecule detection and imaging is given in Chapter 1. Chapter 2 describes the fluorescence microscope custom-built for the single-molecule imaging experiments. Selected results obtained with this instrument are presented in Chapter 3. Chapter 4 discusses ongoing research and possible future single-molecule experiments.

In this first chapter, the methods used for the observation of single fluorescent dye molecules in solution are explained, and then some of the possible applications are discussed. Section 1.1 gives a brief overview of the theory of

laser-induced fluorescence for detection of organic dye molecules in solution. In Section 1.2, the major sources of background in fluorescence-detection experiments are explained. Section 1.3 reviews the history of single-molecule detection experiments, and shows how the detection has become more sensitive over the years. Section 1.4 gives a summary of past single-molecule imaging experiments and some applications.

1.1 Laser-Induced Fluorescence

The most common method used for studying single dye molecules is laser-induced fluorescence. In this technique, laser light is used to excite the organic dye molecule from its ground state to an excited singlet state. Once in this upper state, the molecule may return to the ground state by one of several mechanisms. Figure 1 shows a Jablonski diagram for a typical organic dye molecule, which displays the possible transitions between the excited singlet state and the ground state.

One possibility is that the molecule undergoes an electronic dipole transition to the ground state with the emission of a fluorescence photon. The molecule may also decay directly to the ground state by non-radiative means, resulting in the generation of heat or energy transfer to another molecule. There is also a possibility that the molecule passes to an excited triplet state without emitting a photon. From the triplet state, the molecule can also decay with or without photon emission. This process takes place on a much longer timescale

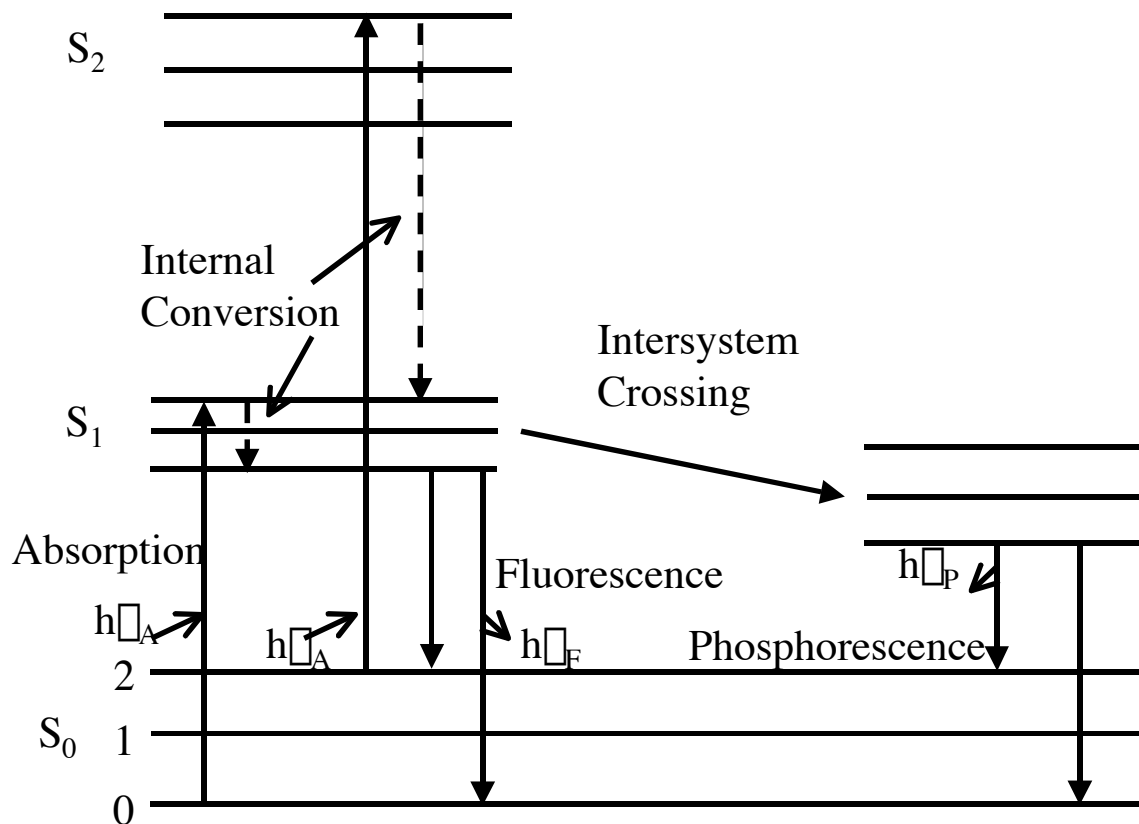


Figure 1: Jablonski diagram for an organic dye molecule.

than fluorescence, and if a photon is emitted, it is known as phosphorescence.

While in the more reactive excited state, the molecule may be chemically altered. This change in the dye results in its inability to produce further fluorescence, and is known as photobleaching.

Another feature of fluorescence is an energy loss between excitation and emission, resulting in a shift in emission towards longer wavelengths [3]. This Stokes' shift is caused by the rapid decay of the fluorophore to the lowest vibrational level of the first excited manifold. The fluorescence is further shifted since molecules generally decay to higher vibrational levels of the ground state.

1.2 Rayleigh and Raman Scattering

In the observation of single molecules there are many sources of background signal that must be eliminated. These sources include autofluorescence of the glass substrate, the objective lens, and other collection optics. The most significant and problematic sources of background are Rayleigh and Raman scattering of light from solvent molecules.

Raman scattering in solution is an inelastic process in which a frequency shift occurs. If monochromatic laser light of frequency ω_0 is incident on a liquid, light will be generated at new frequencies $\omega = \omega_0 \pm \omega_M$, where ω_M is some constant frequency attributable to transitions among vibrational and rotational

levels of the molecules [4]. For example, in water the Raman spectrum is caused primarily by the O-H stretch mode. Rayleigh scattering, on the other hand, is elastic scattering of light from molecules, and occurs at the same frequency as the incident radiation.

1.3 Single-Molecule Detection

The first single-molecule studies are generally credited to Hirschfeld, who was able to observe, both visually and with a photomultiplier detector, individual biological molecules stained with as few as 80 fluoresceine isothiocyanate molecules [5]. In later experiments, the detection limit was reduced by an order of magnitude using hydrodynamically focused flows, more sensitive detection optics, and photon counting [6,7]. Detection of individual single chromophores was finally realized by the utilization of time-gated electronics for rejection of Raman scattered light in the fluorescence band [8]. Time-gating requires a pulsed excitation source, but allows for the rejection of certain background signals, such as Rayleigh and Raman scattering, which occur instantaneously, whereas fluorescence is delayed with respect to the laser pulse.

Single-molecule detection allows for the study of a fluorophore's diffusional motion, but is limited in the sense that the observations take place one molecule at a time. In some applications, however, it is desired to observe many individual molecules simultaneously.

1.4 Single-Molecule Imaging Applications

The large number of pixels on a Charge Coupled Device (CCD) allows for the observation of many individual molecules in parallel. This feature is ideal for observing molecular motion in two dimensions, and is needed for newly suggested methods for rapid DNA sequencing, which involve imaging individual dye molecules nearby an array of nanofabricated features [9,10]. Such applications have become even more feasible with recent improvements in the quantum efficiency of the CCD.

Past experiments have shown the viability of using single molecule imaging to observe molecular motion [11]. These and similar experiments have given information on how the nearby environment of a molecule influences such motion, which is an important factor to consider in the area of nanotechnology.

Several schemes exist for the rapid sequencing of DNA by methods that employ single-molecule detection or imaging. One such scheme involves the cleaving of a fluorescent tag from a DNA base as it becomes incorporated into the double helix [9]. Another plan is the opposite of this; each fluorescently labeled base is sequentially removed from a single DNA strand, and then flows through the laser beam for detection [10]. In principle, both of these schemes could be multiplexed or performed in parallel by replacing single-molecule detection at a point with single-molecule imaging.

2 Experimental Setup

In this chapter, the arrangement for single-molecule imaging experiments is described. Section 2.1 summarizes the laser system used to induce fluorescence. Section 2.2 outlines the arrangement of the collection optics, and describes the analysis that was performed on the resultant images. Section 2.3 explains the two forms of excitation that were utilized, wide field epi-illumination (§2.3.1) and total internal reflection (§2.3.2). Section 2.4 describes how the experimental setup can be modified to perform fluorescence correlation spectroscopy measurements.

2.1 Laser System

The laser system that was used is a synchronously pumped dye laser (Coherent 702-2) operating at a wavelength of 585 nm, which is close to the peak absorption wavelengths of the Bodipy Texas Red fluorophore (588 nm) and Alexa 590 fluorophore (590 nm). The dye laser was pumped with a frequency doubled, mode-locked Neodymium:YAG laser (Coherent Antares), which utilizes a KTP crystal to produce emission at the second harmonic (532 nm) of the Nd:YAG primary 1064 nm wavelength. The mode locking and synchronous pumping causes the lasers to produce trains of pulses at the repetition rate of 76 MHz. A microliter sample of Bodipy Texas Red (BTR) dye (Molecular Probes) at a concentration of 10^{10} M in water, placed on a glass cover slip, was illuminated

with the beam.

2.2 Optical Configuration

The dye laser beam was brought to the sample along one of two possible paths, allowing the use of two types of excitation, wide field epi-illumination and total internal reflection (TIR). Figures 2 and 3 illustrate the two paths and a close-up of the sample area, respectively. The laser power at the sample-coverslip interface differed significantly for these two types of illumination. For TIR the incident power was on the order of 10 mW, while for wide field epi-illumination 50 μ W was used. TIR allows for the use of a much higher laser power, since the spot size is much larger (see §2.3.2). Schematics for the wide field epi-illumination and TIR setups are presented in Figures 4 and 5, respectively.

In both cases, the fluorescence was collected by a water immersion objective lens (Olympus UPLAP060XW; ϕ 60, 1.2 N.A.; OL in Figures 4 & 5) used in conjunction with a 150 mm achromat tube lens (Newport, PAC058; L1 in Figures 4 & 5). The light was then recollimated by another lens with focal length of $f = 150$ mm (Newport, PAC058; L2 in Figures 4 & 5) and imaged with a $f = 250$ mm achromat lens (Newport, PAC067; L3 in Figures 4 & 5) onto a back illuminated CCD (Roper Scientific Micromax; 512 \times 512 pixels; 90% Quantum Efficiency). The camera has the ability to group pixels together to form “super

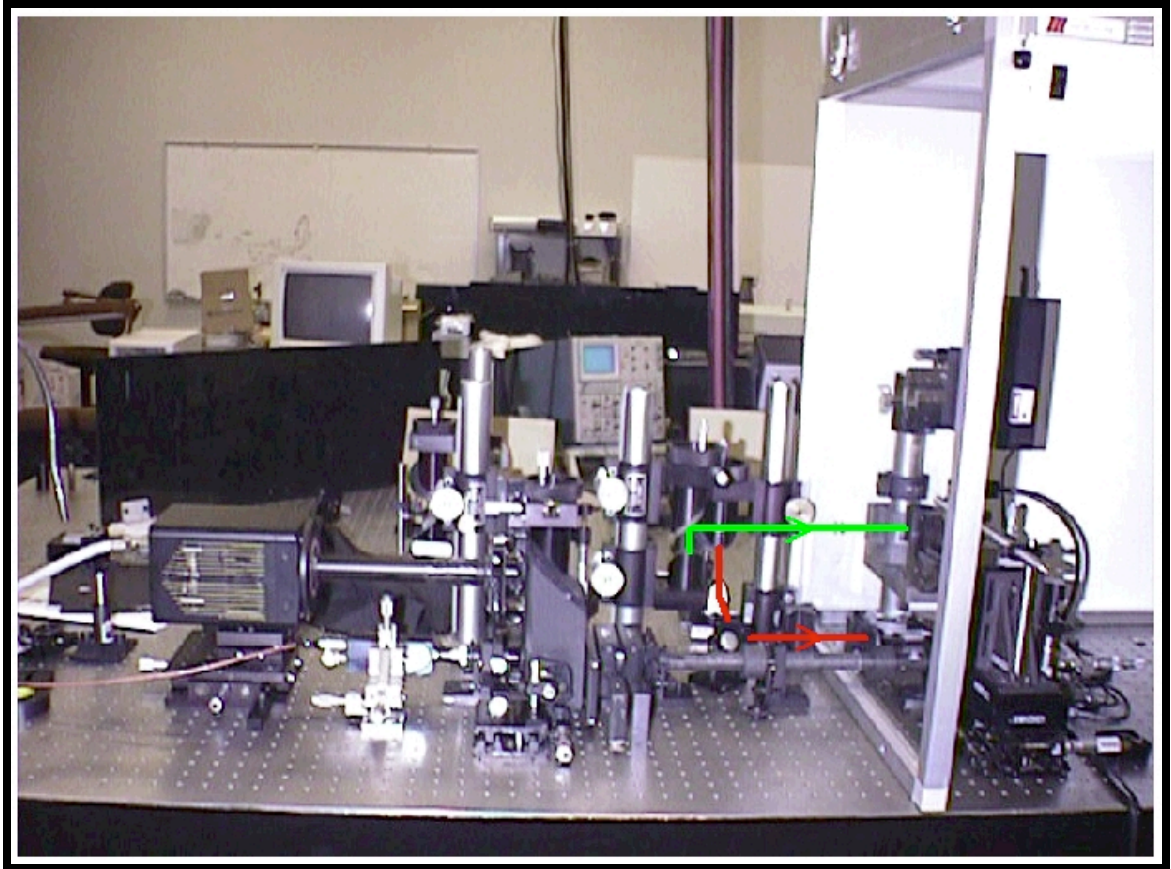


Figure 2: Photograph of experimental setup. The green and red lines show the beam path for TIR and wide field epi-illumination, respectively.

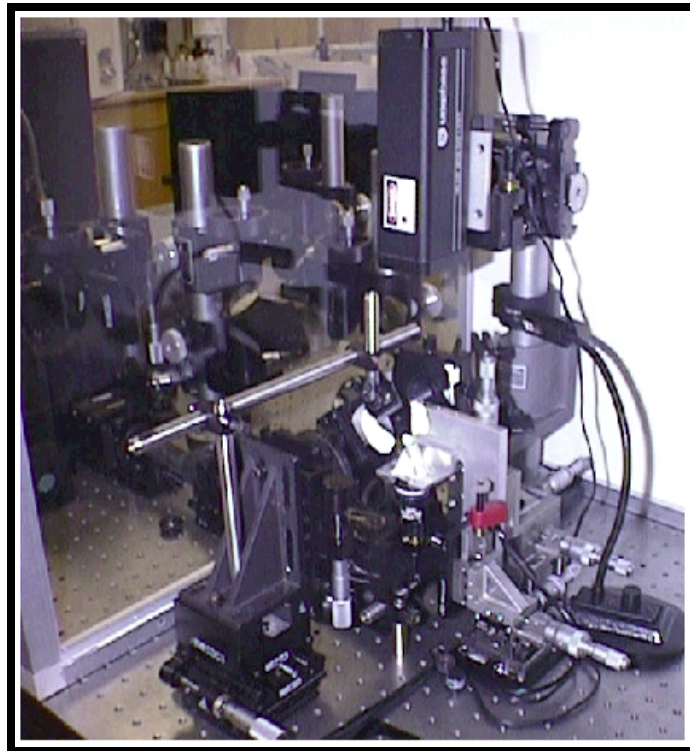


Figure 3: Photograph of sample area of experimental setup.

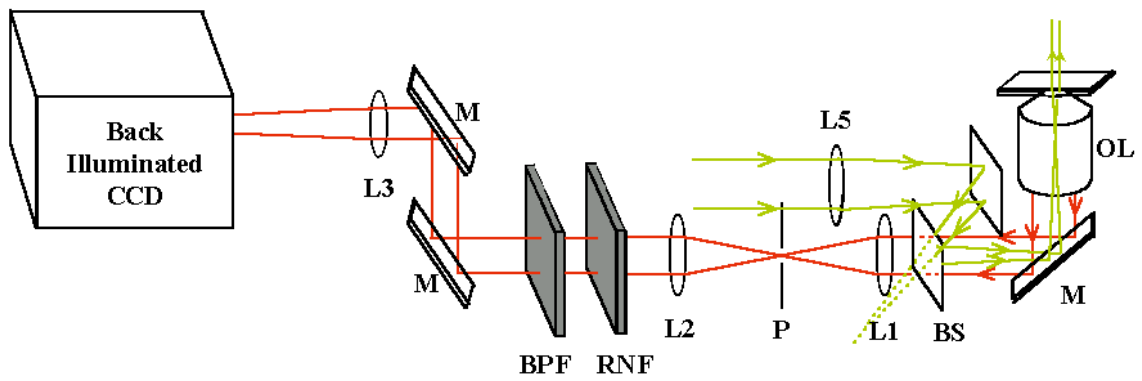


Figure 4: Schematic of experimental setup utilizing wide field epi-illumination.

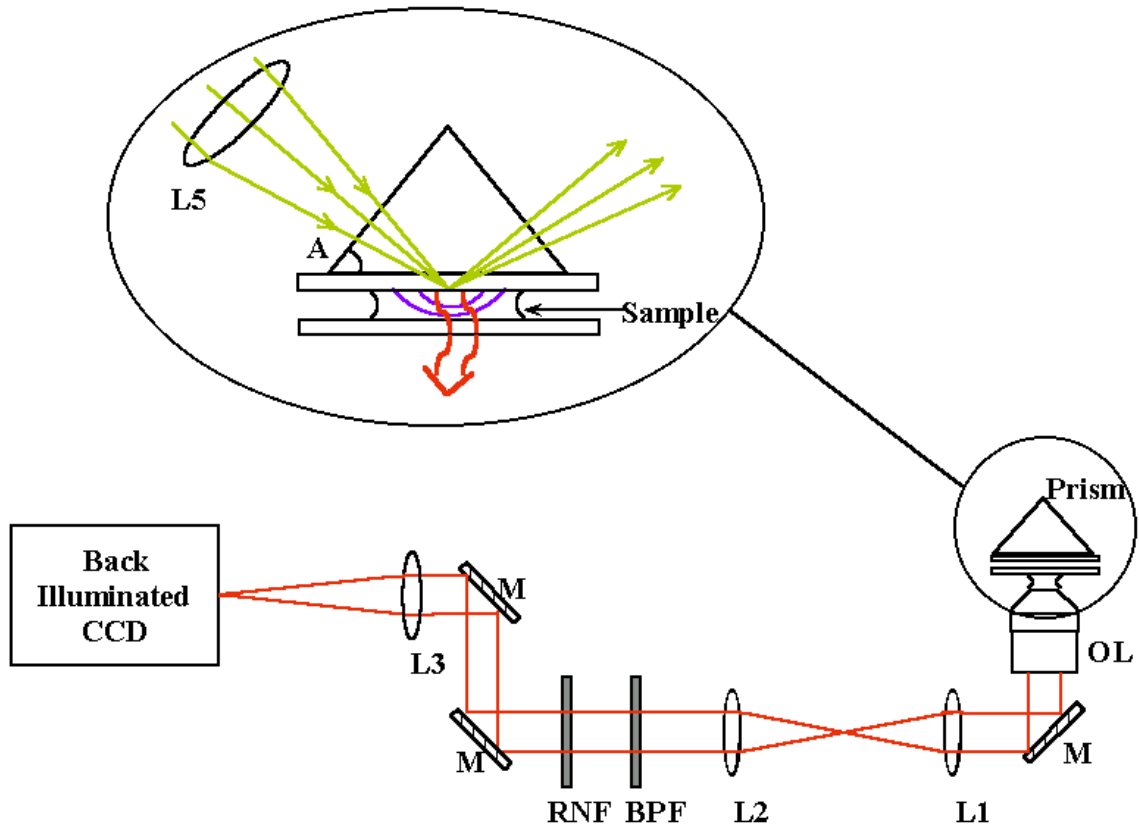


Figure 5: Schematic of experimental setup with TIR excitation. The inset shows a close-up of the prism, and the sample, with total internal reflection occurring and the resultant evanescent field.

pixels”. This allows for significantly faster readout times, but has the obvious disadvantage of reducing the camera resolution. The overall optical resolution will not be compromised provided that the optical magnification is sufficient so that a super pixel is smaller than the Airy disk image of a molecule.

Most of the data was collected with the maximum 512 × 512-pixel collection area with a readout time of 280 ms. Before operation the CCD was cooled to -40° C to reduce darknoise. The camera has a readout speed of 1 MHz, and an approximate noise of 5 electrons (root mean squared).

The tube lens in a commercial Olympus microscope has a focal length of 180 mm rather than 150 mm, and hence the effective magnification at the intermediate focal plane in our instrument is 50 rather than 60. The magnification at the CCD plane is simply related to the ratio of the imaging lens’ focal length to the focal length of the lens which recollimates the light after the tube lens, which in this case is 250/150, multiplied by the magnification of the objective with the 150 mm tube lens (50) giving an overall magnification of 83.3. This value was checked using calibration experiments in which latex beads were immobilized on the glass slide, which was then translated by a known distance. The corresponding displacement of the bead in the CCD image was then compared to the translation distance to verify the magnification. A Raman Notch filter (RNF) and a bandpass filter (BPF) were used to reduce the amount of background from both Rayleigh and Raman Scatter.

2.3 Forms of Excitation

2.3.1 Total Internal Reflection (TIR)

For TIR Illumination, the beam was focused (L5 in Figure 5) into a prism that was placed on top of the sample. The prism was a fused silica Brewster prism with an apex angle A of 68 degrees, and the beam was normal to the prism surface, producing an angle of incidence of $\theta = 68$ degrees at the fused silica-sample interface. The critical angle, θ_c , at this interface is given by:

$$\theta_c = \sin^{-1} \left(\frac{n_2}{n_1} \right) \approx 65.6^\circ, \quad (1)$$

where n_1 and n_2 are the refractive indexes of fused silica ($n_1 = 1.46$) and the sample ($n_2 = 1.33$), respectively. The result of TIR is an evanescent field whose intensity decays exponentially from its peak value I_0 with the distance z from the interface:

$$I(z) = I_0 e^{-z/d}, \quad (2)$$

with the penetration depth, d , given by [12]:

$$d = \frac{\lambda_0 / n_1}{4 \sqrt{\sin^2 \theta - (n_2 / n_1)^2}}, \quad (3)$$

where λ_0 is the wavelength of the light in vacuum. The penetration depth of the evanescent field with the above parameters is approximately $d = 0.2 \mu\text{m}$.

The polarization of the incoming beam in the experiments reported in Chapter 4 is perpendicular to the plane of incidence, resulting in an evanescent

field also polarized perpendicular to this plane. The total internal reflection of the laser at the fused silica-sample interface produces a standing wave inside the prism, and results in an enhancement of the field at the surface within the sample [12], such that

$$\frac{I_y}{I_s} = \frac{4 \cos^2 \theta}{1 - (n_2/n_1)^2}, \quad (4)$$

where I_y is the peak irradiance of the evanescent field (polarized in the y-direction, perpendicular to the plane of incidence), and I_s is the irradiance of the incident s-polarized laser beam. For an incident angle of 68° at the fused silica-sample interface, the enhancement factor is approximately 3.3. The beam profile is elliptical because the laser comes in at an angle, and the area illuminated is approximately $1.9 \times 10^{-4} \text{ cm}^2$. With a laser power of 10 mW, and taking the enhancement factor into account, the estimated mean sample irradiance is $170 \text{ W} \cdot \text{cm}^{-2}$ at the fused silica-sample interface.

2.3.2 Wide Field Epi-Illumination

When wide field epi-illumination was desired, the beam was directed through the objective lens underneath the sample, illustrated in Figure 4 with the yellow lines. Before passing through the objective, the beam was first expanded, and then focused inside the objective with a 250 mm achromat lens (Newport, PAC067; L4), thereby producing a larger, approximately collimated beam at the sample. This is accomplished by focusing

the incoming laser 6 mm prior to the sample, since the effective focal length of the objective, obtained by dividing the 180 mm tube lens focal length by the 60 \times objective magnification, is 3 mm. By examination of the resulting images, it was determined that the radius of the beam at the sample was 30 μ m, making an illumination area of approximately $3 \times 10^5 \text{ cm}^2$, and with a laser intensity of 50 μ W, the estimated irradiance was $1.7 \text{ W} \cdot \text{cm}^2$.

A fused silica window was used as a beam splitter (BS) to bring the excitation illumination to the sample without obstructing the collection optics. At 45° , the reflection coefficient of fused silica is 10% for s-polarization. Thus 10% of the laser power was used for excitation, while the 90% that was transmitted was dumped. Although this significantly reduces the excitation intensity, the fused silica window allows for 90% of the S-polarization component and 99% of the p-polarization component of the fluorescence to be transmitted, compared to the 90% transmitted by a typical dichroic beamsplitter. Further, the same component can be used for any excitation wavelength.

2.3.3 Epi-Illumination With Confocal Detection

Using the same basic configuration as depicted in Figure 4, with some minor adjustments, the experimental setup can be changed to a confocal epi-illumination system for use with FCS. The modifications necessary for this transformation include the removal of the lens (L4), so that the beam is tightly focused at the

sample, and the insertion of a 100 μm pinhole (Melles Griot, 04PIP015; P in Figure 4) between the tube lens (L1) and the recollimating lens (L2). The collected fluorescence is then directed to a polarizing beam-splitter (rather than the CCD), and the two polarization components are sent to two separate single photon avalanche diodes (SPADs) (Custom modules obtained from EG&G Optoelectronics, Canada).

To align the confocal microscope, the pinhole is removed and the coverslip is moved towards the objective, such that the back-reflected laser spot is focused close to where the aperture would be located. The coverslip is then translated back and forth through focus to determine if the back-reflected spot remains optically centered. If not, the incoming laser beam is not aligned along the axis of the instrument, and the beam path is then altered slightly (by adjusting one of the mirrors that brings the beam into the objective) until the spot remains centered. The distance between the objective lens and the coverslip is then changed so the back-reflected spot is again focused near the pinhole. The pinhole is reinserted and adjusted until the back-reflected beam passes completely through the aperture. Finally, the coverslip is translated down approximately 15 μm , to ensure that the excitation volume is at an adequate distance from the coverslip surface.

3 Results and Conclusions

3.1 Fluorescence Correlation Spectroscopy

Analysis of data obtained in single-molecule detection experiments by correlation methods, as used in Fluorescence Correlation Spectroscopy (FCS) [13], can yield information about the motion of molecules through the sample volume. FCS utilizes the autocorrelation function, $G(\tau)$, of a data stream given by [14]:

$$G(\tau) = \lim_{T \rightarrow \infty} \frac{1}{2T} \int_0^T i(t+\tau)i(t)dt, \quad (5)$$

where $i(t)$ is the number of photons detected at time t . In practice, however, the photon intensity is recorded at discrete times and the data is collected for a finite duration T , so that the autocorrelation function becomes a sum,

$$G(\tau) = \frac{1}{T} \sum_{t=1}^T i(t+\tau)i(t). \quad (6)$$

The collected normalized autocorrelation function may be curve-fitted with a model developed by Aragon and Pecora [15],

$$G(t) = 1 + a_1(1 + a_2 t)^{-1}(1 + a_2 a_3 t)^{-1/2}, \quad (7)$$

where the probe volume is approximated to be a three-dimensional Gaussian ellipsoid, $1/a_1 = N$ is the mean number of fluorophores in the probe volume, $1/a_2 = \tau_D$ is the mean diffusional residence time, and $a_3 = \tau_D^2/z_0^2$, with τ_D being the probe

volume radius, and z_0 the probe volume axial radius, or depth. Figure 6 shows the normalized autocorrelation function for Bodipy Texas Red at a concentration of 10^{10} M, along with the curve fit that was performed on it. The fitting parameters for this solution were $a_1 = 1.2939$, corresponding to an average of 0.77 fluorophores in the sample volume, $a_2 = 3354$, resulting in a diffusional residence time of approximately 300 μ s, and $a_3 = 0.0026442$. This value of a_3 is smaller than the expected value of approximately 0.0625, but the fit is relatively insensitive to this parameter, as illustrated by the green curve in Figure 6, obtained by curve-fitting with a fixed value of 0.0625 for a_3 . Spherical aberration or other causes for deviation from the assumption of a 3-D Gaussian ellipsoidal probe volume may account for the small deviations between the data and the fit.

3.2 Image Analysis

Images from the back-illuminated CCD were collected on a PC with WinView software (Roper Scientific), and analyzed using a custom program, which was written for this thesis in Matlab. The Matlab program is a Graphical User Interface (GUI), which reads in the individual frames of a movie collected with WinView. The GUI is then used to select a region of interest, zoom in as desired, and plot the integrated intensity of this region as a function of movie frame. The data from the intensity plot can then be saved as a text file for later use.

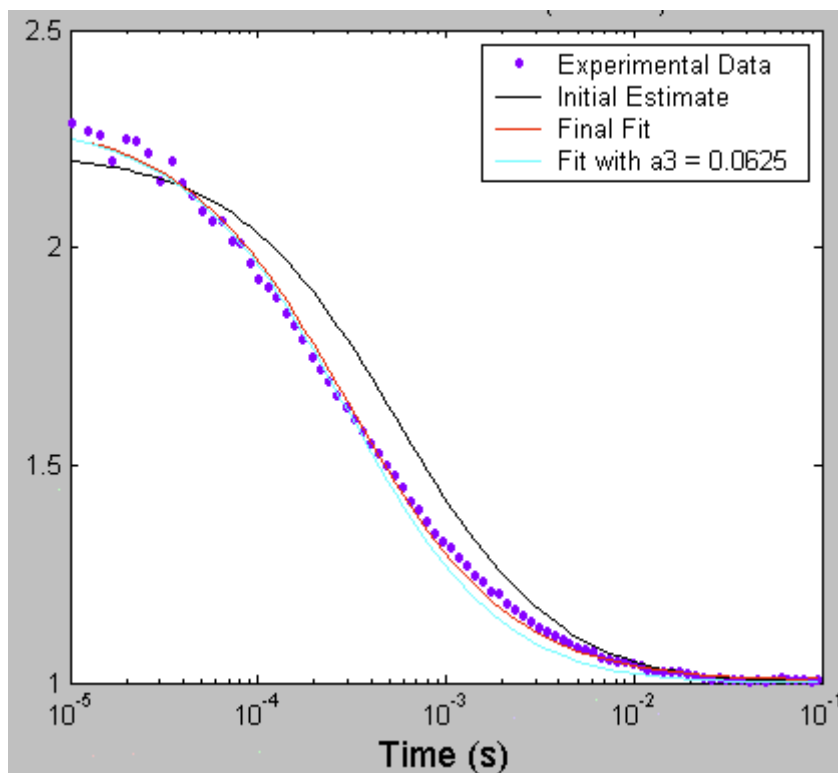


Figure 6: Normalized autocorrelation function for 10^{10} M Bodipy Texas
 Red and curve fit.

The GUI is shown in Figure 7, and the Matlab code is given in the Appendix.

3.3 Single-Molecule Imaging Results

The images, shown in Figures 8 and 9 obtained using wide-field epi-illumination and TIR excitation respectively, exhibit many interesting features. Some molecules (yellow arrows) appear suddenly and remain in the same place for several frames, but the intensity fluctuates in this time period. Others appear for only a single frame (red arrows). The latter are most probably freely diffusing through the sample volume, while the former are molecules that apparently stick to the glass coverslip. For these, the fluctuation in fluorescence intensity is most probably caused by a wobbling or fluctuation of the transition dipole moment of the molecule with respect to the excitation polarization [16].

The intensity of the molecules indicated by the yellow arrows is plotted in Figures 10 and 11. While the fluctuations shown here could be the result of fluctuations in the dipole orientation, there are also other mechanisms that could account for this blinking. The molecules could be making transitions to the triplet manifold, which would result in an elimination of fluorescence for part of a CCD frame, and hence reduced apparent brightness. Another common cause for blinking is that the molecules undergo an isomerization, whereby the molecule folds into different configurations, which emit different amounts of fluorescence. However, Bodipy Texas Red is not known to undergo such isomerization changes.

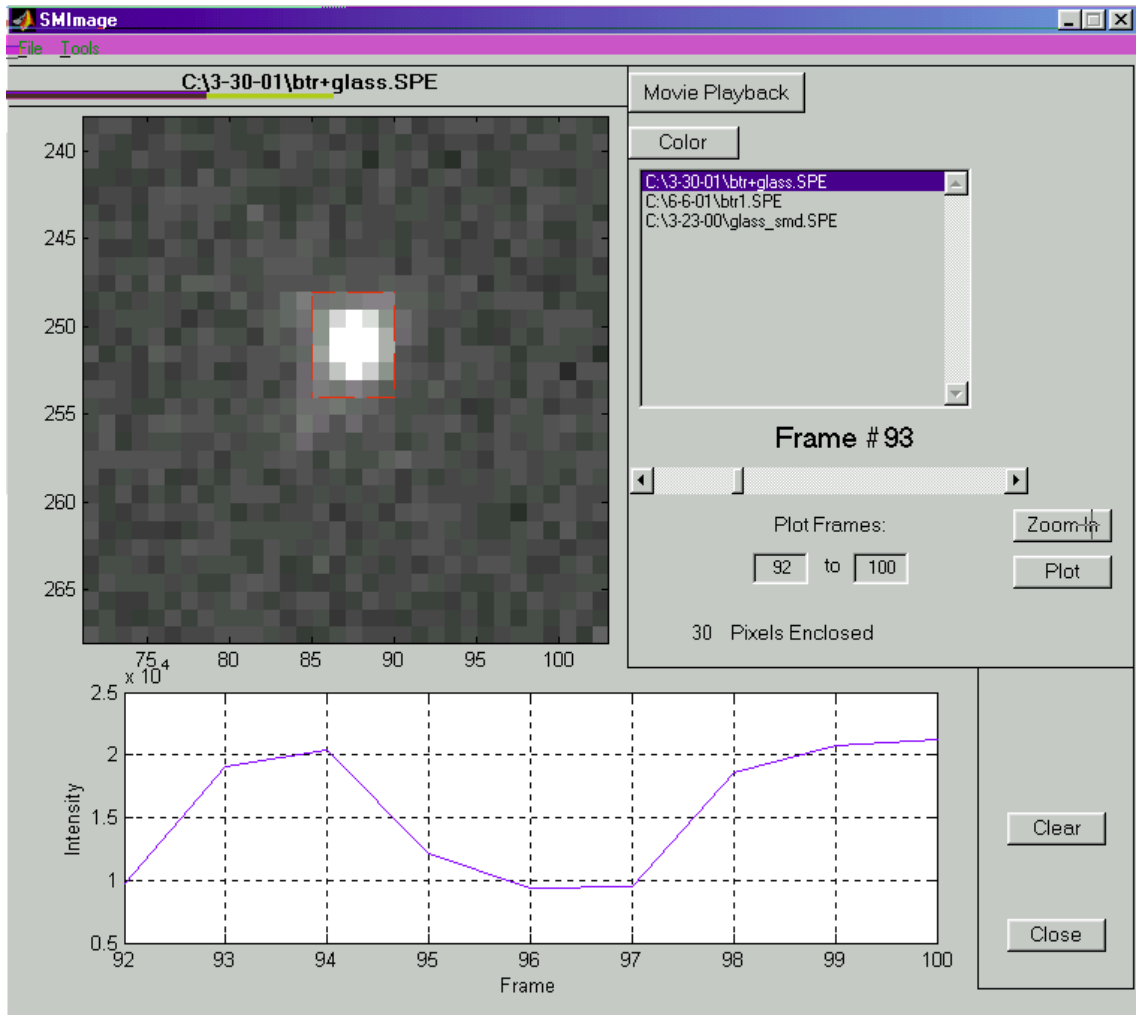


Figure 7: Screenshot of Matlab GUI used for image analysis.

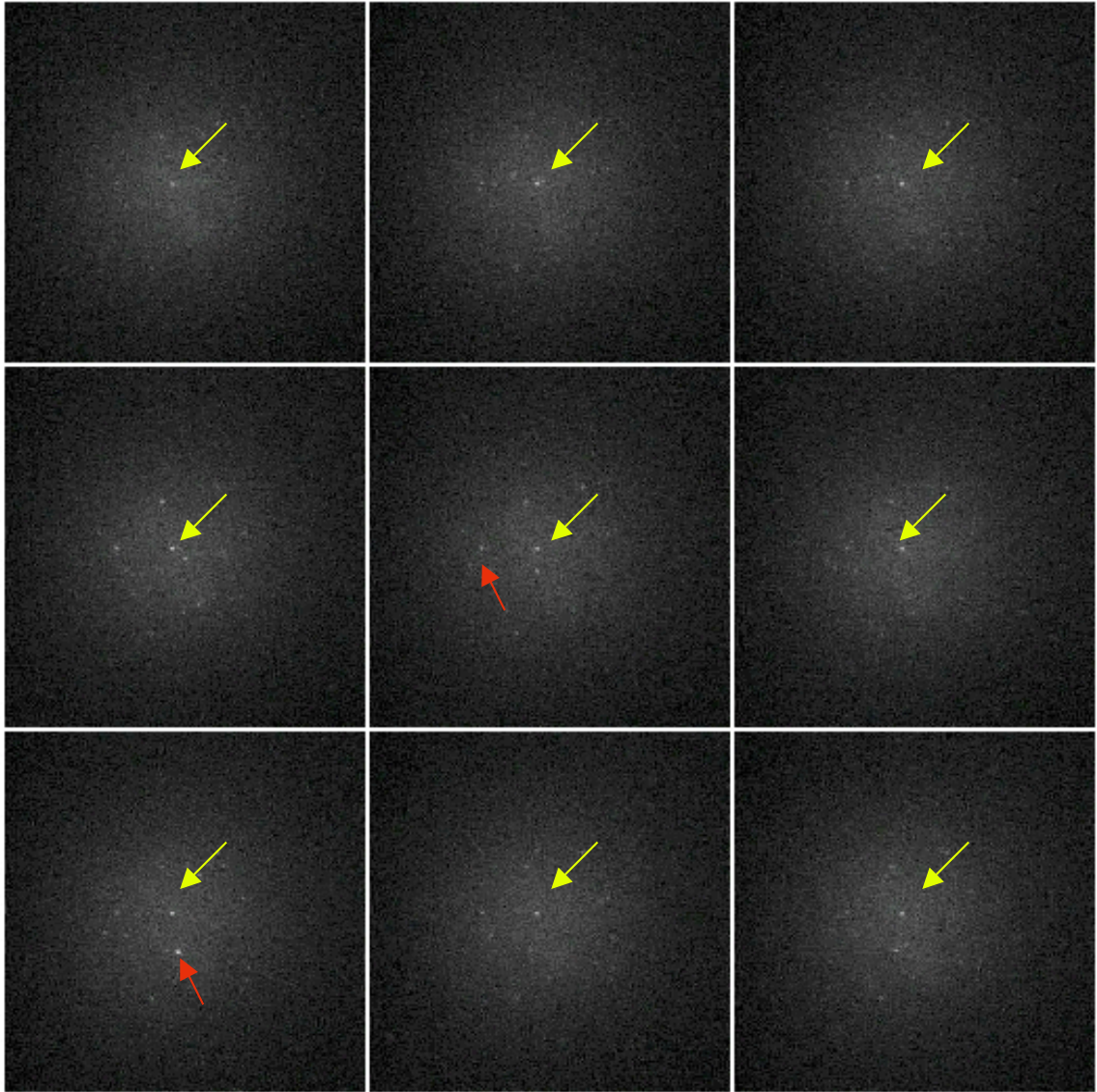


Figure 8: WinView images collected using wide field epi-illumination. Each image is 512×512 pixels and is approximately $80 \mu\text{m} \times 80 \mu\text{m}$ in object space, while the Airy disk of a single molecule is approximately $0.595 \mu\text{m}$ diameter in object space, or 3.8 pixels in image space.

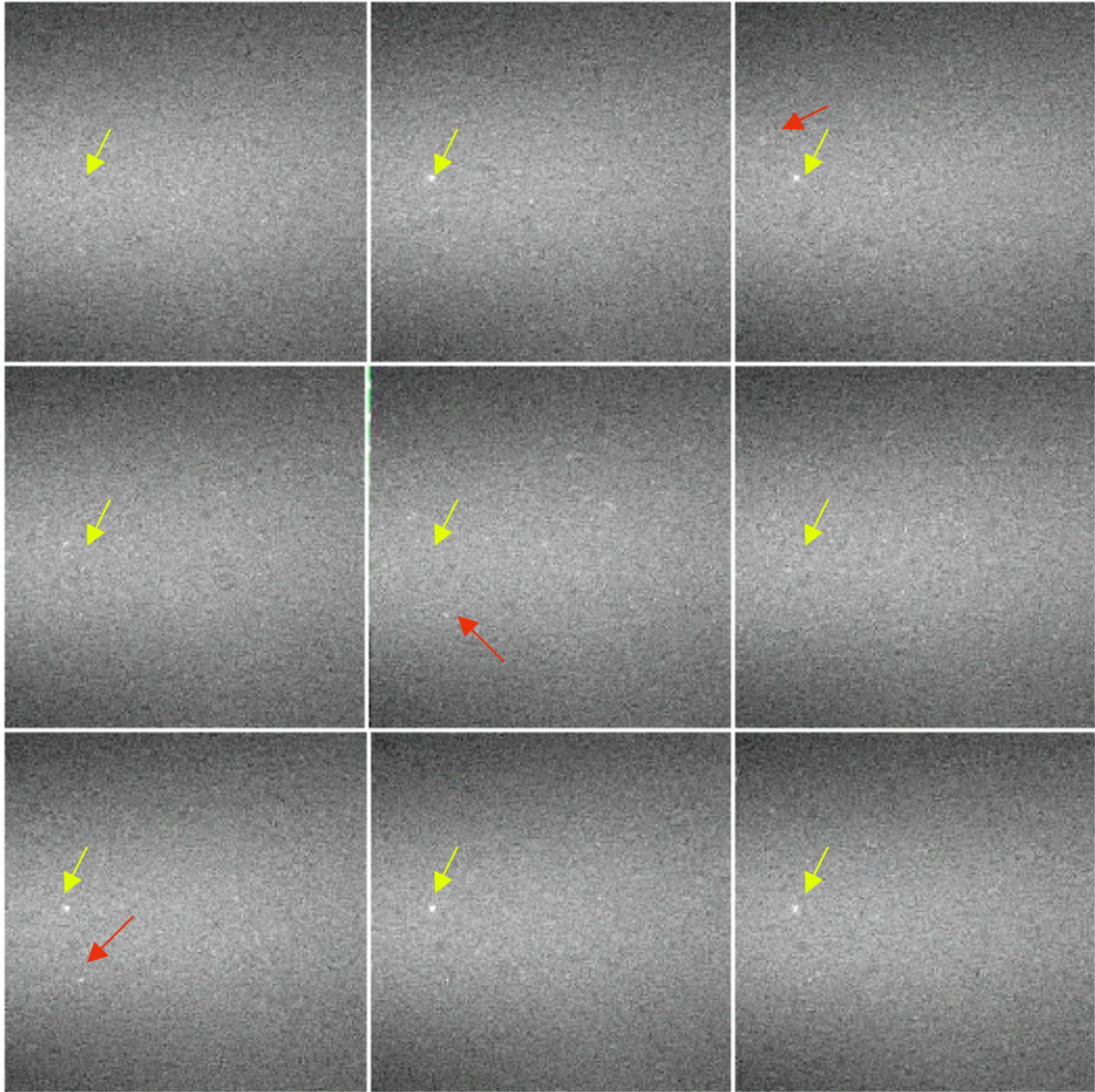


Figure 9: Images obtained with WinView using TIR illumination. The resolution and magnification are the same as in Figure 8, i.e., the image is 512 × 512 pixels and is approximately 80 μm × 80 μm in object space.

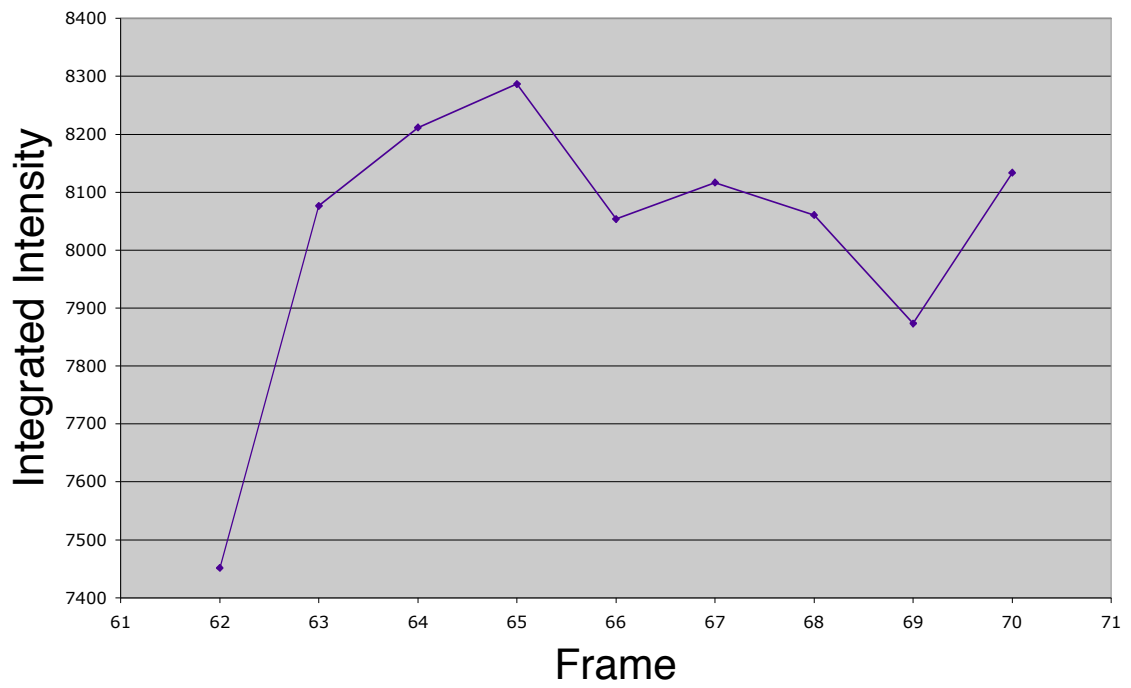


Figure 10: Intensity of the molecule indicated by the yellow arrows in Figure 8. The intensity is integrated over 25 pixels.

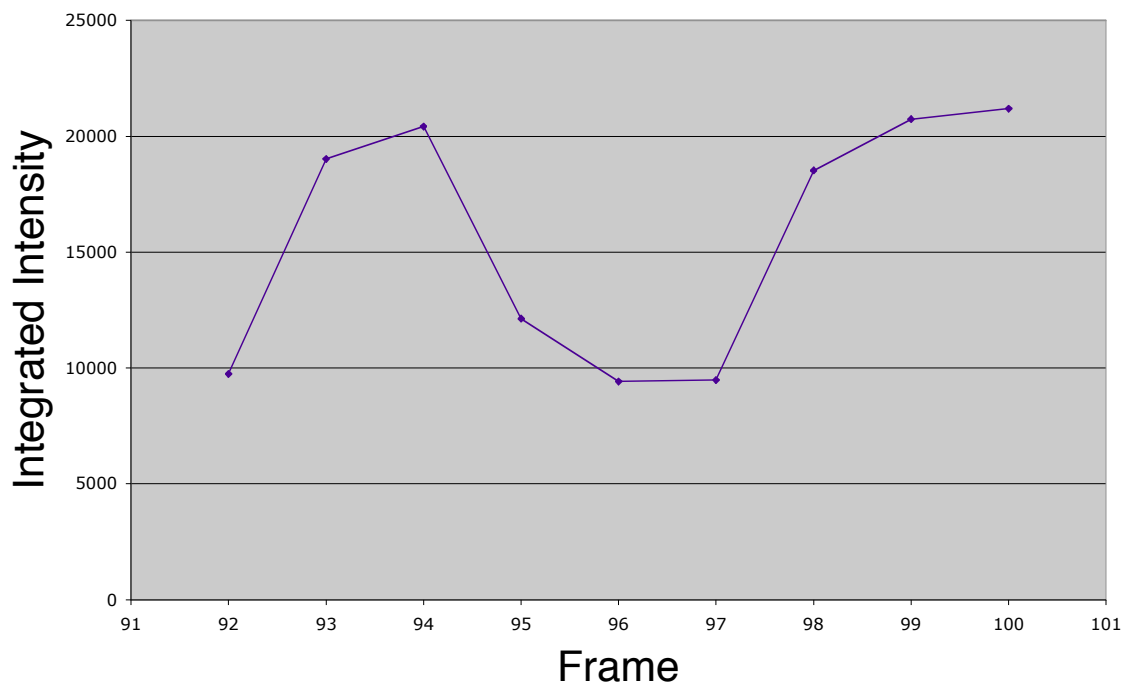


Figure 11: Intensity of the molecule indicated by the yellow arrows in Figure 9.

The intensity is integrated over 25 pixels.

3.4 Conclusions

Single-molecule imaging has become a very important scientific tool because of the many possible applications in many areas of biology. Here, it has been shown that it is feasible to construct an apparatus capable of performing single-molecule imaging, and also single-molecule detection. Single-molecule imaging has the ability to multiplex observations, so that many experiments, can be performed in parallel. Aside from potential biotechnology applications such as rapid DNA sequencing, single-molecule imaging is proving to be helpful in studying the ways in which cells communicate [17].

4 Ongoing and Future Research Possibilities

Current research projects involve the use of FCS for high-throughput pharmaceutical drug screening [18-20]. In these investigations a fluorescently labeled molecule bound to a target protein or receptor becomes displaced when a drug-like compound binds to the target. The result of this should be an autocorrelation function that exhibits two distinct diffusion coefficients, one for the free fluorescently labeled molecule, and one for the bound.

One possibility for future research in single-molecule imaging is the study of cellular communication. In preliminary experiments, epidermal growth factor (EGF) ligands labeled with Alexa 590 dye were observed interacting with corresponding receptors on the surface of 32D blood-line cells, which were provided by collaborators at Vanderbilt University. Such studies can result in a better understanding of the detailed mechanisms by which biological cells transfer information between one another, which, in turn, can lead to improved treatments for certain disorders.

Some future investigations in the area of single-molecule detection could include the study of molecules close to metal particles, and the detection of single fluorophores enclosed in nanometer-sized particles. Preliminary work has begun in both of these areas by other groups, but more studies are required to explore their possible biological applications.

It has been shown that metallic particles enhance the electric field incident on a nearby molecule (the “lightning rod effect”), and also decrease the fluorescence lifetime [21]. The enhancement of the electric field allows the same emission intensity to be detected with a much lower excitation intensity, which results in less photochemical damage to the sample. Because the photon flux of a fluorophore is approximately limited by the inverse of the fluorescence lifetime, a decrease in lifetime results in more photons, and also an increased photostability.

The molecules examined in [21] include fluorophores with both high and low quantum efficiencies. Although there was little improvement in the fluorescence collected from Rhodamine B (high quantum efficiency) near metal particles, the fluorescence of Rose Bengal (low intrinsic quantum efficiency) was significantly enhanced by the presence of the metal. Because the metal particles allow for the detection of molecules with low quantum efficiencies, this method could be used for the study of unlabelled DNA.

Srivastava, et al. have shown that when fluorophores are enclosed in silica nanoparticles, the molecules do not exhibit photobleaching [22]. This is due to the fact that the molecules are isolated from the oxygen molecules in the solution, which chemically alter the fluorophore when it is in the more reactive, excited state. The size of these nanoparticles, along with their improved photostability, makes them ideal for labeling biomolecules, for the study of such things as molecular motors.

References

References

- [1] L.M. Davis, W.C. Parker, D.A. Ball, J.G.K. Williams, G.R. Bashford, P. Sheaff, R. Eckles, D.T. Lamb, and L.R. Middendorf. *Imaging of single-chromophore molecules in aqueous solution near a fused-silica interface*, SPIE BIOS '01 (International Symposium on Biomedical Optics), San Jose, January 2001; p. 301 in 'Multiphoton Microscopy in the Biomedical Sciences', ed. by Ammasi Periasamy, Peter T.C. So, Proceedings of SPIE, Vol. 4262 (2001);
(<http://spie.org/scripts/abstract.pl?bibcode=2001SPIE%2e4262%2e%2e301D&page=1&q=spie>)
- [2] L.M. Davis, W.C. Parker, D.A. Ball, J.G.K. Williams, G. Bashford, D.L. Grone, R.D. Eckles, and L.R. Middendorf. *Imaging single molecules in solution near a fused-silica interface*, 45th Annual Meeting of the Biophysical Society, February 17-21, 2001, Boston, MA.; Biophysical Journal, Vol. 80, p. 160a (2001).
- [3] Joseph R. Lakowicz. **Principles of Fluorescence Spectroscopy**, 2nd ed., Kluwer Academic/Plenum Publishers, New York (1999).
- [4] Liqiang Li. *Single molecule detection in solution*, Ph.D. Dissertation, University of Tennessee, Knoxville (1994).
- [5] T. Hirschfeld. *Optical microscopic observation of single small molecules*,

- Appl. Opt., **15**, 2965-2966 (1976).
- [6] Norman J. Dovichi, John C. Martin, James H. Jett, Michael Trkula, and Richard A. Keller. *Laser-induced fluorescence of flowing samples as an approach to single molecule detection in liquids*, Anal. Chem., **56**, 348-354 (1984).
- [7] Dinh C. Nquyen, Richard A. Keller, and Mitchell Trkula. *Ultrasensitive laser-induced fluorescence detection in hydrodynamically focused flows*, J. Opt. Soc. Am. B, **4**, 138-143 (1987).
- [8] E. Brooks Shera, Newton K. Seitzinger, Lloyd M. Davis, Richard A. Keller, and Steven A. Soper. *Detection of single fluorescent molecules*, Chem. Phys. Lett., **174**, 553-557 (1990).
- [9] L.M. Davis, F.R. Fairfield, J.H. Hahn, C.A. Harger, J.H. Jett, R.A. Keller, L.A. Krakowski, B.A. Marrone, J.C. Martin, H.L. Nutter, R.L. Ratliff, E.B. Shera, D.J. Simpson, and S.A. Soper. *Rapid DNA Sequencing Based Upon Single Molecule Detection*, Genetic Analysis: Techniques and Applications, **8**, 1 (1991).
- [10] John G. K. Williams *Heterogeneous assay for pyrophosphate detection*, United States Patent #6,232,075 (2001).
- [11] Takashi Funatsu, Yoshie Harada, Makio Tokunaga, Kiwamu Saito, Toshio Yanagida. *Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution*, Nature, **374**, 555-559 (1995).

- [12] Lukas K. Tamm. *Total internal reflection fluorescence microscopy*, **Optical Microscopy: Emerging Methods and Applications**, Ed. B Herman and J. J. Lemasters, Academic Press, San Diego (1993).
- [13] R. Rigler, U. Mets, J. Widengren, and P. Kask. *Fluorescence correlation spectroscopy with high count rate and low background: analysis of translational diffusion*, Euro. Biophys. J., **22**, 169-175 (1993).
- [14] Rudolf Rigler, Jerker Widengren, and Ulo Mets. *Interactions and kinetics of single molecules as observed by fluorescence correlation spectroscopy*, **Fluorescence Spectroscopy: New Methods and Applications**, Ed. Otto S. Wolfbeis, Springer-Verlag, Berlin (1992).
- [15] S. R. Aragón, R. Pecora. *Fluorescence correlation spectroscopy as a probe of molecular dynamics*, J. Chem. Phys., **64**, 1791-1803 (1976).
- [16] W. E. Moerner and Michel Orrit. *Illuminating single molecules in condensed matter*, Science, **283**, 1670-1675 (1999).
- [17] Yoshiharu Ishii and Toshio Yanagida. *Single molecule detection in life science*, Single Molecules, **1**, 5-16 (2000).
- [18] L.M. Davis, P.E. Williams, H.M. Cain, D.A. Ball, C.G. Parigger, E.D. Matayoshi, and K.M. Swift. *Comparison of fluorescence correlation spectroscopy and other single-molecule data analysis methods for assay of protein-ligand interactions*, 46th Annual Meeting of the Biophysical Society, San Francisco, California, February 23-27, 2002; Biophysical J. **82**, p. 43a

(2002).

- [19] L. M. Davis, D. A. Ball, P. E. Williams, K.M. Swift, and E. D. Matayoshi. *Data Reduction Methods for Application of FCS to High-Throughput Pharmaceutical Drug Screening*, 6th International Carl Zeiss Workshop on Fluorescence Correlation Spectroscopy & Related Methods, St. Louis, MO, May 21-22, 2002; ([http://www.zeiss.de/C12567BE00472A5C/EmbedTitelIntern/ProgramFCSWorkshop2002/\\$File/Program_FCS_Workshop_2002.pdf](http://www.zeiss.de/C12567BE00472A5C/EmbedTitelIntern/ProgramFCSWorkshop2002/$File/Program_FCS_Workshop_2002.pdf), Page 21.)
- [20] L.M. Davis, D.A. Ball, P.E. Williams, K.M. Swift, and E.D. Matayoshi. *Fluorescence Correlation Spectroscopy data reduction and application to peptide binding studies with BCL-xL*, 5th International Weber Symposium on Innovative Fluorescence Methodologies in Biochemistry and Medicine, Lihue, HI., June 25-29, 2002; (<http://fms.physics.uiuc.edu/LFD/Weber/FMPro?-DB=weberevent.FP5&-Format=speakersonly.html&-SortField=LAST&-max=200&-FindAll>, Record 19.)
- [21] Joseph R. Lakowicz, Ignacy Gryczynski, Yibing Shen, Joanna Malicka, and Zygmunt Gryczynski. *Intensified fluorescence*, Photonics Spectra, **35**, 96-104 (2001).
- [22] Mamta Srivastava, Hooisweng Ow, Daniel Larson, David Holowka, Ulrich Wiesner, Watt Webb, and Barbara Baird. *Single particle tracking of*

fluorescent silica nanoparticles bound to IgE receptors on RBL mast cells, 46th
Biophys. Soc. Meeting, 2436-Pos, Board #B675 (2002).

Appendix

Graphical User Interface (GUI) for Image Analysis

This program, which is described in Section 3.2, was written in Matlab version 6 (release 12) for Microsoft Windows 98. The program utilizes a sub-program called `read_spe.m`, which was written by Greg Bashford of LI-COR and hence is not included. The sub-program reads the data files from movies created by Roper Scientific's WinView software and places the data into a three-dimensional array. It determines the number of pixels in the x-direction, M , the number of pixels in the y-direction, N , and the number of frames in the movie, F . The size of the array that is created is hence $M \times N \times F$, and each element is the intensity of the corresponding pixel. The sub-program allows for the specification of the number of frames to be used, i.e. `x=read_spe('movie1', 1:30)` would read in the first 30 frames of 'movie1'.

```
function varargout = SMImage(varargin)

% SMIMAGE Application M-file for SMImage.fig

% FIG = SMIMAGE launch SMImage GUI.

% SMIMAGE('callback_name', ...) invoke the named callback.

% This program is intended for use with images collected using %WinView,(.SPE
extensions). The purpose of the program is to look at intensity fluctuations for a
```

group of pixels

% D. Ball 8-01-01

% Last Modified by GUIDE v2.0 19-Jul-2001 15:16:01

if nargin == 0 % LAUNCH GUI

fig = openfig(mfilename,'reuse');

% Use system color scheme for figure:

set(fig,'Color',get(0,'defaultUicontrolBackgroundColor'));

% Generate a structure of handles to pass to callbacks, and store it.

handles = guihandles(fig);

guidata(fig, handles);

if nargout > 0

varargout{1} = fig;

end

elseif ischar(varargin{1}) % INVOKE NAMED SUBFUNCTION OR

%CALLBACK

try

[varargout{1:nargout}] = feval(varargin{:}); % FEVAL switchyard

catch

disp(lasterr);

```

    end

end

% First we need to load a list of files created from previous sessions of

    %SMImage

load ListBox.mat

smimage = get(0,'Children');

LB = findobj(smimage,'Tag','listbox1');

set(LB,'String',List)

Open_File = findobj(smimage,'Tag','Open_File');

set(Open_File,'Accelerator','o');

Save_File = findobj(smimage,'Tag','Save_File');

set(Save_File,'Accelerator','s');

Exit_SMImage = findobj(smimage,'Tag','Exit_SMImage');

set(Exit_SMImage,'Accelerator','q');

Exit_MATLAB = findobj(smimage,'Tag','Exit_MATLAB');

set(Exit_MATLAB,'Accelerator','x');

```



```
Next_Frame = findobj(smimage,'Tag','Next_Frame');
```

```
set(Next_Frame,'Accelerator','n');
```

```
Previous_Frame = findobj(smimage,'Tag','Previous_Frame');
```

```
set(Previous_Frame,'Accelerator','p');
```

```
Zoom_In = findobj(smimage,'Tag','Zoom_In');
```

```
set(Zoom_In,'Accelerator','i');
```

```
Zoom_Out = findobj(smimage,'Tag','Zoom_Out');
```

```
set(Zoom_Out,'Accelerator','z');
```

```
Plot_Frames = findobj(smimage,'Tag','Plot_Frames');
```

```
set(Plot_Frames,'Accelerator','l');
```

```
% -----
```

```
function varargout = Open_File_Callback(h, eventdata, handles, varargin)
```

```
% Creates a dialog box to find files with .spe extension, and displays the movie in
```

```
%the upper axes (Currently only the first 10 frames are read in).
```

```
set(handles.axes1,'NextPlot','replace'); % Clears any previous image that may be
```

```

%displayed

Zoom_In = findobj(gcf,'Tag','Zoom_In');
Zoom_Out = findobj(gcf,'Tag','Zoom_Out');
Plot_Frames = findobj(gcf,'Tag','Plot_Frames');
set(Zoom_In,'Enable','off');
set(Zoom_Out,'Enable','off');
set(Plot_Frames,'Enable','off');

global NFRAMES %
global xdim % global variables retrieved from READ_SPE
global ydim

% Open the dialog box, and search for .SPE files
[fname,pname] = uigetfile({'*.spe', 'WinX Data (*.spe)'},'Open');
FILENAME = [pname fname];

if isequal(fname,0)|isequal(pname,0)

    FILENAME = get(handles.listbox1,'UserData');

    FileLim = get(handles.axes1,'UserData');

    if isempty(FILENAME)|isempty(FileLim)

```

```

return
else
    File_MIN = FileLim(1);
    File_MAX = FileLim(2);
    frame = get(handles.text5,'String');
    Frame = str2num(frame);
    file = read_spe(FILENAME,Frame);
    imagesc(file,'parent',handles.axes1,[File_MIN,File_MAX]);
end
set(handles.listbox1,'UserData',FILENAME);
set(handles.axes1,'Tag','axes1');
set(handles.axes1,'UserData',[File_MIN File_MAX]);
return

```

else

```

% Add the file opened with the dialog box to the listbox, and save the updated
% list.
List = get(handles.listbox1,'String');
for Lng = 2:length(List)+1
    New{Lng} = List{Lng - 1};
end

```

```

New{1} = FILENAME;

List = New;

set(handles.listbox1,'String',List)

save ListBox List

% If toggle button is pressed the image will appear in Pseudo color.

button_state = get(handles.togglebutton1,'Value');

if button_state == 1

    colormap(jet);

elseif button_state == 0

    colormap(gray);

end

% Read in the first frame of the data to set the color scale, then delete.

File1 = read_spe(FILENAME,50);

File_MIN = min(min(File1));

File_MAX = max(max(File1));

set(handles.axes1,'CLim',[File_MIN File_MAX]);

clear File1;

set(handles.text4,'String',FILENAME) % Sets the title above the image to the

```

```

file name

% Read in the data

for j = 1:10

    File = read_spe(FILENAME,j,j);

    set(handles.slider1,'Max',NFRAMES,'SliderStep',[1/NFRAMES
10/NFRAMES]);

    IMG = imagesc(File,'parent',handles.axes1,[File_MIN,File_MAX]);

    M(j) = getframe(handles.axes1);

    set(handles.slider1,'Value',j);

    set(handles.text5,'String',j);

end

% Re-set the info that was cleared from altering the axes

set(handles.axes1,'Tag','axes1')

set(gcbo,'UserData',M)

set(gcbo,'Tag','Open_File')

set(handles.listbox1,'UserData',FILENAME);

set(handles.axes1,'UserData',[File_MIN File_MAX]);

set(handles.pushbutton2,'Enable','on');

end

% -----

```

```

function varargout = Save_File_Callback(h, eventdata, handles, varargin)

%Saves a text file (Data.txt) in the current directory. This text file contains %the
filename of the movie, the range of pixels, and the data displayed in
%handles.axes2

Sigma = get(handles.pushbutton1,'UserData');

iframe = get(handles.edit1,'String');

Iframe = str2num(iframe);

fframe = get(handles.edit2,'String');

Fframe = str2num(fframe);

frame_vec = Iframe:Fframe;

Data = [frame_vec;Sigma];

EditMarker = findobj(gcf,'Tag','EditMarker');

x = get(EditMarker,'XData');

y = get(EditMarker,'YData');

xmax = max(x);

xmin = min(x);

ymax = max(y);

ymin = min(y);

File = get(handles.listbox1,'UserData');

```

```

fid = fopen('Data.txt','w');

fprintf(fid,'Filename: %s\n', File);

fprintf(fid,'Range of pixels in x: %6.2d to %6.2d\n', xmin, xmax);

fprintf(fid,'Range of pixels in y: %6.2d to %6.2d\n', ymin, ymax);

fprintf(fid,'%6.2d\t %6.2d\n',Data);

fclose(fid);

% -----

function varargout = togglebutton1_Callback(h, eventdata, handles, varargin)

% Callback for togglebutton1 (labeled 'Color' in GUI). Choices are gray
%scale(button not selected), and jet (button pressed).

button_state = get(gcbo,'Value');

if button_state == 1

    colormap(jet);

elseif button_state == 0

    colormap(gray);

end

% -----

function varargout = listbox1_Callback(h, eventdata, handles, varargin)

% Callback for the uicontrol handles.listbox1. A file can be opened directly
%from the listbox without the use of the Open dialog.

% Note: A single click on a filename is sufficient to open it.

```

```

set(handles.axes1,'NextPlot','replace');

global NFRAMES

Zoom_In = findobj(gcf,'Tag','Zoom_In');
Zoom_Out = findobj(gcf,'Tag','Zoom_Out');
Plot_Frames = findobj(gcf,'Tag','Plot_Frames');

set(Zoom_In,'Enable','off');
set(Zoom_Out,'Enable','off');
set(Plot_Frames,'Enable','off');

List = get(gcbo,'String');
index = get(gcbo,'Value');
File = List{index};
File1 = read_spe(File,50);
File_MIN = min(min(File1));
File_MAX = max(max(File1));
set(handles.axes1,'CLim',[File_MIN File_MAX]);

% Again, check the toggle button to decide which color scheme to use
button_state = get(handles.togglebutton1,'Value');

if button_state == 1

```



```

        colormap(jet);
elseif button_state == 0
    colormap(gray);
end

set(handles.text4,'String',File); % Sets the title above image to filename

% Read in data
for j = 1:10
    y = read_spe(File,j);

    set(handles.slider1,'Max',NFRAMES,'SliderStep',[1/NFRAMES
        10/NFRAMES]);

    imagesc(y,'parent',handles.axes1,[File_MIN,File_MAX]);

    M(j) = getframe(handles.axes1);

    set(handles.slider1,'Value',j);

    set(handles.text5,'String',j);
end

% Re-set items cleared from changes to axes
Open_File = findobj(gcf,'Tag','Open_File');
set(Open_File,'UserData',M);

```

```

set(handles.axes1,'Tag','axes1');

set(handles.listbox1,'UserData',File);

set(handles.axes1,'UserData',[File_MIN File_MAX]);

set(handles.pushbutton2,'Enable','on');

% -----

function varargout = slider1_Callback(h, eventdata, handles, varargin)

% Callback for the uicontrol handles.slider1. Controls which frame is %displayed
in the axes

% Note: The slider can be used to look at frames that may not have been
%displayed when the file was first opened.

set(handles.axes1,'NextPlot','replace');

% Get needed info, ie. name of file and min & max intensities
FILENAME = get(handles.listbox1,'UserData');

FileLim = get(handles.axes1,'UserData');

File_MIN = FileLim(1);

File_MAX = FileLim(2);

% Get the current slider value, round it down, and read in only that frame
f = get(handles.slider1,'Value');

F = floor(f);

```

```

File = read_spe(FILENAME,F);

imagesc(File,'parent',handles.axes1,[File_MIN,File_MAX]);

set(handles.text5,'String',F);

set(handles.axes1,'Tag','axes1');

set(handles.axes1,'UserData',[File_MIN File_MAX]);

% -----

function varargout = pushbutton5_Callback(h, eventdata, handles, varargin)

% Callback for the uicontrol handles.pushbutton5 (labeled 'Zoom In' in GUI)

% Note: A region of the image must be selected before 'Zoom In' can be

%pressed.

% Get needed info, ie. name of file and min & max intensities

FILENAME = get(handles.listbox1,'UserData');

FileLim = get(handles.axes1,'UserData');

File_MIN = FileLim(1);

File_MAX = FileLim(2);

% Set properties of the image axis, and get the position of the editmarker

set(handles.axes1,'NextPlot','replace');

F = get(handles.slider1,'Value');

EditMarker = findobj(gcf,'Tag','EditMarker');

```

```

x = get(EditMarker,'XData');
y = get(EditMarker,'YData');

xmax = max(x);
xmin = min(x);
ymax = max(y);
ymin = min(y);

% Read in data, but display only that which lies in the editmarker box
file = read_spe(FILENAME,F);
zoom_file = file(ymin:ymax,xmin:xmax);
set(handles.axes1,'XLim',[xmin xmax]);
set(handles.axes1,'YLim',[ymin ymax]);
set(handles.axes1,'NextPlot','add');
imagesc(zoom_file,'parent',handles.axes1,[File_MIN,File_MAX]);
set(handles.axes1,'Tag','axes1');
set(handles.pushbutton5,'UserData',[F xmin ymin]);
set(handles.text8,'String','0');
set(gcbo,'Enable','off');
set(handles.pushbutton1,'Enable','off');
Zoom_In = findobj(gcf,'Tag','Zoom_In');
Zoom_Out = findobj(gcf,'Tag','Zoom_Out');

```

```

Plot_Frames = findobj(gcf,'Tag','Plot_Frames');

set(Zoom_In,'Enable','off');

set(Zoom_Out,'Enable','on');

set(Plot_Frames,'Enable','off');

% -----

function varargout = pushbutton1_Callback(h, eventdata, handles, varargin)

% Callback for the uicontrol handles.pushbutton1 (labeled 'Plot' in GUI).

% Note: A region, and a range of frames must be selected before 'Plot' will work.

FILENAME = get(handles.listbox1,'UserData');

% Get the range of frames from edit boxes

iframe = get(handles.edit1,'String');

fframe = get(handles.edit2,'String');

Iframe = str2num(iframe);

Fframe = str2num(fframe);

% Find the highlighted region

EditMarker = findobj(gcf,'Tag','EditMarker');

x = get(EditMarker,'XData');

y = get(EditMarker,'YData');

```

```

xmax = max(x);

xmin = min(x);

ymax = max(y);

ymin = min(y);

% Read in data and plot it in the lower axes

file = read_spe(FILENAME,Iframe:Fframe);

sigma = sum(sum(file(ymin:ymax,xmin:xmax,:)));

for page = 1:Fframe - Iframe + 1

    Sigma(page) = sigma(:, :,page);

end

frmvec = Iframe:Fframe;

Ax2Kids = get(handles.axes2,'Children');

if length(Ax2Kids) == 0

    plot(frmvec,Sigma,'b','parent',handles.axes2);

elseif length(Ax2Kids) == 1

    plot(frmvec,Sigma,'r','parent',handles.axes2);

elseif length(Ax2Kids) == 2

    plot(frmvec,Sigma,'g','parent',handles.axes2);

else

    plot(frmvec,Sigma,'k','parent',handles.axes2);

```

```

end

axes(handles.axes2)

xlabel('Frame')

ylabel('Intensity')

set(handles.axes2,'NextPlot','add');

set(handles.axes2,'XGrid','on');

set(handles.axes2,'YGrid','on');

set(handles.pushbutton3,'Enable','on');

set(handles.pushbutton1,'UserData',Sigma);

% -----

function varargout = pushbutton2_Callback(h, eventdata, handles, varargin)

% Callback for the uicontrol handles.pushbutton2 (labeled 'Movie Playback' % in
GUI).

% Get the frames for the movie which were stored when the data was first read in
Open_File = findobj(gcf,'Tag','Open_File');
M = get(Open_File,'UserData');

% Delete the current image that is displayed
IMG = findobj(gcf,'parent',handles.axes1);
delete(IMG);

```

```

%Select the color scheme

button_state = get(handles.togglebutton1,'Value');

if button_state == 1
    colormap(jet);
elseif button_state == 0
    colormap(gray);
end

axes(handles.axes1);

% Play the Movie

movie(M);

set(handles.axes1,'Tag','axes1');

set(handles.axes1,'NextPlot','replace');

val = get(handles.text5,'String');

Val = str2num(val);

FILENAME = get(handles.listbox1,'UserData');

FileLim = get(handles.axes1,'UserData');

File_MIN = FileLim(1);

File_MAX = FileLim(2);

```



```

Frame = read_spe(FILENAME,Val);

imagesc(Frame,'parent',handles.axes1,[File_MIN,File_MAX]);

set(handles.axes1,'Tag','axes1');

set(handles.axes1,'NextPlot','add');

set(handles.axes1,'UserData',[File_MIN File_MAX]);

set(handles.listbox1,'UserData',FILENAME);

% -----

function varargout = pushbutton3_Callback(h, eventdata, handles, varargin)

% Callback for the uicontrol handles.pushbutton3 (labeled 'Clear' in the %GUI).

% Clears all plots in the lower axes

AxKids = findobj(gcf,'parent',handles.axes2);

delete(AxKids);

set(gcbo,'Enable','off');

% -----

function varargout = Next_Frame_Callback(h, eventdata, handles, varargin)

% Callback for the uimenu handles.Next_Frame.

% Get needed info, ie. name of file and min & max intensities

FILENAME = get(handles.listbox1,'UserData');

FileLim = get(handles.axes1,'UserData');

File_MIN = FileLim(1);

```

```

File_MAX = FileLim(2);

set(handles.axes1,'NextPlot','replace');

% Get the current frame and replace it with the next frame
OldFrame = get(handles.slider1,'Value');
NewFrame = OldFrame + 1;
file = read_spe(FILENAME,NewFrame);
imagesc(file,'parent',handles.axes1,[File_MIN,File_MAX]);
set(handles.axes1,'Tag','axes1');
set(handles.slider1,'Value',NewFrame);
set(handles.text5,'String',NewFrame);
set(handles.axes1,'UserData',[File_MIN File_MAX]);
set(handles.listbox1,'UserData',FILENAME);
% -----
function varargout = Prev_Frame_Callback(h, eventdata, handles, varargin)
% Callback for the uimenu handles.Previous_Frame.

% Get needed info, ie. name of file and min & max intensities
FILENAME = get(handles.listbox1,'UserData');
FileLim = get(handles.axes1,'UserData');

```

```

File_MIN = FileLim(1);

File_MAX = FileLim(2);

set(handles.axes1,'NextPlot','replace');

% Get the current frame value and replace it with the previous frame

OldFrame = get(handles.slider1,'Value');

NewFrame = OldFrame - 1;

file = read_spe(FILENAME,NewFrame);

imagesc(file,'parent',handles.axes1,[File_MIN,File_MAX]);

set(handles.axes1,'Tag','axes1');

set(handles.slider1,'Value',NewFrame);

set(handles.text5,'String',NewFrame);

set(handles.axes1,'UserData',[File_MIN File_MAX]);

set(handles.listbox1,'UserData',FILENAME);

% -----

function varargout = Zoom_Out_Callback(h, eventdata, handles, varargin)

% Callback for the uimenu handles.Zoom_Out.

% Get needed info, ie. name of file and min & max intensities

FILENAME = get(handles.listbox1,'UserData');

```

```

FileLim = get(handles.axes1,'UserData');

File_MIN = FileLim(1);

File_MAX = FileLim(2);

% Find out what frame we zoomed in on

Zoomed_frame = get(handles.pushbutton5,'UserData');

% Read in the data for the whole frame

Unzoom_frame = read_spe(FILENAME,Zoomed_frame(1));

set(handles.axes1,'NextPlot','replace');

imagesc(Unzoom_frame,'parent',handles.axes1,[File_MIN,File_MAX]);

set(handles.axes1,'Tag','axes1');

set(handles.pushbutton5,'UserData',[]);

set(handles.axes1,'UserData',[File_MIN File_MAX]);

set(handles.pushbutton1,'Enable','off');

Plot_Frames = findobj(gcf,'Tag','Plot_Frames');

set(Plot_Frames,'Enable','off');

set(handles.pushbutton5,'Enable','off');

set(gcbo,'Enable','off');

% -----

function varargout = pushbutton4_Callback(h, eventdata, handles, varargin)

```

```

% Callback for the uicontrol handles.pushbutton4 (labeled 'Quit' in GUI).

% Open a dialog box to confirm exit of GUI
button = questdlg('Quit SMImage?', ...
                 'Exit','Yes','No','No');

switch button
case 'Yes',
    close('SMImage') % If 'yes' is selected, close figure and clear all
variables
    clear all
case 'No',
    return;
end

% -----
function varargout = Exit_Mat_Callback(h, eventdata, handles, varargin)

% Callback for the uimenu handles.Exit_Mat.

% Open dialog box to confirm exit of MATLAB
button = questdlg('Quit MATLAB?', ...
                 'Exit','Yes','No','No');

switch button

```

```

case 'Yes',
    disp('Exiting MATLAB');
    quit
case 'No',
    quit cancel;
end

```

```

function SMIWBFs(arg,newX,newY)

%Window Button Down Function for SMImage GUI

%D. Ball

Axhndl = findobj(gcf,'Tag','axes1');

axes(Axhndl)

currentPoint = get(Axhndl,'CurrentPoint');

newx = currentPoint(1);

newX = floor(newx);

newy = currentPoint(3);

newY = floor(newy);

set(Axhndl,'NextPlot','add');

set(Axhndl,'NextPlot','add');

switch arg

```

```

case 'down'

    editmarker = findobj(gcf,'Tag','EditMarker');

    if editmarker

        delete(editmarker)

    end

    rectX = repmat(newX, [1 5]);

    rectY = repmat(newY, [1 5]);

    EditMarker = plot(rectX, rectY,'r--');

    set(EditMarker, 'Tag', 'EditMarker');

    set(gcf,'WindowButtonUpFcn', 'SMIWBFs up');

    set(gcf,'WindowButtonMotionFcn', 'SMIWBFs motion');

case 'motion'

    EditMarker = findobj(gcf,'Tag','EditMarker');

    rectX = get(EditMarker,'XData');

    rectY = get(EditMarker,'YData');

    rectX(2:3) = newX;

    rectY(3:4) = newY;

    X = max(rectX);

    x = min(rectX);

```

```

Y = max(rectY);
y = min(rectY);
npixel = (X - x)*(Y - y);
set(EditMarker, 'XData', rectX, 'YData', rectY);
text8 = findobj(gcf,'Tag','text8');
set(text8,'String',npixel);
case 'up'
    EditMarker = findobj(gcf,'Tag', 'EditMarker');
    rectX = get(EditMarker, 'XData');
    rectY = get(EditMarker, 'YData');

    Zoom = findobj(gcf,'Tag','pushbutton5');
    Zoom_In = findobj(gcf,'Tag','Zoom_In');
    Plot = findobj(gcf,'Tag','pushbutton1');
    Plot_Frames = findobj(gcf,'Tag','Plot_Frames');
    set(Zoom,'Enable','on');
    set(Zoom_In,'Enable','on');
    set(Plot,'Enable','on');
    set(Plot_Frames,'Enable','on');
    set(gcf,'WindowButtonMotionFcn','');
    set(gcf,'WindowButtonUpFcn','');

```



```
set(Axhdl,'Tag','axes1');  
end
```

Vita

David A. Ball was born in Cortland, New York on May 1, 1978. He was raised in Cortland until the sixth grade, when his family moved to nearby Homer. He was always curious about how things worked, and was constantly taking things apart, and *trying* to put them back together. It wasn't until his senior year at Homer High School, however, that he was shown the fascinating world of physics by Mr. Hal Fuller. David then attended The University at Albany (NY), graduating cum laude in 2000 with a bachelor's degree in Physics. While in Albany, he worked on such projects as the restoration of the university's 16" Cassegrain telescope. In the fall of 2000, David accepted a Graduate Research Assistantship at The University of Tennessee Space Institute, where he earned a masters of science degree in Physics in July 2002. He is currently working towards a Ph.D. in Physics at UTSI.