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Determining the Rate of Transcription of T7 RNA Polymerase Using Single Molecule Fluorescence Imaging

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DETERMINING THE RATE OF TRANSCRIPTION OF T7 RNA POLYMERASE USING SINGLE MOLECULE FLUORESCENCE IMAGING

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Abstract

It is important to understand the many factors impacting the rate at which an RNA polymerase incorporates nucleotides. The transcription rate of T7 RNA polymerase has been determined using single molecule fluorescence microscopy. A Cy3 labeled circular 45nt ssDNA molecule was used to monitor the transcription process. T7 RNA polymerase was used because it is a single subunit polymerase that does not need any cofactors and will transcribe single-stranded DNA circles that do not contain a promoter.

The transcription was monitored by measuring the quasi-periodic change in intensity associated with the transit of the probe through the polymerase as the DNA is transcribed. The time between these intensity changes of the Cy3 molecule represents the time it takes the polymerase to transcribe the circle once. Transcription rates were determined at a variety of NTP concentrations. Because glass can affect how the enzyme works, the surface of the glass was coated with poly-L-lysine in some of the experiments. The poly-L-lysine was used to keep the T7 RNAP from touching the glass surface. In order to extend the observation time, factors affecting the photostability of the Cy3 probe were evaluated using determinations of the photochemical half-life.

Introduction

RNA and Transcription in Cells

In cells, transcription is a biological process in which complementary RNA (ribonucleic acid) is made from genomic DNA via an enzyme called an RNA polymerase. RNA is made up of four main bases: adenine, cytosine, guanine, and uracil. Figure 1 shows the structure of the 4 nucleic acid bases in RNA.[1] Each of these bases is connected to a ribose sugar that is attached to a phosphate group that comprises the backbone of the RNA. The RNA will base pair with another complementary RNA strand. Each base has a complement and will pair through two or three hydrogen bonds. Adenine pairs with uracil and cytosine pairs with guanine. These base pairings create double stranded RNA or DNA:RNA hybrids. During transcription the growing RNA chain forms a DNA:RNA hybrid in the polymerase. The RNA base complementary to the base in the template DNA is added to the growing RNA polymer. This is how the DNA passes along the information it carries.

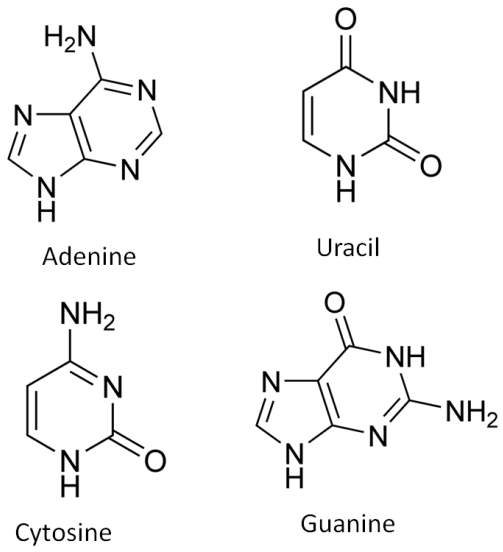


Figure 1: The four nucleic acid bases that make up RNA.[1]

There are three main types of cellular RNA: messenger RNA, ribosomal RNA, and transfer RNA. Each of these three RNA types has different size and secondary characteristics that help with its specific jobs.[2] The messenger RNA comprises about 5% of the RNA in a cell. Messenger RNA is the most heterogenous type of RNA in

regard to size and sequence. This RNA carries information from the DNA and is used as the template for protein synthesis. The messenger RNA travels from the nucleus where it was synthesized into the protein synthesis sites in the cytosol.[(2)]

The ribosomal RNA forms part of the ribosomes that are responsible for synthesizing proteins. Ribosomal RNA accounts for about 80% of the RNA in a cell. In eukaryotic cells there are four species of ribosomal RNA, and in the prokaryotic cells there are three species of ribosomal RNA. These differences in the number and size of the ribosomal RNA account for the difference in structure of the ribosome in eukaryotic and prokaryotic cells.[(2)]

The last type of RNA is the transfer RNA. Transfer RNA are the smallest of the three types of RNA. There is a different type of tRNA for each of the twenty amino acids. The transfer RNA carries its amino acid to the ribosome where it adds the amino acid according to the messenger RNA code. The transfer RNA contains unusual bases such as N4-Acetylcytosine. The unusual bases help the RNA be recognized by specific enzymes and to keep it from being digested by RNases. The secondary and tertiary structure of the transfer RNA is important to its ability to carry its specific amino acid.[(2)]

In a cell, transcription is closely regulated by proteins. The proteins guide the polymerase to where transcription should occur. There is usually a specific sequence called a promoter region immediately before the sequence that needs to be transcribed. The promoter region signals to the polymerase that it needs to bind to it and start transcribing. Transcription can be divided into three distinct sections: initiation, elongation, and termination. In initiation, the polymerase binds to the DNA and starts making short pieces of RNA, usually less than 10 bases long. Elongation occurs when the

polymerase releases the promoter and starts sliding along the DNA making the complementary RNA. Termination occurs when the polymerase releases the RNA it was making and the DNA it was bound to.

T7 RNA Polymerase

The RNA polymerase that we used was T7 RNA polymerase (RNAP). T7 RNAP is from the T7 bacteriaphage virus. This polymerase is a single subunit enzyme that is only about 100kDa[3] compared to RNA polymerase II from yeast that consists of 12 subunits and is 500kDa[4]. T7 RNAP is one of the most studied RNA polymerases due to its relative simplicity and has been used in many rolling circle transcription studies.[5][6][7][8] Numerous crystal structures in various stages have also been made and are in the protein data bank.[9]

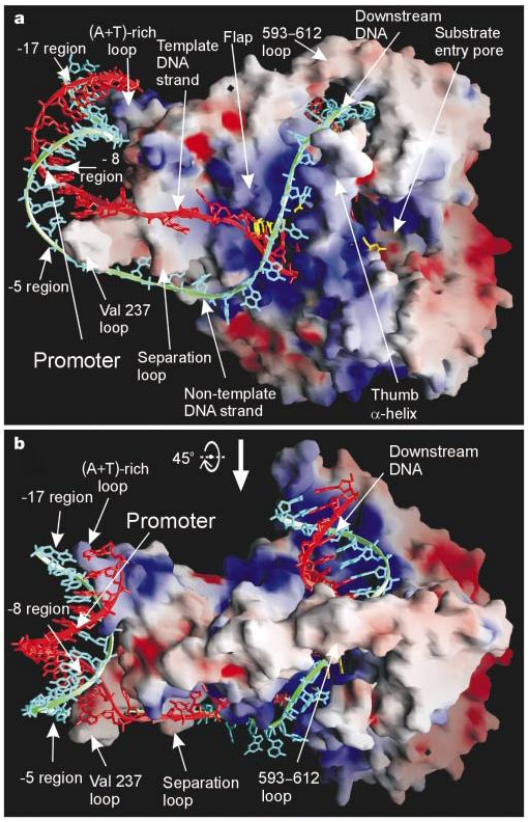


Figure 2: Representation of the conformation of T7 RNA polymerase during initiation (top) and elongation (bottom).[11]

During the initiation stage, the RNAP loosely binds to the DNA template strand. The RNAP starts to transcribe the template strand. The RNAP undergoes abortive synthesis in which it transcribes many short (8-12 nt) RNA strands before the RNAP starts the second stage of transcription, elongation. While the short RNA strands are being made, the RNAP

remains bound to the promoter region and does not move along the DNA strand, which is why the RNA that is made is less than 12nt long.[10]

Once the T7 RNAP has transcribed 8-10 nucleotides and released the promoter, if there is one, the elongation stage has started. During this stage the RNAP can produce RNA over 15,000 nucleotides in length. During this stage the elongation process is very stable. To obtain this stability the T7 RNAP undergoes a conformational change. The N-terminal end of the protein (residues 2-266) reorients to form three structural entities. Residues 2-71form an N-terminal extension, residues 152-205 form a central flap, and residues 258-266 form a C-terminal linker that connects the N-terminal and C-terminal.[11]

Figure 2 (top) shows the T7 RNAP in the initiation conformation. Figure 2 (bottom) shows the T7 RNAP in the elongation conformation. In the elongation conformation, the polymerase folds around the DNA making a pocket where transcription occurs. The template strand goes through the pocket, is transcribed, and is part of a DNA:RNA hybrid for 10-12 bases. The non-template strand goes around the outside of the RNA polymerase and binds with the template strand after the RNA has been separated from the DNA.[11]

The number of nucleotides the polymerase attaches to the RNA strand per second is called the transcription rate. Numerous studies have been performed to determine the transcription rate of polymerases, and these techniques will be discussed in the next section. Determining the transcription rate can be difficult due to pausing. Pausing occurs when the RNAP temporarily stops transcribing during elongation. Different polymerases pause for different periods of time. T7 RNAP pauses less often and for a shorter period of

time than some other RNA polymerases, which makes it a good RNA polymerase to use for this study.[12]

The last stage of transcription is termination. Termination occurs when the polymerase releases the RNA it was transcribing and the template DNA. For T7 RNAP the termination is sequence dependent. A sequence in the template DNA causes the RNA to form a hairpin that destabilizes the structure. Usually there are multiple uracil bases in the RNA before termination. The uracils destabilize the RNA:DNA hybrid inside of the polymerase.[13]

Rolling Circle Transcription

RNA transcription is the cellular process in which complementary RNA is made from DNA through the use of an enzyme called an RNA polymerase. Rolling circle transcription (RCT) is a special type of transcription in which the DNA template that is being transcribed is a circular DNA molecule that does not possess a termination sequence. This omission of the termination sequence results in a repeating RNA strand many bases longer than the original DNA template being produced. Because there is no termination sequence, there is no set place for termination to occur. Having no set place for termination, it can occur at multiple places on the circle and at multiple revolutions, so many different sizes of RNA are produced from one circular template.[5] Rolling circle transcription was first seen in viruses. [14][15][16] The virus would have circular DNA or RNA that would be replicated by a polymerase. The polymerase would transcribe the circle multiple times, making the resulting RNA much longer than the original template. The RNA would be cleaved, leaving monomeric linear complements of the circle.[17]

Dr. Eric T. Kool et al. first used RCT to transcribe small single-stranded DNA circles with no promoter region using T7 RNA polymerase.[5] It was thought that the shape of the small circle allowed it to be such an efficient template, as the sequence of the circle did not make a difference as to whether or not the DNA template was transcribed. Rolling circle transcription has since been used to produce catalytic RNA's in which the long repeating RNA strand self-cleaved to make shorter nonrepeating RNA[6][19], to produce circular RNA[18], and to make short hairpin RNA strands.[7]

For most RNA polymerases certain cofactors need to be present in order for transcription to occur. Most polymerases need a promoter sequence, a 15-20 base sequence of nucleotides that signals the polymerase to start transcribing, and certain ions or small molecules such as Mg^{2+}. For circular ssDNA templates, however, no promoter sequence is needed for T7 RNAP to transcribe the DNA. The exact reason for this is not known, however; some researchers have hypothesized that the promoter regions are not due to a specific sequence, but they are promoter regions because they are areas of ssDNA in a dsDNA strand.[8]

Single Molecule Fluorescence Imaging

Single molecule fluorescence imaging allows individual fluorescent molecules to be seen. There are many different types of single molecule fluorescent imaging. The first single molecule fluorescence experiment was published in 1990 by Orrit and Bernard. Orrit and Bernard studied the fluorescence of pentacene molecules in a p-Terphenyl crystal.[20] This experiment showed the first single molecule fluorescence detection, but

the molecules were observed in a crystal at extremely low temperatures, which limited the scope of the technique.

In 1994 Chu et al. recorded videos of individual DNA molecules stained with YOYO-1 dye and attached via a strepavidin biotin bond to a polystyrene bead. The bead was held in place with optical tweezers and the DNA was stretched. Optical tweezers are made by focusing an infrared laser through the objective of the microscope and making an attractive or repulsive force to hold onto and manipulate the polystyrene bead. Then the relaxation of the DNA was measured. Images of the single DNA molecules as they relax were taken with a silicon-intensified target camera.[21] This experiment is one of the first single molecule fluorescence experiments that observed DNA. In order to keep the DNA in place while it was stretched and then while it was relaxing, optical tweezers were used. One problem with optical tweezers is that DNA is too small for the tweezers to hold onto, so a bead has to be attached to the DNA. The optical tweezers then hold onto the bead, which, in turn, keeps the DNA in place. Although this works well for many experiments, in a complex system, the bead could get in the way. It would be much better to image only the molecules in the system that one is interested in.

In 2003[22], the Selvin group published a paper in which they discussed the use of a new single molecule fluorescence technique called FIONA, Fluorescent Imaging with One Nanometer Accuracy. FIONA was first used to watch labeled myosin walk along actin filaments in 2003. An episcopic fluorescence microscope with a 60x objective was used to view the sample. A prism style total internal reflection fluorescence (TIRF) system was used to ensure that only fluorescence from the surface of the coverslip was in focus and sent to the detector. TIRF was used to decrease noise. A charge coupled device

(CCD) camera was used to detect the fluorescence. It is known that a well localized spot forms an airy disk due to diffraction. The images were fit to the 2D Gaussian function using a least squares method. From this fit, the sub-pixel positions of each spot could be determined down to 1.5nm resolution. This resolution is much lower than the Abbe resolution of about 200nm. This resolution allowed one to look at fluorescently labeled single molecules that were not attached to anything larger, such as a bead.

Since FIONA in 2003, many other sub resolution single molecule techniques have been invented. In 2006, papers describing two other techniques, stochastic optical reconstruction microscopy (STORM)[23] and photo-activated localization microscopy (PALM) [24], were published. These two techniques can be used to gain better image resolution. Single molecule FRET uses two fluorescent molecules whose fluorescence will change when they are within a certain distance of separation from each other.[25] This technique shows that more information can be gathered from single molecule fluorescence images than location. Changes in how the fluorescent molecule acts (blinking or increased fluorescent signal) or photobleaching (faster or slower) can show that the environment directly around the fluorescent molecule is changing. This is especially true for fluorescent molecules that are sensitive to their environment such as Cy3.

Figure 3: Picture of a microscope set up to use TIRF. The prism directs the light from the laser to hit the surface at an angle that can create the evanescent wave needed for TIRF.[24]

Some single molecule fluorescence experiments use Total Internal Reflection Fluorescence (TIRF) style microscopy. TIRF is

used to reduce the amount of fluorescence background from out-of-focus regions. Using TIRF only molecules within 100-200nm of the surface fluoresce.[26] TIRF is great for solution experiments in which there might be other fluorescent molecules out of focus that can overwhelm the fluorescence of a single molecule in solution. Because the molecules are in solution, the molecules of interest may have to be tethered to the surface to keep them from floating away. TIRF has been done on experiments with living cells as well as in vitro experiments.

In TIRF the excitation light travels through the glass coverslip at a high incident angle creating an evanescent wave at the glass/ water or buffer interface. This evanescent wave excites the fluorophores close to the surface (less than 200nm). The strength of the evanescent wave decays as it travels farther from the surface.[27] The easiest way to add TIRF to a microscope is to add a prism. The prism directs the excitation light toward the interface of the glass/ liquid at an angle that is slightly larger than the critical angle for total internal reflection.[28] Figure 3 shows a typical microscope set up for TIRF. Part of the excitation light from the laser is directed into the prism. The prism directs the light at the correct angle to create an evanescent wave. The light from the excited fluorescent molecules are directed to the detector (in this case the CCD camera and PMT cabinet) in the same way as non-TIRF microscopy.

Overview of this project

We are determining the rate of T7 RNA polymerase by recording the changes in intensity of a fluorescent molecule during transcription. We used the fluorescent molecule Cy3 shown in Figure 4. Cy3 is a fluorescent molecule that is used in many

single molecule studies because of its brightness and stability. Cy3 is also sensitive to its environment, which makes it a good reporter molecule. Luo et al. showed that Cy3's intensity will increase when T7 DNA polymerase binds to it during replication.[29] They hypothesized that this was due to the polymerase limiting the range of motion of the fluorophore and therefore not allowing the Cy3 molecule to get rid of the energy through vibrations, only by releasing a photon. The constraints on Cy3 should work similarly in the case of T7 RNA polymerase because the RNAP will put the same constraints on the Cy3 molecule that the DNAP would.

A circular DNA template was chosen because the polymerase will transcribe the circle multiple times, thus giving more data because there will be multiple interactions with the Cy3 before termination. The multiple interactions also omit the need to label the DNA in multiple places. To image the Cy3 labeled DNA as it is being transcribed, the T7 RNAP, NTPs, DTT, Trolox, and Cy3 labeled circular DNA is loaded into a flow cell in 0.5x transcription buffer. Transcription buffer is a mixture of salts and ions that produce a good ion and pH environment for the enzyme. The flow cell is imaged under a fluorescent microscope and the images are recorded sequentially, making movies. Each movie is composed of 1250 frames with 48 milliseconds between frames. The movie is then processed using a Matlab script, and the individual fluorescent spots are selected and their intensity over time is

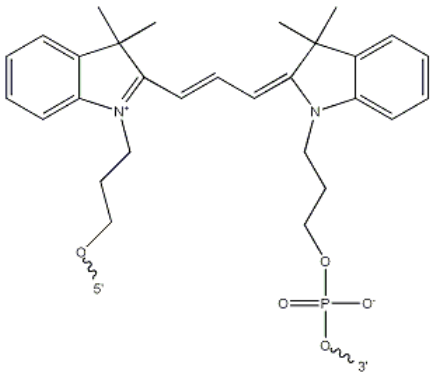


Figure 4: Cy3 molecule/ pseudobase that is inserted into the DNA strand. The image is from idtdna.com.

graphed. The intensity graphs show quasi-periodic modulations that are due to the intensity changes of the Cy3 as the T7 RNAP transcribes the circle.

The RNAP resting directly on a glass surface has been shown to decrease the efficiency of the enzyme,[30] probably due to steric effects. If the polymerase is adsorbed on the surface of the glass, there will be fewer degrees of freedom for the polymerase to move while transcribing. The polymerase will adsorb onto a glass surface, unless the glass is protected by a coating of another protein. Although the intensity modulations did occur when the polymerase is directly on glass, the surface of the flow cell was coated to determine whether the processivity of the enzyme would increase. Two coatings, bovine serum albumin (BSA) and poly-L-lysine, were used to determine which, if either, worked better than uncoated glass. A thin layer of the protein was formed between the glass of the enzyme, and then the reaction was run in the coated flow cell and recorded.

Determination of Transcription Rate

Many experiments have been performed to determine the transcription rate of RNA polymerases. If we can measure the rate of transcription for a particular polymerase then we can figure out what environmental factors we can use to slow down or speed up the polymerase. Ensemble techniques used to be the only ones available to determine the rate. The problem with ensemble techniques is one only obtains an average rate for all of the polymerases in the sample. With single-molecule techniques one can look at each individual polymerase's rate and pausing. Generalizations for a particular polymerase can still be made; however, more information will be known about the differences of individual polymerases.

When single molecule techniques were used to determine the transcription rate, a variety of pausing times were found, especially for E.coli RNA polymerase, a very well studied multi-subunit RNAP. Pausing times from 1 second to 30 minutes were found for this enzyme.[31] T7 RNAP does not pause as often or for as long as E.coli RNAP, so, when determining the transcription rate, pausing is not a large concern. Certain DNA sequences such as 5'- ATCTGTT-3' are known to cause pausing. However, these sequences are not in the circular template that we will be using.[5]

A variety of experiments has been done to determine the rate of T7 RNAP. Some of the techniques will be described below, but a discussion on some of the published rates will be discussed here. Table 1 shows four published rates and the technique and some

Technique	Concentration of NTPs	DNA template	Published Rate	Reference Number
Estimated using computer simulation	Excess	pT3/T7luc (linear)	97nt/sec	32
Single molecule force measurements	30-590μM	λ DNA	129 nt/sec	33
Single molecule FRET	Very low	Multiple T7 promoters (linear)	20-60 nt/sec	34
Single molecule fluorescent tracking of T7 RNAP	0.2mM	λ DNA	42 nt/sec	35

Table 1: Table of different transcription rates and the techniques used to calculate them for T7 RNAP.

parameters used to calculate the rate. As shown in the table, there is a wide range of rates depending on the NTP concentration and the technique used. The highest rate, 129nt/sec, was calculated from a single molecule force measurement of T7 RNAP transcribing λ

DNA. There are two single molecule fluorescence techniques shown in Table 1. One used single molecule FRET and published a rate of 20-60 nt/sec, and the second used a fluorescently labeled T7 RNAP and published a rate of 42 nt/sec.

Ensemble Techniques

The rate of a polymerase has historically been determined by quantifying the amount of RNA that was produced. One method for making this determination was described in a paper by Guerniou et al. in 2005. RNA was transcribed in vitro then purified. The purified RNA was annealed with a radioactive phosphate labeled DNA primer. The labeled DNA was extended by reverse transcription, and then the DNA was run in a polyacrylamide gel. The DNA was imaged using phosphorimager screens and quantified.[31] The amount of transcript obtained after a certain amount of time by a certain quantity of enzyme was used to determine the transcription rate.

A radioactive label was used because DNA can be more precisely quantified using radioactive labels than by using fluorescent dyes such as ethidium bromide and SYBER green. One drawback to this technique is that it is an indirect approach. DNA made from the RNA that was transcribed is measured. Another drawback of this technique is that the process is assumed to be homogeneous. Every enzyme of the same type is assumed to behave the same way, and every DNA template is assumed to be the same. Although enough enzymes are used that one or two slow ones will not affect, the overall group rate, the individual nuances from each enzyme is missed. Pausing is another concern. The pausing can be counted in the transcription time giving an overall lower transcription rate. Overall a lot of information is lost or overlooked in ensemble techniques.

Single Molecule Techniques

Single molecule techniques show the individual nuances of the polymerases, and can indicate the difference between pausing and elongation. A high variability of transcription rates, standard deviation of about 30%, have been found for T7 RNAP using single molecule techniques.[13] This variability is due to the variation of the single polymerases that are usually averaged in ensemble experiments. One of the most popular single molecule techniques, especially in the early part of the decade, was to connect the DNA to beads and measure the changes in the beads. Bustamante et. al. used a flow controlled optical trap with fluorescence microscopy to determine the rate of E. coli RNAP. The template DNA was tethered between two beads. As the RNAP transcribes the DNA, it will bring the two beads closer together. The distance between the two beads was measured with fluorescence microscopy.[36]

Another way to gain information on the transcription of single RNA polymerase molecules is to measure the force of the RNA polymerase during transcription. When a RNA polymerase transcribes DNA to RNA, the energy from breaking the triphosphate bond in the nucleotide triphosphate that it added to the RNA chain propels the RNAP down the DNA strand. Gelles et. al. attached the DNA template to a polystyrene bead that was held in place using an optical trap. As the RNAP transcribed the DNA template, it pulled the DNA template, which, in turn, pulled the polystyrene bead. The polystyrene bead could only move a little because it was in the optical trap, so the force that was applied to the optical trap to keep the bead in there was related to the transcription of the RNAP.[33]

Some have used single molecule fluorescence techniques to observe RNAP transcription. Forgoing an optical trap means that one does not need to add a bead to the reaction, and that one will be measuring or visualizing the actual components of transcription (DNA, RNA, or RNAP) instead of a bead that is attached to one of the components (usually DNA). Berge et al. combed the template DNA onto a glass surface and added fluorescently labeled uracil triphosphates along with the other nucleotide triphosphates in order to fluorescently label the RNA. They allowed transcription to occur and then caused it to pause by removing the nucleotide triphosphates. Any fluorescence that was not attached to RNA would be removed and then the glass was imaged. Fluorescent lines from the RNA were made, and they could determine how many bases were added into the growing RNA polymer by measuring the length of the fluorescent line. Multiple RNAs could be seen in the same field of view allowing more data to be collected at once. [37]

Experimental

Microscope Setup

We used an episcopic fluorescence microscope that was set up for single molecule imaging studies. The microscope configuration is shown in Figure 5. The fluorescence microscope has a high pressure mercury lamp (Hg lamp) as the excitation source, which is the white box to the right of the microscope in Figure 5. The Hg lamp was used instead of a laser because of the wide range of wavelengths it could excite. Filters were then used to narrow the range of wavelengths that were used to illuminate the sample. The fluorescence from the illuminated sample is passed through another filter set and then

directed through the optical splitter (black box to the left of the microscope pictured in Figure 5). The emitted light is then directed to the CCD camera (red and silver camera to the left of the optical splitter in pictured in Figure 5), which detects the fluorescence and sends the image to the computer.

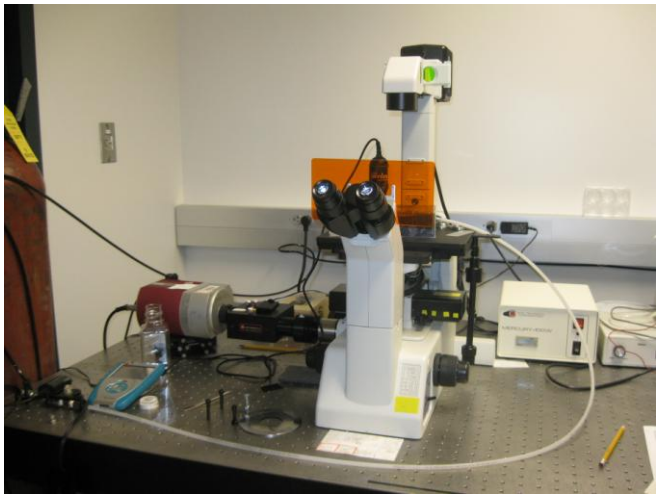


Figure 5: Picture of fluorescence microscope used in these experiments.

TIRF was not used in this microscope because our background was low enough to image single molecules in solution without it. TIRF is often used in fluorescent studies of cells in which one does not have as much control over the solutions. For our experiments, all of our solutions except our labeled DNA were shown not to be fluorescent.

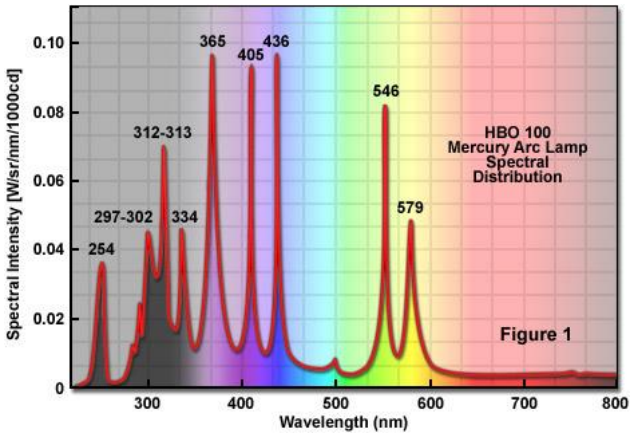


Figure 6: Hg lamp spectra. There is a large peak at 546nm which corresponds well with Cy3.[38]

Figure 6 shows the spectra of the mercury lamp. Hg lamps produce a high luminance compared to other continuous light sources. The Hg lamp also emits some amount of light continuously from the UV to the IR region. About one third of the light it irradiates is in the visible region. [38] The Hg lamp is a good choice for Cy3, especially because of the high peak at 546nm, which is close to the 550nm excitation maximum for Cy3.

There are two different bandpass filters and an optosplit filter attached to the fluorescence microscope. One of the bandpass filters only passes light of wavelength 475/25nm from the lamp to the sample and only lets light 525/25nm from the sample to pass through to the detector. The other bandpass filter set allows 550/20nm light from the lamp to illuminate the sample and 595/50nm light from the sample to reach the detector.

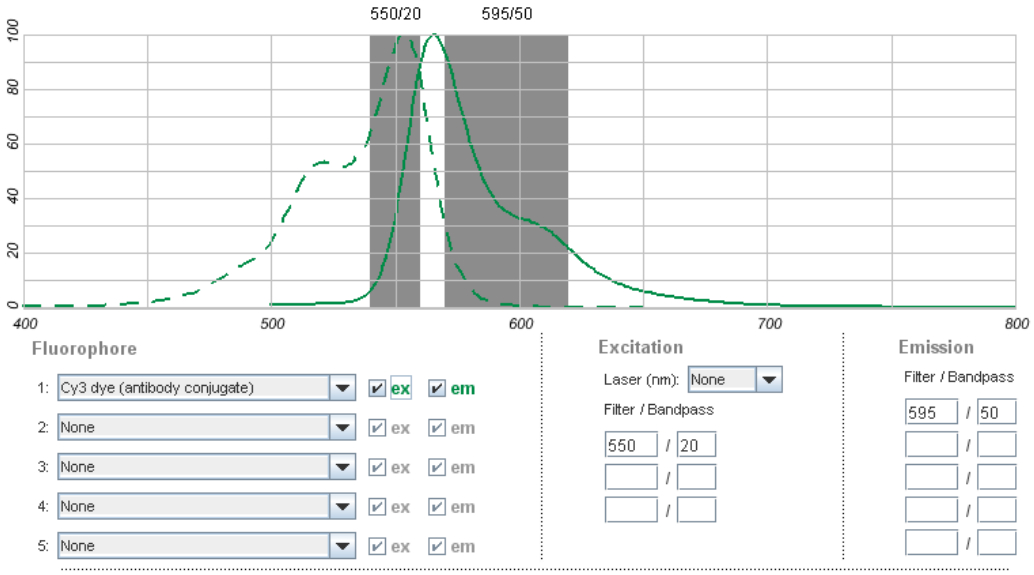


Figure 7: Excitation and emission spectra of Cy3 using Spectra Viewer from Invitrogen.com. The dashed line is the excitation and the solid line is the emission. The grayed areas are the filter sets that are used for the Cy3. The first grayed area, 550/20, is the excitation filter, and the second grayed area, 595/50, is the emission filter.

This second bandpass filter set is the one that we used for our experiments because it matched so closely with the spectroscopic properties of the Cy3 probe. Figure 7 shows Cy3's spectral data with the excitation and emission filter ranges in gray. The dashed line is the excitation profile of Cy3 and the solid line is the emission profile of Cy3. This graph was made using Spectra Viewer in Invitrogen's website.[39]

The optical splitter allows images using the green filter set (excitation 475/20nm and emission 525/20nm) and the red filter set (excitation 550/20nm and emission

595/50nm) to be seen in the same field of view in two separate channels. On the right side of the image is the red channel and on the left side is the green channel. The optical splitter was helpful in experiments because it showed that the spots appeared only in the red channel and therefore would not likely be adventitious (impurity) fluorescence.

The CCD camera employed was a Rolera-MGi plus from Qimaging and is shown in Figure 8. The camera has a 512x512 array of sensor pixels that detect the light. The amount of light detected from each pixel sensor is changed to an electrical signal every frame. This signal is then amplified according to the gain settings. This amplified image is what is sent to the computer and displayed. CCD cameras are used in many single molecule fluorescence experiments because of their sensitivity.[40]

Figure 8: (Top) Image of CCD camera used to detect the fluorescence of the single Cy3 molecules..[40]

Circularization of Cy3 labeled DNA

Linear 45 base single-stranded DNA with a Cy3 internal modification and a phosphate group at the 5' end was ordered from IDT. The sequence of the DNA was 5'-phosphate-CTG GAG GAG ATT TTG TGG TA(Cy3)T CGA TTC GTC TCT TAG AGG AAG CTA- hydroxyl- 3'. The DNA was resuspended in UV-treated ddH_2O to a concentration of 100mM, which was used as the stock solution. The UV-treated ddH_2O was distilled H_2O distilled again using our distillation apparatus then irradiated with UV light for 10 minutes. An aliquot of the stock solution was removed and diluted to a concentration of 10μM using UV-treated ddH_2O. The Circligase II kit from Epicenter Bio was used to circularize the DNA by combining on ice 2.0μL of 10x Transcription buffer,

1.0µL of $MnCl_2$, 120pmol of linear DNA, and 2.0µL of CircligaseII ssDNA ligase. The solution was incubated at 60°C for 2 hours and 80°C for 10 minutes. After the solution cooled to room temperature, it was ethanol precipitated and resuspended in UV-treated ddH_2O.

In Vitro Rolling Circle Transcription:

RNA was made by combining on ice 2pmol of circular DNA with Cy3 internal label, 2µL 10x transcription buffer (Ambion), 2µL 100mM DTT, 3.5µL 10mM NTP mix (Invitrogen), 20U Scriptguard RNase inhibitor (Epibio), and 40U T7 RNAP (Ambion) in a final volume of 20µL. The solution was incubated at 37°C for 3 hours. The product was treated with Baseline Zero DNase(Epicenter Bio) to remove the DNA. The remaining RNA was ethanol precipitated and resuspended in UV treated ddH_2O. The RNA was analyzed through an agarose gel and fluorescence microscopy.

The RNA was run in a 1.2% Agarose RNA flashgel (Lonza) along with RNA Century Marker (Ambion) and the transcription solution before incubation at 37°C as a control. The gel was run at 225V for 5 minutes or until the components of the ladder were sufficiently separated. The flashgel was imaged using filter set 2 (for ethidium bromide stained gels) of the Alpha Innotech gel imager. The gel was then allowed to set in the dark for 15 minutes and then imaged again using filter set 2 of the gel imaging system. The dye that is in the flashgel does not bind to RNA as well as DNA, and many times the RNA bands will be invisible while the gel is running. Letting the flashgel sit for 15-30 minutes before being imaged helped to intensify the bands so they can be imaged.[41]

The purified RNA was annealed with a 45nt DNA complement labeled with rhodamine at the 5' end (IDT) by combining the RNA and the DNA in a microcentrifuge

tube in a 1:5 ratio of RNA to DNA then heating the solution to 70°C for 5 minutes in a digital dry bath then allowing it to slowly cool to room temperature. The annealed DNA:RNA was filtered with a Microcon YM-30 centrifuge filters (Millipore) to remove the excess 45nt rhodamine labeled DNA. The RNA:DNA hybrid was combed onto a clean glass coverslip and imaged under N_2 using a Hg lamp as the excitation source with excitation filters at 550/20nm and emission filters at 595/50nm. The movies were taken using QCapture Pro software. An EM gain of 2500 was used to take the images and the time between each frame was 140msec.

Cleaning Glass and Making a Flowcell

Glass coverslips (Fisher Scientific cat # 12-548-B) were lightly scratched using a diamond scribe to produce fiducial marks and then sonicated in acetone for 20 minutes. The glass coverslips were next rinsed with UV treated ddH_2O, dried with N_2 and then irradiated with UV light for 15 minutes. The coverslip was imaged scratch side up. The scratch was used to quickly focus on the surface of the coverslip. To make the flow cell, two holes were drilled in a glass slide (Fisher Scientific catalog #12-544-6) using a Dremel tool with a diamond bit. The glass slide was sonicated in acetone for 20 minutes then rinsed in UV treated ddH_2O, dried with N_2 then irradiated with UV light for 15 minutes. A schematic of the construction of the flow cell is shown in Figure 9 (left). Double-sided tape was placed on the long edges of the clean glass slide. A clean glass coverslip was placed on top of the tape over the drilled holes scratch side down and the excess tape was removed. Epoxy was used to seal the places between the glass slide and glass coverslip where there is no tape. The epoxy was allowed to completely dry before any solution was added.

Later in the project a second, simpler design for the flow cell was made that omitted the epoxy. A clean glass coverslip was placed on top of the tape over the drilled holes scratch side down and the excess tape was removed. A schematic for the construction of this flow cell is shown in Figure 9 (right). The liquid still stayed inside of the flow cell because the total volume was such a small amount (40µL), and the omission of the epoxy allowed liquid to be flowed in and out by wicking with a Kim wipe.

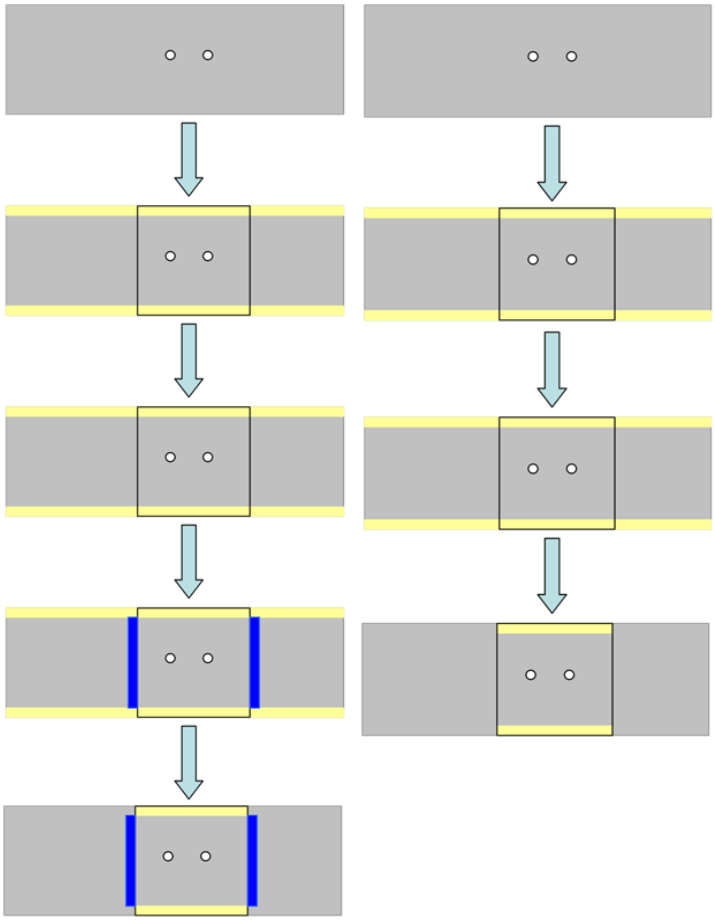


Figure 9: Cartoon of the making of the two types of flow cell. To the left is the first where the glass coverslip is attached to the slide with double-sided tape and the other two ends are sealed with epoxy. To the right is the flow cell where the glass coverslip is attached to the slide with the double-sided tape and the other two ends are left open to allow liquid to be wicked through.

Photostability of Cy3 labeled DNA:

To image the Cy3 labeled DNA on glass, 10µL of 100nM Cy3 labeled DNA was deposited onto a clean glass coverslip and allowed to incubate for 10 minutes. The coverslip was rinsed once with UV treated ddH_2O and dried with N_2. To image the Cy3

labeled DNA in H_2O and in 1x transcription buffer (40 mM Tris pH 7.8, 20 mM NaCl, 6 mM $MgCl_2$, 2 mM Spermidine HCl, 10 mM DTT), 50 fmol of Cy3 labeled DNA and 8.0μL of H_2O or transcription buffer was pipetted into the flowcell. The Cy3 labeled DNA in either H_2O or 1x transcription buffer and Trolox solution was made by combining and adding to the flow cell 2μL of 150nM Cy3 labeled DNA, 6.0 μL H_2O or transcription buffer, and 2.0 μL 0.5mM Trolox. All images were taken under N_2 using a Hg lamp as the excitation source with excitation filters at 550/20nm and emission filters at 595/50nm. The movies were recorded using QCapture Pro software. An EM gain of 2500 was used to take the images, and the time between each frame was 141msec for the Cy3 labeled DNA on glass, in water and in a water Trolox solution; the time between each frame for the Cy3 labeled DNA in 1x transcription buffer and in 1x transcription buffer with Trolox is 141msec. The movies were converted to AVI files using Image J. The AVI files were then run through a Matlab script where each individual spot's intensity was graphed over time. Using the graph and the chart of the intensities, the frame in which each spot photobleached was determined. A graph was made of the photobleaching times using Microsoft Excel, and the photochemical half-life of the Cy3 labeled DNA was determined from the graphs.

Fluorescent Imaging of Transcription process

2μL of 1U/μL T7 RNAP(Ambion) was pipetted into a clean flow cell and allowed to incubate for 10 minutes. The flow cell was then rinsed with UV-treated ddH2O twice and 0.5x transcription buffer twice. DTT and NTP mix was added to the flowcell. 2μL of 150nM Cy3 labeled circular DNA was added to the flow cell and immediately imaged. The movies were taken on an episcopic fluorescent microscope with a Hg lamp as the

excitation source and a CCD camera as the detector. An excitation filter at 550/20nm and an emission filter at 595/50nm allowed only certain wavelengths through to the sample and the detector respectively. Movies were taken, using QCapture Pro software with an EM gain of 2500 and an exposure time of 25milliseconds. The image was 512x256 pixels. The time between each frame of the movie was 48 milliseconds. The exposure time and the size of the image could be changed to obtain a lower time between frames. The lowest possible time between frames that the CCD camera with the QCapture Pro software can obtain is ~33 milliseconds between frames.[33] The lowest exposure time that could be used and still obtain images of single molecules was 25milliseconds. The size of the image was reduced by half to obtain a better time between frames while still showing both channels from the optical splitter.

BSA Coating Flow cells

A clean flow cell was constructed as described above. 25mg of BSA(Promega) was dissolved in 10mL PEM-80 buffer. The BSA solution was filtered using YM-100 centrifuge filter from Millipore. 40µL BSA solution was flowed into the flow cell and was incubated at room temperature for 10 minutes. The flow cell was rinsed with 100µl of PEM-80 buffer to remove excess BSA.[42]

Poly-L-lysine coated flow cells

A glass coverslip was cleaned as described above. The coverlip was placed in a new clean plastic Petri dish. Then 1mL of 0.1% w/v Poly-L-lysine solution in water (Sigma Aldrich) was pipetted onto the glass coverslip and incubated at room temperature for 5 minutes. The poly-L-lysine solution was pipetted off of the coverslip and the coverslip dried overnight in the petri dish. The coverslip was then rinsed with UV-treated

ddH_2O and dried with N_2. The flow cell was then made by using double sided tape to attach the poly-L-lysine coated coverslip, coated side down to the clean glass slide along the long edge of the glass slide.

Results

Circularizing 45nt single-stranded DNA with internal Cy3 modification

We ordered a 45 base single-stranded oligonucleotide with an internal Cy3 modification from IDT. The sequence of the DNA oligo was 5'- phosphate-CTG GAG GAG ATT TTG TGG TA(Cy3)T CGA TTC GTC TCT TAG AGG AAG CTA-hydroxyl-3'. The DNA was suspended in UV treated ddH_2O to a final concentration of 100μM that was used as the stock solution. An aliquot of the stock solution was diluted to a concentration of 10 μM with UV treated ddH_2O. This solution was used as the working solution (the solution that was circularized). The UV-Vis spectrum of the working solution was taken using the NanoDrop 2000. The UV treated ddH_2O was used as the blank. The UV-Vis spectrum of the linear DNA with the Cy3 internal modification is shown in Figure 10 (top). There was a good absorbance peak at 260nm (where DNA absorbs) and another smaller peak at 550 where Cy3 absorbs. Using Beer's law, the concentration of the DNA was found to be 0.013M or 13mM.

$$0.455 = (33 cm^{-1} \cdot M^{-1})(1cm)(c) \quad \text{[Equation 1]}$$

The DNA oligo was circularized using the Circligase II ssDNA kit from Epicenter Bio. The circularization solution was incubated at 60°C for 2 hours and 80°C for 10 minutes. The first incubation temperature is the optimum temperature for the enzyme to work and is the time that it should circularize the ssDNA, and the second temperature should deactivate the enzyme. An aliquot of the circularized solution was removed and stored at -20°C and the rest was treated with Exonuclease I to digest any remaining linear DNA so all that should remain in our solution was circular DNA. The DNA was ethanol precipitated and then resuspended in 1x TE buffer. The ethanol precipitation concentrated the DNA and removed excess salts. Now, only the DNA and the 1x TE buffer was in the solution.

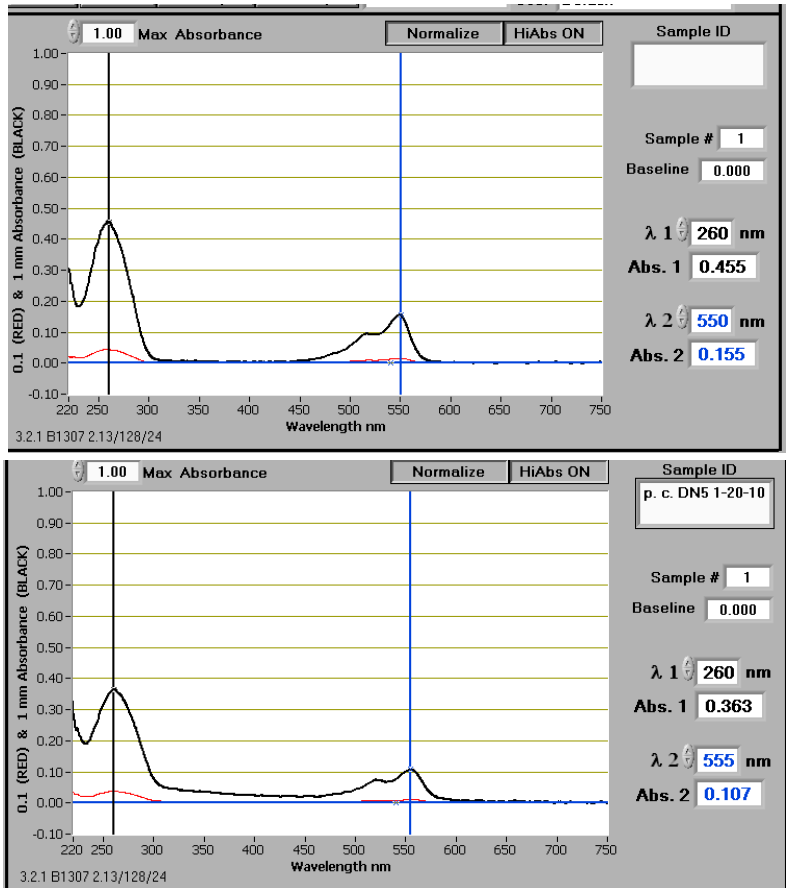


Figure 10: (Top) UV-Vis spectra of linear ssDNA with Cy3 inserted into the phosphate backbone. (Bottom) UV-Vis spectra of circular ssDNA with Cy3 inserted into the phosphate backbone.

A UV-Vis spectrum of the resuspended circular ssDNA was taken using the Nanodrop. 1x TE buffer was used as the blank and the spectra is shown in Figure 10

(bottom). There was a good absorbance peak at 260nm, where DNA absorbs, of 0.363. The Cy3 absorbed at 555nm instead of 550nm. This red shift of the dye could be due to the added constraints from the circularization of the DNA. This shift shows how sensitive the Cy3 dye is to changes in its environment.

Using Beer's law, the concentration of the DNA was found to be 0.011M or 11mM when the extinction coefficient was $33cm^{-1} \cdot M^{-1}$.

$$0.363 = (33cm^{-1} \cdot M^{-1})(1cm)(c) \text{ [Equation 2]}$$

The linear ssDNA, the DNA after the Circligase reaction, and the DNA after the Circligase reaction and treatment with Exonuclease I was run on a 15% denaturing polyacrylamide gel shown in Figure 11. An Ultra low range DNA ladder from Fermentas was run in Lane D of the gel. This gel shows the linear 45nt DNA (Lane A), the 45nt DNA after the Circligase reaction (Lane B) and the circular 45nt ssDNA after Exonuclease I treatment and ethanol precipitation (Lane C).

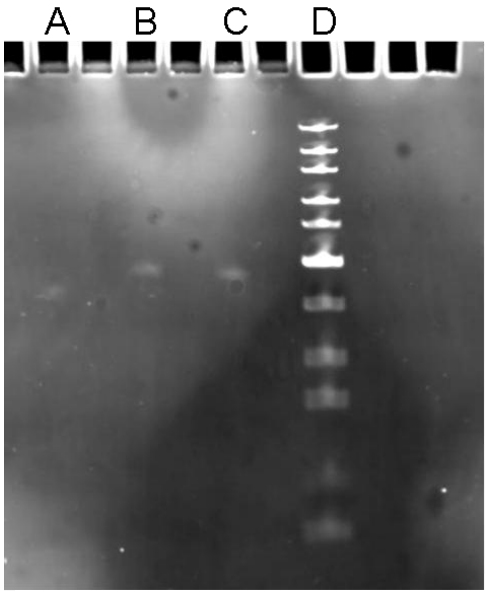


Figure 11: 15% polyacrylamide gel stained with ethidium bromide. Lane A contains the 45nt linear ssDNA, lane B contains the DNA after the Circligase reaction, lane C contains the 45nt circular DNA, and lane D contains the Ultra low range DNA ladder.

Only linear DNA was loaded into lane A, so lane A is the reference point to where linear 45nt ssDNA with Cy3 should run. The circligase reaction after incubation was loaded into lane B. This lane shows how much of the DNA was circularized. Only one band was seen in lane

B and it ran slower than the band in lane A. This shows that the majority of the linear DNA was circularized in the reaction. The band in lane C ran slower than the band in lane A and the same as the band in lane B. Because lane C contained the DNA after treatment with Exonuclease, this is further proof that the bands in lanes B and C are circular because Exonuclease only digests linear DNA.

The gel in Figure 11 shows that the internal Cy3 modification in the DNA did not affect the circularization of the DNA. Almost all of the linear DNA that was added to the circularization reaction was circularized. The addition of the Cy3 probably did not affect the circularization because it is inserted internally, not on the ends. The Cy3 was added in the middle of the linear DNA at least 20 bases away from either one of the edges. The purified circular DNA was used as the DNA template in RNA transcription.

InVitro Transcription of Cy3 modified circular ssDNA with T7 RNAP

To ensure that the T7 RNAP would transcribe the modified circular DNA, we first ran the transcription experiment in vitro and visualized it with agarose gel electrophoresis. An aliquot of the components of the transcription reaction were stored at -20ºC before incubation. This aliquot was used as the control, as the RNAP should not transcribe at -20ºC, so all of the reaction components are in there, but no RNA, so only a DNA band should be seen in the gel. The circular DNA was transcribed with T7 RNAP at 37ºC, and an aliquot of this mixture was stored at -20ºC. This aliquot should have both the template DNA and the RNA. The rest of the transcription mixture was treated with DNase to remove the DNA template, and then the remaining RNA was ethanol precipitated and resuspended in 1x TE buffer. The concentration of the RNA was

determined through UV-Vis spectroscopy using the Nanodrop. 1x TE buffer was used as the blank. The spectrum of the RNA is shown in Figure 12.

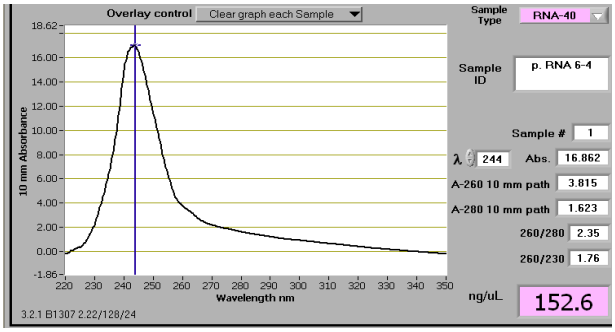


Figure 12: UV-Vis spectra of purified RNA in 1x TE buffer.

The transcription control, the solution from the transcription reaction and the transcribed RNA after DNase treatment was visualized on a 1.2% Agarose RNA flashgel (Lonza). Figure 13 shows the image of the FlashGel. Lane A shows the RNA Millennium marker from Ambion. This RNA marker shows RNA bands from 500-9000 bp and was used as we were expecting very long RNA transcripts to be made from the DNA template. Lane B contained the control which, was all of the solutions in the transcription solution stored at -20ºC so the polymerase could not work. Lane C contained the transcription solution after incubation. Lane D contained the RNA made from transcription and purified using DNase and concentrated by ethanol precipitation.

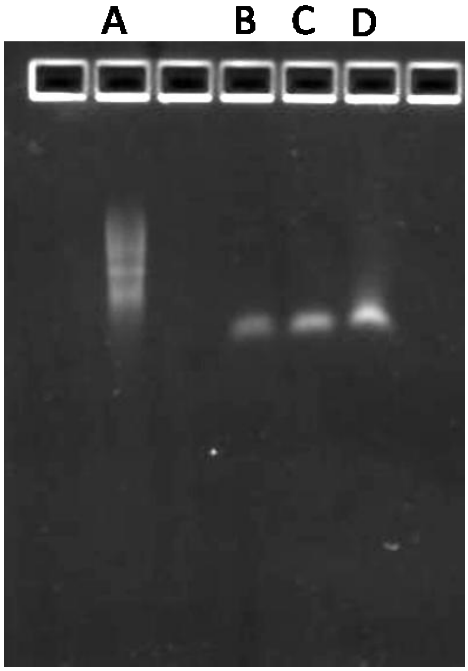


Figure 13: 1.2% FlashGel with RNA Millennium Marker (Lane A), control reaction (Lane B), transcription reaction after incubation at 37°C (Lane C), and purified concentrated RNA from transcription (Lane D).

The circular ssDNA template, seen in Lane B, ran faster than the lowest band in the RNA marker, which is 500bp. It looks close to the bottom of the ladder because a 1.2%

Agarose gel cannot separate small DNA or RNA molecules well. Lane C shows the RNA that was made during transcription and the template DNA. The band ran the same as the one in Lane B but was brighter, indicating that there were more nucleic acids in the band. Lane D shows the purified and concentrated RNA. A band is at the same position as the other two lanes, but there is a smear above the band. This smear is due to the different lengths of RNA that were produced during rolling circle transcription (RCT). The different lengths of RNA are too close together to be separated into distinct bands by this gel. This smear is seen in Lane D but not in Lane C because the RNA in Lane D is more concentrated than the RNA in Lane C. The same RNA is in the lane but there is not enough RNA to be visualized.

According to the gel, most of the RNA produced is 1000 bases or less, but some of the RNA is up to 6000 bases in length. In terms of how many revolutions around the DNA circle the polymerase traveled, the majority of the time the RNAP transcribed the circle 21 times or less and, in some cases, transcribed the circle up to 130 times. The Cy3 in the phosphate backbone of the DNA did not stop transcription. For the single molecule fluorescence studies, 21 revolutions would be plenty of time to watch the changes in intensity and find a pattern.

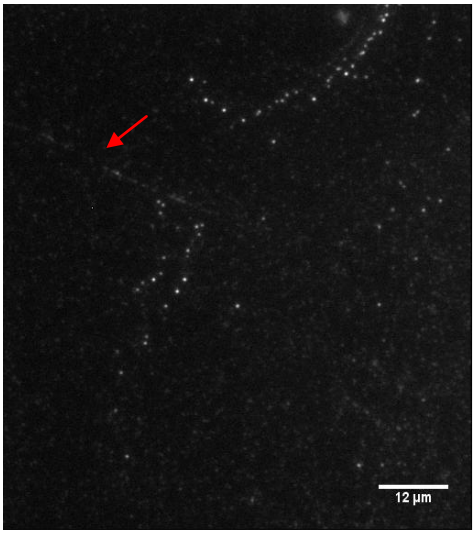


Figure 14: Fluorescence image of RNA annealed with short rhodamine labeled DNA and combed onto a clean glass surface. The red arrow shows a long RNA strand.

To further prove that RNA much longer than the template DNA was produced the RNA was purified with gel extraction and

ethanol precipitation and then annealed with a 45nt DNA complement labeled with rhodamine at the 5' end. The DNA:RNA hybrid was combed onto a clean glass surface and imaged using the fluorescence microscope.

Figure 14 shows the first frame of a movie of the combed DNA:RNA hybrid. A red arrow shows a long line of fluorescence diagonal in the top left side of the image. This is a long RNA strand with complement rhodamine labeled DNA. The breaks in the line are places on the RNA where DNA did not bind. It was not expected that the DNA would bind to every piece of the RNA. Excess labeled DNA was filtered out to remove free labeled DNA from the solution. Every fluorescent dot on the image should correspond to an RNA strand bound to a labeled DNA molecule. Other long lines and small fluorescent lines, only one or two pixels, appear in the image, with the latter were most prevalent on the coverslip. This finding corresponds to our gel data, which showed that most of the RNA produced was small (under 500 nt), but there were some much larger RNA strands produced. These larger strands will be what we mostly want to image under the fluorescent microscope because they will give more data before termination. The fluorescent line in the left side of the image, shown by the arrow in Figure 14, is 32.8 μm long. This length corresponds to a RNA strand that is 994 bases long. To make a RNA strand this long the T7 RNAP would have to transcribe the circle 21 times.

Photostability of Cy3 labeled DNA

We needed to know how stable the Cy3 molecule inside of the circular DNA would be in the transcription conditions because, if something in the transcription buffer causes the Cy3 molecule to photobleach very quickly, it will not make a good probe for this experiment. Ideally, one would need the majority of the probes to stay active for at

least 30 seconds. This is about half of the length of other recordings reported in this thesis. To measure the photochemical half-life, we determined the photostabilty of the Cy3 in three different environments: dry on glass, in water, and in 1x transcription buffer.

Transcription experiments cannot be performed dry on glass or in water. The salts that are in the transcription buffer are required for the polymerase to transcribe efficiently. Therefore, while determining the photochemical half-life times for the Cy3 labeled DNA water and dried on glass is useful for comparison, the environment that needs to have a long photochemical half-life is the Cy3 labeled DNA in transcription buffer. If the photochemical half-life was found to be too short then parameters, including salt concentration, could be modified to lengthen the photochemical half-life.

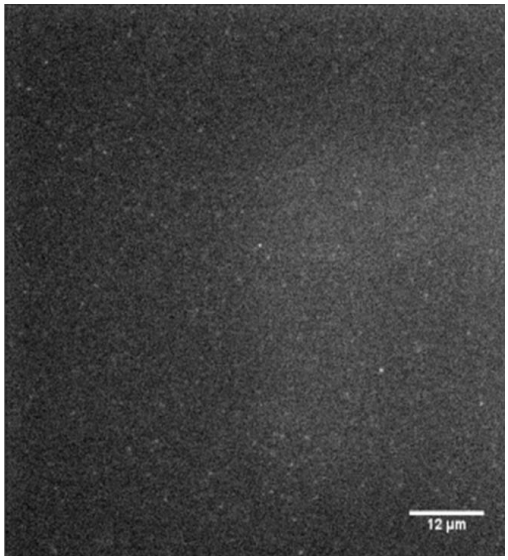


Figure 15: Fluorescence image of Cy3 labeled DNA in transcription buffer. This image is the 1st frame of a 500 frame movie.

To determine the photostabilty of Cy3, movies were taken of the Cy3 labeled DNA in different environments: on glass, in H_2O, and in transcription buffer. Figure 15 shows the first frame of a movie of Cy3 labeled DNA in transcription buffer. The dots of intensity are single fluorescent molecules. Using SpotSelect, a script run in Matlab, places of intensity are labeled as spots, and the intensity over time for each of the spots is graphed. The intensity over time data for each spot is filtered using a Weiner2 filter. The Weiner2 filter reduces the noise that is seen, so the photobleaching step can more clearly be seen. The time that each fluorescent molecule photobleached is recorded and graphed.

In this way the photochemical half-life of the Cy3 in each environment can be determined.

When photobleaching occurs the fluorescent intensity of the Cy3 molecule should decrease to background levels in one frame. Figure 16 shows two graphs of two different spots in the movie. Both show one-step photobleaching. In one-step photobleaching, the intensity drops to zero or background in one step. One-step photobleaching is a characteristic of single molecule fluorescence techniques and a way to prove that what one is looking at is a single molecule. Some of the molecules fluoresced throughout the entire movie but most photobleached sometime during the movie. Each movie was 750 frames long and 140 milliseconds between frames, so each movie was a total of 105 seconds long.

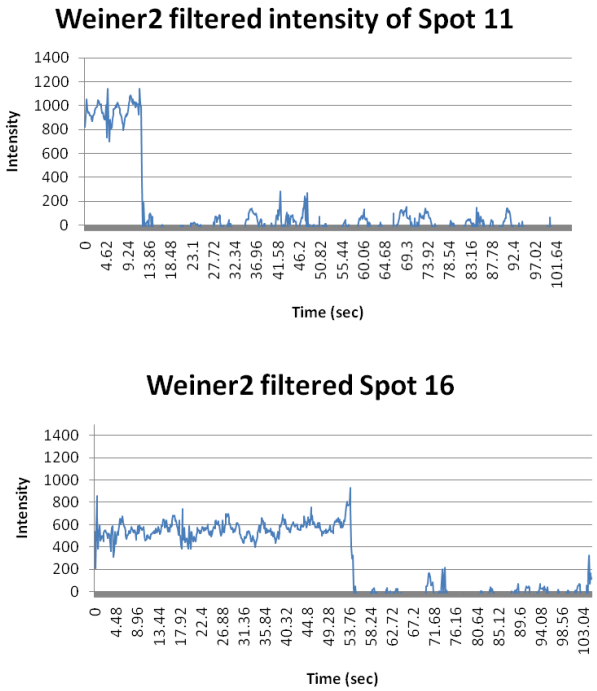


Figure 16: Two graphs showing the intensity over time (seconds) of a Cy3 labeled DNA in transcription buffer. Both graphs show one-step photobleaching which proves that there was only one molecule producing the light.

Each graph of each individual spot in a movie was run through a set of criteria to be certain it was a single molecule. The first criteria is that the spot was above the minimum threshold value determined by the SpotSelect script. A description of the threshold can be found in Appendix D. The second criterion is that the spots did not show two-step or multistep photobleaching. If they showed single-step photobleaching, it

proved that they were single molecules. The last criterion was that the spots' intensities were not larger than 2000. Most of the single molecule spots had intensities between 500 and 1200. If the spot passed the test to be a single molecule, the time that it photobleached was recorded. If the molecule did not photobleach during the movie, the end time of the movie was recorded. This process was done for spots in movies for each environment tested.

For each environment, the number of molecules active at increments of 5 seconds was graphed and fit to a decaying exponential curve. The total number of molecules that were analyzed for a particular environment is shown in the number of molecules at time zero. Figure 17 shows the exponential graphs for Cy3 labeled DNA in three different environments: dry on glass under N_2, in UV-treated ddH_2O under N_2, and in 1x

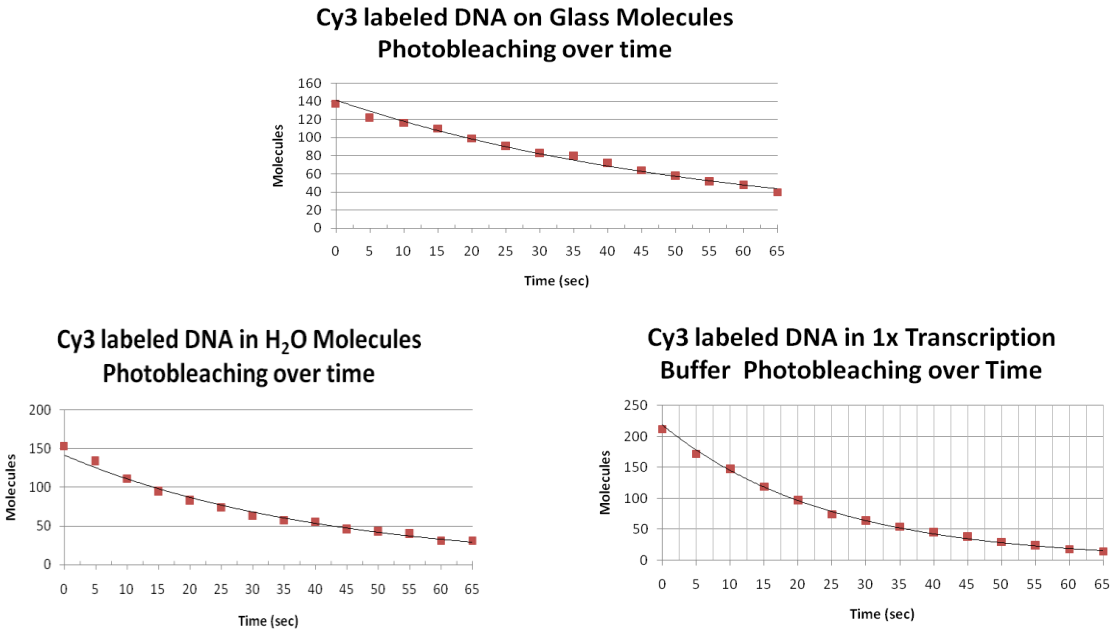


Figure 17: Graphs showing the number of fluorescent molecules active after a given time in a movie. These data points were fit to an exponential curve. The top graph shows the number of molecules active at varying times for the Cy3 labeled DNA dried on a glass coverslip, the bottom left graph shows the number of molecules active at varying times for the Cy labeled DNA in water, and the bottom right graph shows the number of molecules active at varying times for the Cy3 labeled DNA in 1x transcription buffer.

transcription buffer under N_2.

For each of the environments, we looked at over 100 spots to get a good sampling of the times they photobleached. The Cy3 labeled DNA in transcription buffer showed the best exponential decay, and, by the end of the sampling time, the majority of the molecules had photobleached. The exponential fit was not quite as good in the graph of the Cy3 labeled DNA in water as in transcription buffer, but it was good enough to give us a value. For the Cy3 labeled DNA that was dried on glass, the decay was almost linear, not exponential. For all of the environments the majority of the molecules had photobleached by the end of the experimental time.

From the graphs, the photochemical half-life of the Cy3 labeled DNA in each of the environments was calculated. Table 2 shows the calculated half-life. The photochemical half-life of the Cy3 labeled DNA on glass under N_2 was 37 seconds, in water under N_2 was 25 seconds, and in 1x transcription buffer under N_2 was 17 seconds. The photochemical half-life of the Cy3 was longest dried on glass and shortest in 1x transcription buffer, which was unexpected. One reason for this result could be that there was very little oxygen (less than 1%) around the Cy3 molecules that were dried on glass, but for the two samples in liquid, there could still have been a high percentage of O_2 causing the fluorophores to photobleach faster.

Sample	Photochemical Half-life
Cy3 labeled DNA on glass	37 seconds
Cy3 labeled DNA in H_2O	25 seconds
Cy3 labeled DNA in 1x Transcription buffer	17 seconds

Table 2: The photochemical half-life of the Cy3 labeled DNA dried on glass, in water, and in 1x transcriptionbuffer.

Although the photochemical half-life of the Cy3 labeled DNA in transcription buffer was only 17 seconds, which is almost half of the ideal time of 30 seconds. A quarter of the molecules will still be fluorescing after 30 seconds, leaving enough molecules to analyze with enough modulations that we can figure out the time it takes for the polymerase to transcribe the DNA circle.

Single Molecule Fluorescence Imaging of Transcription

Clean glass coverslips and slides were used to fashion the flow cells. The transcription buffer was treated with UV light to photobleach any fluorescent molecules that may have been in the solution. The DTT, NTP mix, transcription buffer, and T7 RNAP were added to the flow cell and imaged under the fluorescence microscope. This step was done to ensure there is no fluorescence in the solution before the fluorescently labeled DNA is added because the only fluorescence in the flow cell should be from the fluorescently labeled DNA.

Fluorescence was seen in the solution, so the source had to be determined. To determine the source, T7 RNAP, NTP mix, DTT, and transcription buffer were studied in separate clean flow cells and imaged using the fluorescence microscope. The only one that showed fluorescence was the NTP mix shown in Figure 18. Two things could have contributed to the fluorescence of the NTP mix: it was over one year old so it could have been contaminated or part of the mix could have broken down making a fluorescent product. Also the mix had ATP, CTP, GTP, and UTP all together, which by itself, would not be expected to cause fluorescence, but an additive could have been added to increase the stability of the solution. New ATP, CTP, GTP, and UTP were ordered from Invitrogen. They were mixed immediately before being imaged in a clean flow cell and no fluorescence was seen.

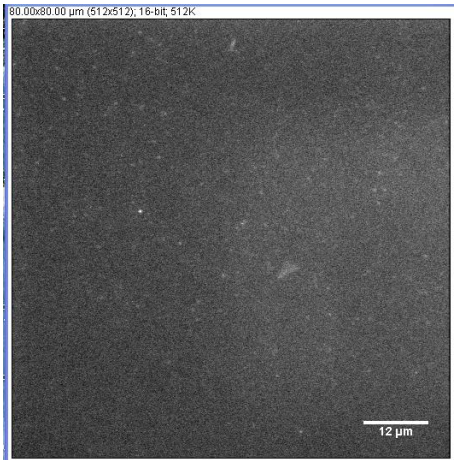


Figure 18: Image of a clean glass flow cell loaded with 2.5mM NTP mix. Many fluorescent spots from the NTP mix can be seen in this sample.

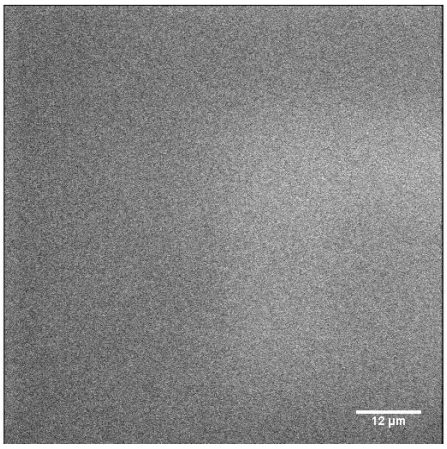


Figure 19: Image of clean glass flow cell with 0.5x transcription buffer, 2.5mM NTPs, 1mM DTT, and 0.01mM Trolox. No fluorescence was seen in this image.

The DTT, NTPs, transcription buffer, and T7 RNAP were flowed into a flow cell and imaged again. This time no fluorescence was seen as shown in Figure 19.

Now that it is certain that the only fluorescence in the flow cell would be from the Cy3 labeled DNA, the experiment could proceed with the addition of the DNA and imaging the labeled DNA with the transcription buffer, NTPs, DTT, and T7 RNAP. Movies were taken of transcription of the Cy3 labeled DNA by T7 RNAP in different NTP concentrations. These studies began with imaging at 2.5mM NTP concentration because that is the same concentration that is used in the in vitro experiments. Then experiments were performed at 5mM, 1.25mM, and 0.75mM NTP concentrations because there should be a change in the rate when the concentration of NTP is changed. Figure 20 shows the background corrected first frame of an image of the Cy3 labeled DNA being transcribed in a solution with 2.5mM NTP. The graph in Figure 21A shows the intensity over time (seconds) of one spot in the movie.

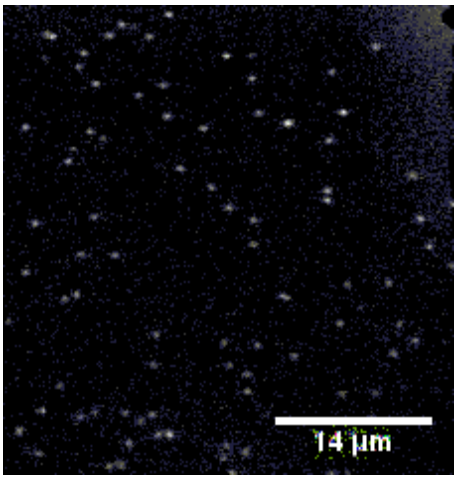
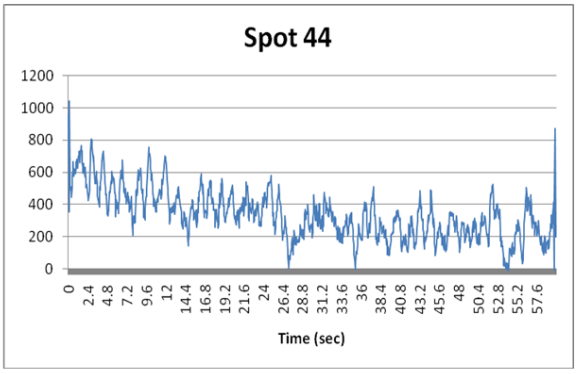


Figure 20: Background corrected 1st frame of a 750 frame movie. The fluorescent spots are the Cy3 labeled DNA imaged during transcription with T7 RNAP in 2.5mM NTP mix

A



B

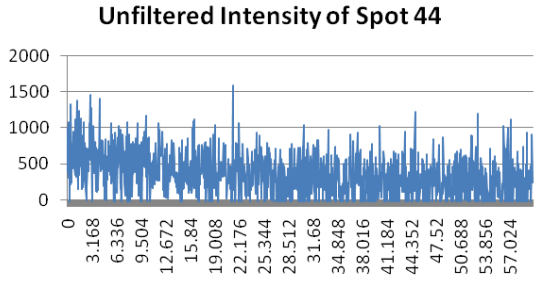


Figure 21: (A) Wiener filtered intensity data from Spot 44 from the transcription of the Cy3 labeled DNA at a concentration of 2.5mM NTP mix. (B) Unfiltered intensity data for the same spot in A.

The intensity data shown on the graph have been filtered using a Wiener filter. The Wiener filter will smooth out the noise by increasing large changes in the intensity and decreasing the small changes. The Wiener filter is described in more detail in Appendix D. These small changes are usually noise, and, by decreasing them, one can obtain a clearer view of the slow variation in the image intensity. The graph of the intensity over time of spot 44 without the Wiener2 filter is shown in figure 21B. The intensity data without the Wiener filter are too noisy to see any modulations of the intensity data.

The background had to be corrected for each movie to reduce the noise and to obtain a clearer plot of the intensity. It is important to reduce any changes that might come from the background and might skew the data. In Figure 21A the intensity modulates fairly regularly. We would not expect the modulations to occur at exactly the same distance in time, but we would expect them to be fairly close for the same spot and the same concentration of NTPs.

Concentration of NTPs in flow cell during movie	Average time between peaks from all of the spots (sec)	Average rate of transcription (nt/sec)
5mM	1.3	36
2.5mM	1.2	39
1.25mM	1.7	28
0.75mM	2.0	23

Table 3: Table of the average time between peaks of the modulations in the intensity data of the Cy3 labeled DNA during transcription with different concentrations of NTPs. The transcription rate of the T7 RNAP was calculated from the average time between the peaks.

Spots were selected based on a set of criteria to remove spots that were not single molecules or were not being transcribed. The first criterion was that the spot's intensity be above the minimum threshold value of the background. The second criterion was that the spot not show 2-step or multiple step photobleaching. If a spot showed 1-step

photobleaching, it proved that the spot was a single molecule. The next criterion is that the intensity has a value of no more than 2000. The majority of the single molecules had intensities between 500 and 1200. The last criterion was that the spot has modulations with increases that are at least 40% higher than the baseline of the spot's intensity. This criterion ensured that the spot is the DNA during transcription by T7 RNAP.

Multiple spots were analyzed for each concentration of NTP and the average time per each modulation was found. From the average time between modulations, the transcription rate for each NTP concentration was determined. The average time between modulations for each of the four NTP concentrations and the resulting transcription rate are shown in Table 3. The average time between modulations for the transcription reaction with 5mM NTP concentration was 1.268 seconds, for the 2.5mM NTP concentration reaction the average time was 1.174 seconds, the average time for the 1.25mM NTP concentration was 1.713 seconds, and the average time for the 0.75mM concentration was 2.036 seconds.

The time between modulations for the 2.5mM and the 5mM was the same within error, but the 1.25mM was less than the 2.5mM or 5mM. The transcription with the 0.75mM concentration had even more time between the modulations than the 1.25mM concentration. 2.5mM of NTP is probably at the saturation level of the reaction, so, in order to see a change, we would need to lower the concentration. The average transcription rate for each sample with the different NTP concentration was determined.

The experiments were begun with a NTP concentration of 2.5mM because that was the concentration that is used for the in vitro transcription. However, this amount of NTP is probably saturating the reaction because, in the in vitro reactions, one wants the

most RNA made in the least amount of time. One of the objectives of these single molecule experiments is to determine the relationship between a change in the concentration of NTPs and a change in the rate of transcription. In order to observe this relationship, the NTP concentration must be reduced below the saturation point. As there was a significant increase in the time between the modulations for the 1.25mM concentration of NTP compared to the 2.5mM concentration, more transcription experiments were run in lower concentrations of NTP. Samples with an NTP concentration of 0.125mM, 0.25mM and 0.50mM were imaged, and the time between frames was determined for these concentrations as well.

Table 4 shows the time between the peaks of the modulations in intensity for the three lower NTP concentrations. The transcription reaction with 0.5mM NTP concentration had an average time between peaks of 2.103 seconds, which equals a transcription rate of 21.76nt/sec. The transcription reaction with 0.25mM NTP concentration had an average time between peaks of 19.91nt/sec, which equals a transcription rate of 19.91. Last, the transcription reaction with 0.125mM NTP concentration had an average time between peaks of 2.483 seconds and a transcription rate of 18.83nt/sec.

Concentration of NTPs in flow cell during experiment	Average time between peaks from all of the spots (sec)	Average rate of transcription (nt/sec)
0.5mM	2.1	22
0.25mM	2.4	20
0.125mM	2.5	19

Table 4: List of time between peaks of modulations and the corresponding transcription rate for transcription with three different NTP concentrations.

The average rates of transcriptions are close to the published rates for T7 RNAP using other single molecule fluorescence techniques. For a NTP concentration of 0.2mM, the published rate using single molecule fluorescence imaging was 42 ±8 nt/sec, which is within 15nt/sec of rate shown in Table 4 for transcription with 0.25mM NTP concentration. Another single molecule fluorescence technique records the T7 RNAP transcription rate between 20 and 60 nt/sec depending on the sequence. The transcription rates that are calculated from the modulations are close to what others are finding.

The time between the peaks of the modulations increases as the concentration of NTPs decrease, which was expected. This increase in time between peaks represents a decrease in the transcription rate as the concentration of NTPs decreases. Because there are less NTPs around, it takes longer for an NTP to be attached to the growing RNA chain by the T7 RNAP. The decrease in rate is not steady. The decrease between NTP concentrations of 1.25mM and 0.75mM was more pronounced than the decrease between NTP concentrations 0.5mM and 0.25mM.

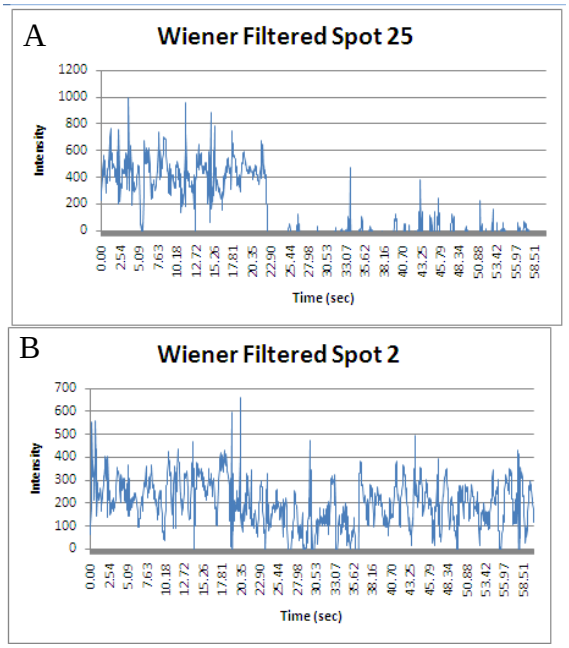


Figure 22: (A) Graph of the intensity over time of Wiener filtered spot in transcription with 2.5mM NTP concentration. (B) Graph of the intensity over time of Wiener filtered spot in transcription with 0.125mM NTP concentration. The peaks in graph A are much closer together than the peaks in graph B.

Figure 22A shows a spot from a movie of the transcription with 2.5mM NTP concentration and Figure 22B shows a spot from a movie of transcription with 0.125mM NTP concentration. The peaks in the graph that had the 0.125mM NTP concentration were much more spread out than the peaks in the graph from 2.5mM NTP concentration. Even from a quick glance, the change in transcription rate can be seen.

So far we have only compared the average transcription rate of the T7 RNAP at the different concentrations. To realize the full use of single molecule techniques, we want to look at the rates for each T7 RNAP transcribing a circle at a known NTP concentration. By looking at individual polymerases, one can see the range of transcription rates at a given NTP concentration.

Histograms of all of the times between the peaks of the modulations for the different spots in the movies at each NTP concentration were made. These histograms show the distribution of the rates of the individual T7 RNAP molecules with different concentrations of NTPs. Figure 23 shows the histogram plots for each of the concentrations of NTPs. There is a large spread in rates for each of the NTP concentrations, but the overall spread is shifted to the right as the concentration of NTPs decreases as shown by the average.

The x-axis scales for all of the histograms are the same so one can see how the time between peaks changes with different concentrations of NTP. The histogram for the transcription with 5mM and 2.5mM NTP concentration are very similar; the highest peak was at 1.008 and 0.864 respectively. The highest peaks for both of these concentrations are clustered around 1 second. The similarity of the histograms of transcription with

5mM and the 2.5mM NTP concentration agreed with the averaged time between the peaks data.

The peaks in the histogram of transcription with 1.25mM NTP concentration were shifted to the right compared to the histogram of the time between peaks of transcription at 2.5mM and 5mM NTP concentrations. The majority of the peaks was clustered around 1.008 and 1.44 seconds. The peaks in the histogram of transcription with 0.75mM NTP concentration were shifted even more to the right compared to the histogram of the time between peaks of transcription at 1.25mM NTP concentration. The majority of the peaks was between 0.96 and 1.872 seconds. There were relatively more peaks in the three and four second area than were seen in any of the transcription reactions with a higher NTP concentration.

The most common range of peaks for transcription with 0.5mM NTP concentration was similar to the range for the transcription reactions with 0.75mM NTP concentrations but still shifted to the right. The range for transcription with 0.5mM NTP concentration was 1.104 to 2.064 seconds. The difference in the time between the peaks for these two concentrations is not as large because the difference between the concentrations is smaller. Most of the other concentrations were decreased by half, but from 0.75mM to 0.5mM only decreased by 1/3.

The histogram showing the time between the peaks of transcription with 0.25mM NTP concentration had most of the peaks between 1.2 and 2.448 seconds. This range is shifted to the right of transcription with 0.5mM NTP concentration. There are also more cycles with times between three and four seconds than in any of the transcription reactions with higher NTP concentrations.

The lowest concentration of NTPs in the transcription reaction tested was 0.125mM . The peaks in the histogram for this concentration were shifted to the right compared to transcription with 0.25mM NTP concentration. The majority of the peaks was between 1.104 and 2.736 seconds. Transcription at this concentration also had more times between four and five seconds than any other concentration of NTPs. There is a wide range of times between peaks for each of the NTP concentrations. Single molecule experiments always have a range of values because, unlike ensemble experiments, there is no inherent averaging. Each selected spot is a labeled DNA molecule where a certain T7 RNA polymerase is transcribing. The difference in the time between the peak of each modulation shows the time it took for one T7 RNAP to transcribe a circle one time from inserted Cy3 molecule to inserted Cy3 molecule.

The histograms showing the individual time between peaks for each spot for transcription at all of the NTP concentrations tested agreed with the averaged data. Both the histograms and the average time between peaks for each NTP concentration showed an increasing time between peaks as the concentration of NTPs decreased.

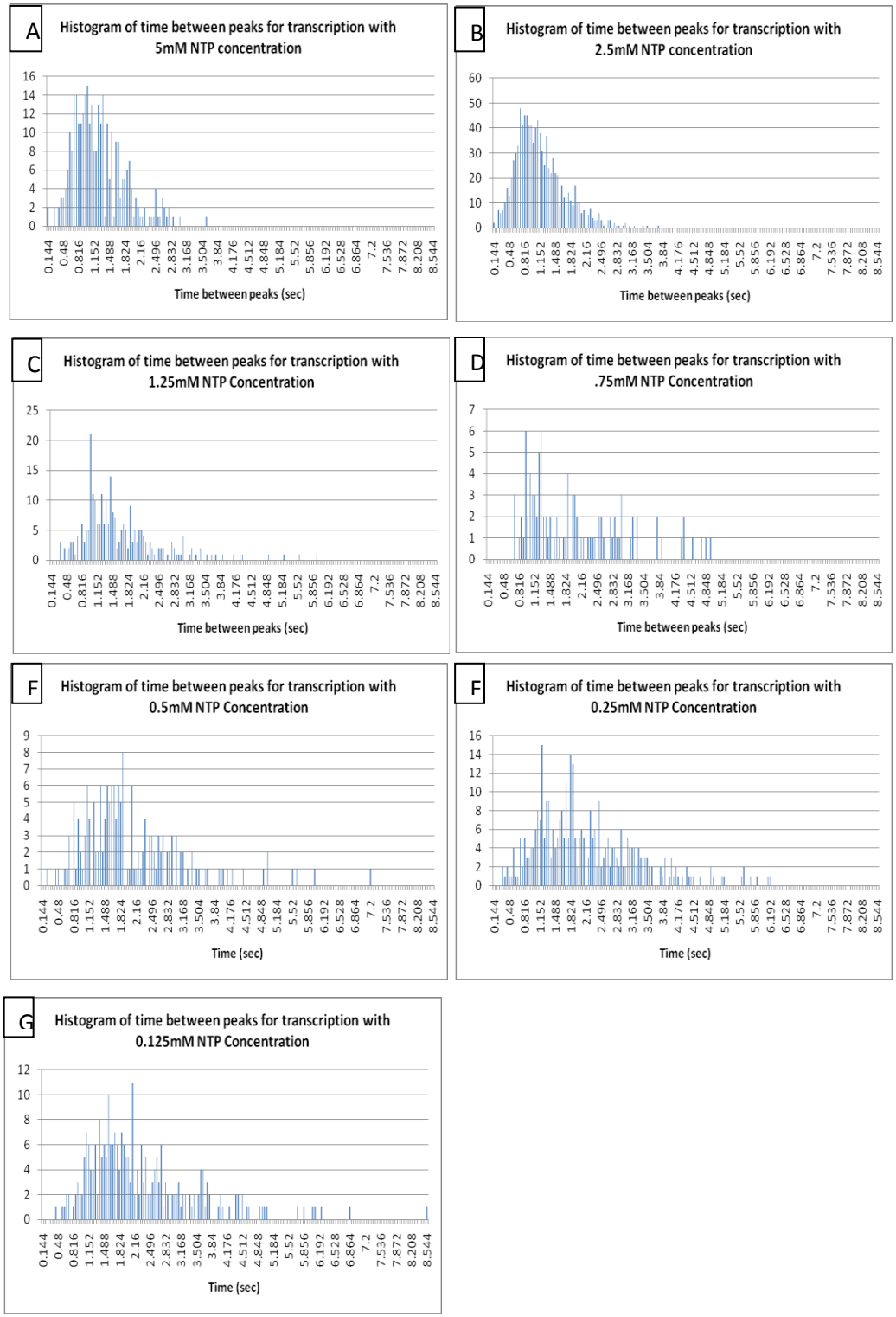


Figure 23: Histograms of the time between the peaks for all of the spots selected at each concentration of NTP. (A) 5mM NTP concentration (B) 2.5mM NTP concentration (C) 1.25mM NTP concentration (D) 0.75mM NTP concentration (E) 0.5mM NTP concentration (F) 0.25mM NTP concentration (G) 0.125mM NTP concentration.

BSA Coated Flowcell

The T7 RNAP transcribed the labeled DNA in the clean glass flow cell. The modulations of the intensity were recorded and graphed. Then the transcription rate was determined from the time between the peaks of the modulations. It has been shown that the efficiency of an enzyme is decreased when it is on a glass surface. Even though the reaction is done in buffer, the polymerase could be on the glass surface. To determine if the glass was, in fact, decreasing the efficiency of the enzyme, the glass was coated with BSA.

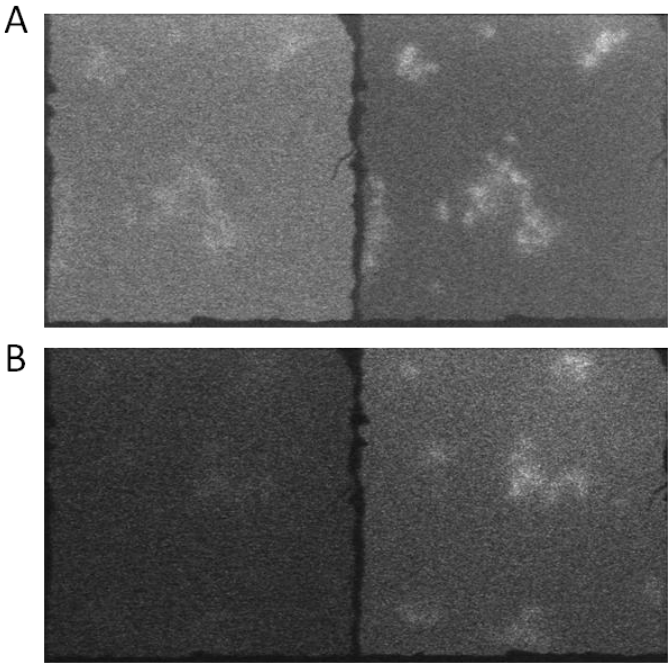


Figure 24: (A) Fluorescent Optosplit image of BSA coated flowcell rinsed with UV-treated PEM-80 buffer. (B) Fluorescent Optosplit image of BSA coated flow cell rinsed with 0.5x transcription buffer with 0.5mM NTP, 1mM DTT, and 0.01mM Trolox.

The BSA-coated flow cell was imaged using the fluorescence microscope. The coated flow cell was imaged to make sure that there would not be background fluorescence that would be too bright to see the single molecules. There was a lot of background fluorescence due to the BSA, mainly in clumps that could be seen in the red channel and the green channel of the optical splitter. The buffer was thought to be causing the fluorescence, so the PEM-80 buffer was irradiated with UV light to remove any fluorescence. Then a new BSA solution was made in the UV treated PEM-80.

A new clean flow cell was coated with BSA and rinsed with the UV treated PEM-80 buffer. The flow cell was imaged using the fluorescent microscope, and the first frame of the 200 frame movie is shown in figure 24A. There was still a significant amount of fluorescence clustered in groups around the slide. As the PEM-80 buffer had been irradiated to remove any fluorescence, the fluorescence must be coming from the BSA. The flow cell was rinsed with 0.5x transcription buffer with 0.5mM NTPs, 1mM DTT, and 1mM Trolox then imaged again (Figure 24B). The flow cell was rinsed with the transcription buffer mixture that is used in the transcription reaction. After the rinse there was less fluorescence, but there was still too much to see the single molecules. The BSA was causing too much background fluorescence to use it for the single molecule experiments, so alternative molecules to coat the glass so that the RNAP would not rest directly on the glass surface were sought.

Poly-L-lysine coated flow cell

Because the BSA was too fluorescent in the flow cell, in the next set of experiments poly-L-lysine was used to coat the cell. The poly-L-lysine coated flow cell was imaged using the fluorescence microscope with the optical splitter.

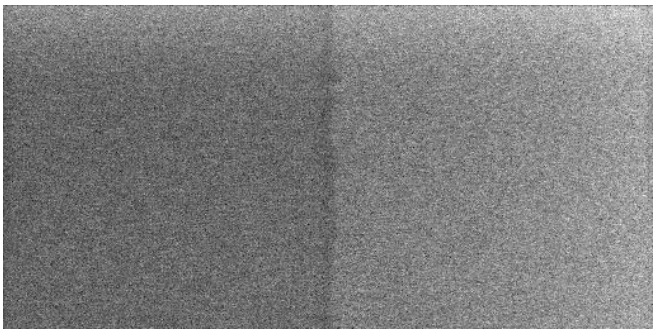


Figure 25: Fluorescent Optosplit image of poly-L-lysine coated flow cell.

A 200 frame 512x256 pixel movie was taken of the poly-L-lysine coated flow cell. The flow cell was imaged to be certain that the poly-L-lysine on the glass was not fluorescent. Figure 25 shows the first frame of the poly-L-lysine coated flow cell movie. No

fluorescence was seen on the flow cell. The pixel intensities were all between 200 and 300, which are in the background range. Because the poly-L-lysine coated flow cells were not fluorescent, transcription movies were recorded with different NTP concentrations.

Transcription in Poly-L-lysine coated flow cells

Transcription of the Cy3 labeled circular DNA was recorded in the poly-L-lysine coated flow cells. Three different concentrations of NTPs were used in the transcription reaction: 0.5mM, 0.25mM, and 0.125mM. These lower concentrations were chosen because the higher concentrations such as 2.5mM were too close to the saturation level for the reaction. Even lower NTP concentrations might be used, in the future, to determine how slow we can make the T7 RNAP transcribe.

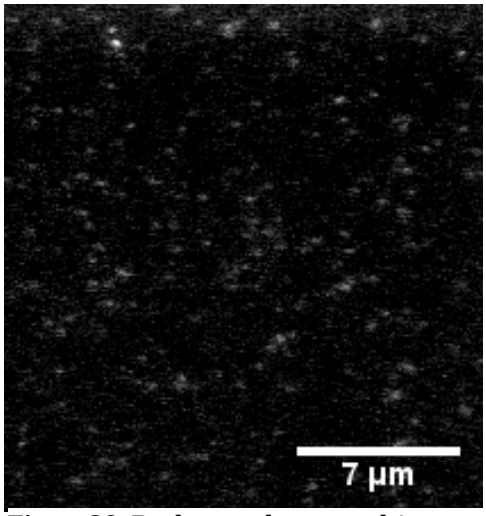


Figure 26: Background corrected 1st frame of transcription of Cy3 labeled DNA in poly-L-lysine coated flow cell with 0.5mM NTP concentration.

Figure 26 shows the background corrected 1st frame of the transcription of the Cy3 labeled circular DNA in a poly-L-lysine coated flow cell with 0.5mM NTP concentration. Just by looking at the 1st

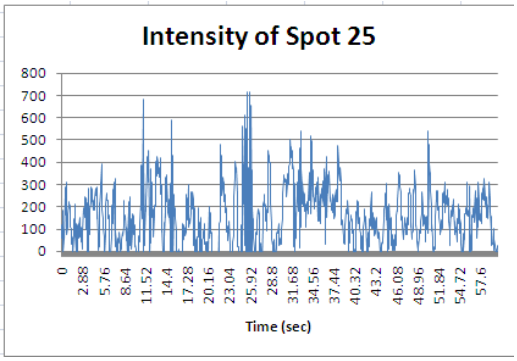


Figure 27: Graph of background and illuminant corrected, weiner2 filtered intensity data of spot 25 in the movie of transcription in a poly-L-lysine coated flow cell with 0.5mM NTP concentration.

frame, no difference can be seen from the uncoated glass flow cell. Figure 27 shows a graph of the weiner2 filtered data for one of the spots.

The time between the peaks of the modulations was calculated for the selected spots for each NTP concentration. From the time between peaks, the transcription rate was determined. The time between the peaks and the transcription rates are shown in Table 5. The transcription with 0.5mM NTP concentration has an average time between peaks of 1.799 seconds, which equals transcription rate of 25.66 nt/sec. The transcription with the 0.25mM NTP concentration has a time between peaks of 2.254 seconds and an average rate of transcription of 20.83 nt/sec. The 0.125mM NTP transcription has an average time between peaks of 3.033

seconds and a transcription rate of 15.24nt/sec.

Concentration of NTPs in flow cell during experiment	Average time between peaks from all of the spots (sec)	Average rate of transcription (nt/sec)
0.5mM	1.8	26
0.25mM	2.3	21
0.125mM	3.0	15

Table 5: Average time between peaks of modulations and the corresponding transcription rate for poly-L-lysine coated flow cell at different NTP concentrations.

The transcription rates of the T7 RNAP in the poly-L-lysine coated flow cell are higher than the uncoated glass flow cell for the 0.5mM and 0.25mM NTP concentrations, which agrees with the hypothesis that the glass was decreasing the efficiency of the T7 RNAP. For the 0.125mM NTP concentration, the transcription rate of the poly-L-lysine coated flow cell was lower than the uncoated glass. More experiments will have to be

done to determine why this is happening; however, one possibility is that the PLL coating is binding some of the NTPs so that, once the concentration of NTPs is below a certain threshold, there is a drastic decrease in the amount of available NTPs in solution.

Histograms of the time between the peaks for all of the spots for transcription in poly-L-lysine coated flow cells for each of the different NTP concentrations were graphed and are shown in Figure 28. The histogram of transcription with 0.5mM NTP concentration had most of the peaks between 1.104 and 2.208 seconds. The histogram of transcription with 0.25mM NTP concentration has most of the peaks between 1.152 and 2.304 seconds. The range of peaks for the transcription experiment with 0.25mM NTP concentration was shifted to the right compared to the 0.5mM NTP concentration experiment.

The histogram of transcription with 0.125mM NTP concentration had most of the peaks between 1.344 and 3.168 seconds. This range is again shifted to the right compared to the transcription experiments with higher NTP concentrations. The increase in the majority of the times between the peaks of the modulations agrees with the averaged time between the peak data. Both show that the time between the peaks increases as the NTP concentration decreases.

Next we compared the ranges of the most common times between the peaks in the experiments using an uncoated glass flow cell versus a poly-L-lysine coated glass flow cell. The range of the most common time between peaks at 0.5mM NTP concentration was 1.104 to 2.064 seconds for the uncoated glass flow cell and 1.104 and 2.208 seconds for the poly-L-lysine coated flow cell. The ranges for both of these are very similar. The poly-L-lysine coated flow cell had a range 0.2seconds longer. The differences in the

ranges of the times between the peaks of the modulations were not as pronounced as the differences between the averaged time between peaks between the poly-L-lysine coated and uncoated flow cell.

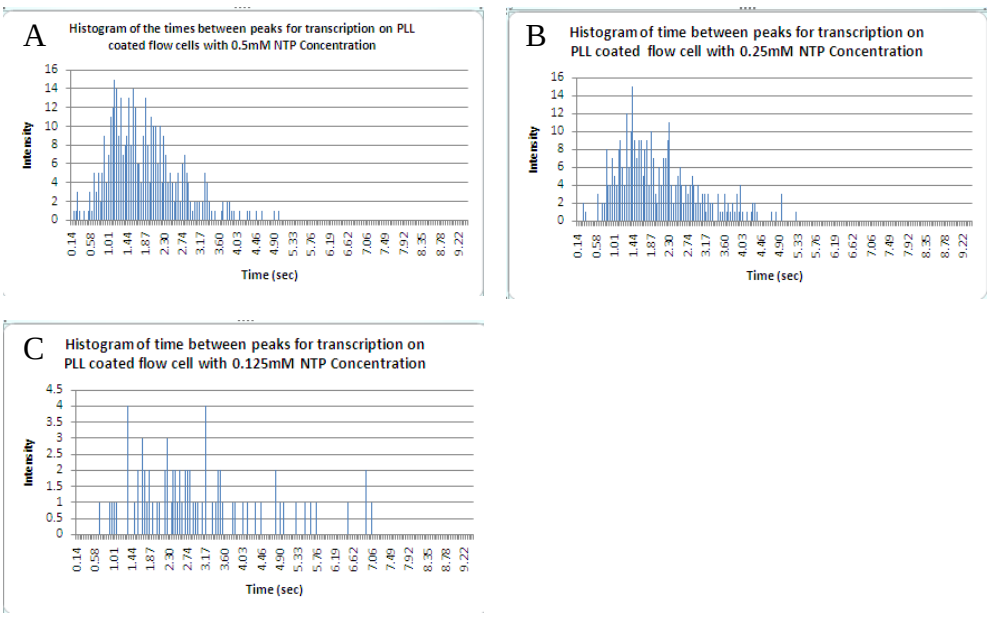


Figure 28: Histograms of the times between the peaks of the modulations in the movies of transcription in poly-L-lysine coated flow cells at various concentrations. (A) 0.5mM NTP (B) 0.25mM NTP (C) 0.125mM NTP

The most common ranges from the movies of transcription with 0.25mM NTP concentration in an uncoated flow cell were 1.2 to 2.448 seconds and in a poly-L-lysine coated flow cell were 1.152 and 2.304 seconds. Similar to the 0.5mM NTP concentration movies, the range was very close to the same for both. The experiment ran in the poly-L-lysine coated flow cell was only less than a tenth of a second faster than the experiment in the uncoated flow cell.

There was a large difference in comparing the transcription experiments with 0.125mM NTP. The experiment with the uncoated flow cell has a range of 1.104 and 2.736 seconds, and the experiment with the poly-L-lysine coated flow cell has a range of 1.344 and 3.168 seconds. The poly-L-lysine coated flow cell had a longer time between

the peaks by 0.2 to 0.4 seconds. This outcome agrees with the averaged time between peaks, in which the average time for the uncoated experiment was 2.483 seconds and the average time for the poly-L-lysine coated flow cell was 3.033 seconds. In both the averaged time between peaks and the histogram of the time between peaks for the experiments in a poly-L-lysine coated flow cell with 0.125mM NTP, the time was longer for the experiment with the poly-L-lysine coated flow cell.

The histograms of the individual times give us a different way at looking at the rate of the T7 RNAP. The general trends are the same for the averaged data and the single data plotted in a histogram; the transcription rate of the T7 RNAP decreases as the NTP concentration decreases. The histogram shows how long each T7 RNAP molecule takes to transcribe the circle once. We can see that there are outliers; sometimes the T7 RNAP transcribes the DNA quickly, less than a second, and sometimes it transcribes it very slowly (or pauses) over 5 seconds. But most of the time the polymerases work at a fairly regular and consistent pace depending on their environment.

Control Experiment

To prove that the modulations in the intensity of the fluorescent spots were due to the proximity to Cy3 of the polymerase during transcription and not something that is inherent to Cy3 or due to the microscope, a negative control experiment was done. A clean poly-L-lysine coated flow cell was loaded with the transcription buffer, T7 RNAP, DTT, Trolox and the labeled circular DNA, and then imaged under the fluorescence microscope. Figure 29

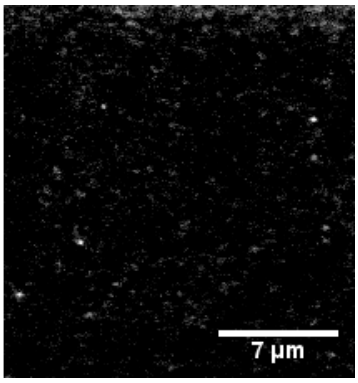


Figure 29: Background corrected 1st frame of negative control for transcription experiment.

shows the background corrected 1st frame of the negative control.

From the spots that were graphed, ones that showed two-step photobleaching were discarded. If there are two molecules, the intensity data recorded are averaged between the two so any increases or decreases in intensity are lessened. A graph of the intensity of the Cy3 labeled DNA for one spot is shown in Figure 30. There are variations in the intensity over the course of the movie, but the increases in intensity are not as regular or as large as the modulations in the transcription experiments. The increases in intensity in this graph are only about 20% on average, where as the modulations in the intensity of the transcription graphs are at least 40%.

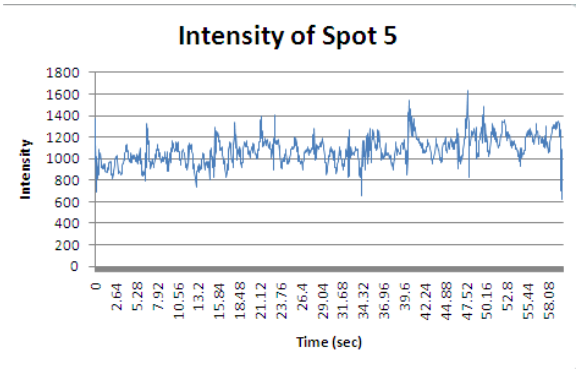


Figure 30: Graph of the intensity of the Cy3 labeled DNA in transcription buffer with T7 RNAP, DTT, and Trolox.

The small changes in the intensity of the Cy3 molecule in the control are probably from noise in the background and the difference in how much light the fluorophore is emitting. The background noise is due to the scattered light and any autofluorescence of the sample[43], as well as the inherent noise from the camera.

Fourier Transform

Fourier transform was explored as a way to determine the most common frequency of the modulations in the intensity plots instead of manually finding each peak and the time it occurred. Using Fourier transform to analyze the data would remove any human error and would be a quicker way to find the transcription rate. Instead of calculating the time between the peaks, the frequency of the peaks would be calculated.

From the frequency, the time and then the rate could be determined. A fast fourier transform was added to the code in the Matlab script, and a power spectrum was graphed with the most frequent instances over frequencies. An example of one of the power spectra for a spot in a movie is shown in Figure 31. No peak stands out as the dominant frequency for three potential reasons: the noise is too high due to the modulations not being regular enough, there are not enough data points in the fourier transform for there to be a strong peak, or because the DC component (the distance of the baseline of the data from the x-axis) of the intensity data is too large. The latter of the two possibilities may be remedied, but there is nothing one can do if the modulations from the Cy3 molecule are not regular.

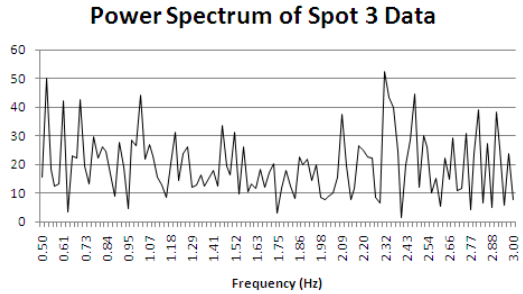


Figure 31: Power spectrum of weiner2 filtered intensity data.

To try to get a better signal to noise ratio, longer movies movies were recorded. The number of frames was increased from 1250 to 2000. This approach did not help because increasing the number of frames also increased the time between frames, and by the end of the 2000 frames there were usually none or only one or 2 fluorophores still active. The increase of the time between frames was due to a computer issue.

Next an attempt was made to obtain a better signal to noise ratio by removing the DC component in the graphs of the intensity. This method would eliminate the empty space in the graph where the intensities, even in the low troughs, are above zero. The power spectrum of a spot with its DC component removed is shown in Figure 32, which is from the same data that was used in Figure 31. As you can see, the signal to noise ratio

in the DC component removed spot is better than the signal to noise in the regular power spectrum, but the signal is still less than double the size of some of the noise.

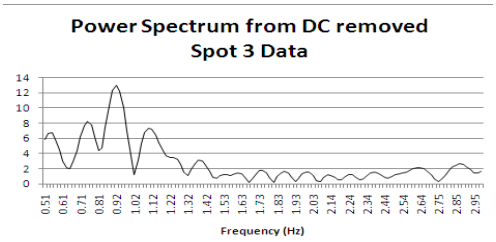


Figure 32: Power spectrum of weiner2 filtered intensity data with DC component removed.

The power spectrum cannot be used to determine the frequency of the modulations as long as the signal to noise ratio is low. Another algorithm will have to be found that can determine the most common frequencies allowing for the quasi-periodic state of the modulations

Conclusions

Cy3 was determined to be a good probe for the single molecule fluorescence analysis of the transcription rate of T7 RNA polymerase. The fluorescent molecule was bright enough to be seen in the single molecule level with short exposure time, and it was stable enough to stay active long enough for the movies of transcription to be recorded. The photochemical half-life of the Cy3 labeled circular DNA was 23.5 seconds and many lasted for over a minute. The Cy3 also made a good probe because it did not inhibit the circularization of the linear DNA or the transcription of the circular DNA it was a part of.

The sensitivity of Cy3 to its environment and its availability were the main reasons it was chosen as the probe. The intensity of the Cy3 molecule increases by at least 40% when it was in the T7 RNAP. Once it was outside of the RNAP, the intensity of the RNAP decreased to baseline levels. These modulations were used to determine the transcription rate of T7 RNAP. All of the transcription experiments were done at 22°C

with 3mM Mg^{+2} concentration. For the 2.5mM NTP concentration, the transcription rate was 39nt/sec; for the 1.25mM NTP concentration, the transcription rate was 28nt/sec; for the 0.75mM NTP concentration, the transcription rate was 23nt/sec; for the 0.5mM NTP concentration, the transcription rate was 22nt/sec; for the 0.25mM NTP concentration, the transcription rate was 20nt/sec, and for the 0.125mM NTP concentration, the NTP concentration was 19nt/sec. The transcription rate was shown to decrease as the NTP concentration decreased, showing a correlation between the modulations and what the polymerase was doing.

The coating of the flow cell with poly-L-lysine changed the transcription rates, but the same overall decrease in transcription rate as the NTP concentration decreased was seen. The experiment with 0.5mM NTP concentration had a transcription rate of 22nt/sec; for the 0.25mM NTP concentration, the transcription rate was 20nt/sec, and for the 0.125mM NTP concentration, the transcription rate was 19nt/sec. Single molecule fluorescence imaging can be used to determine the rate of a RNA polymerase using rolling circle transcription.

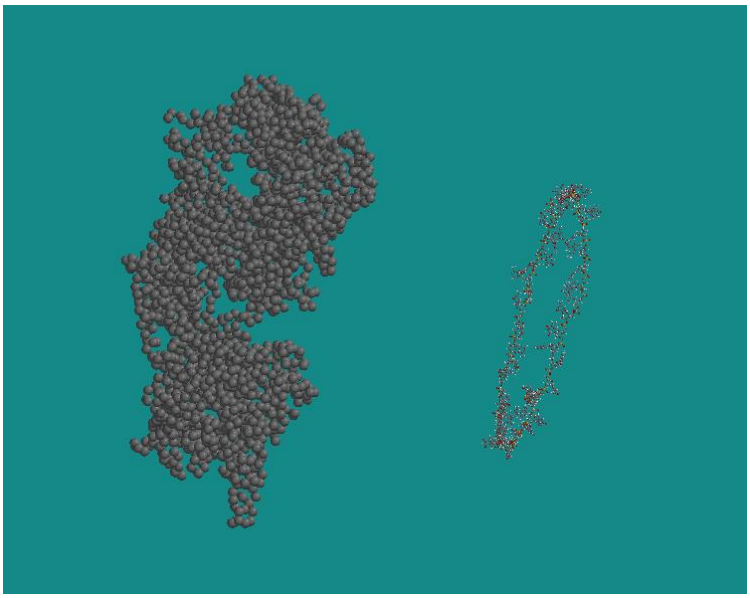
Appendix A

45nt DNA circle

The 45nt DNA has a sequence of 5'-CTG GAG GAG ATT TTG TGG TAT CGA TTC GTC TCT TAG AGG AAG CTA-3'.

The 45nt DNA with the Cy3 internal modification has a sequence of 5'-CTG GAG GAG ATT TTG TGG TA(Cy3)T CGA TTC GTC TCT TAG AGG AAG CTA-3'.

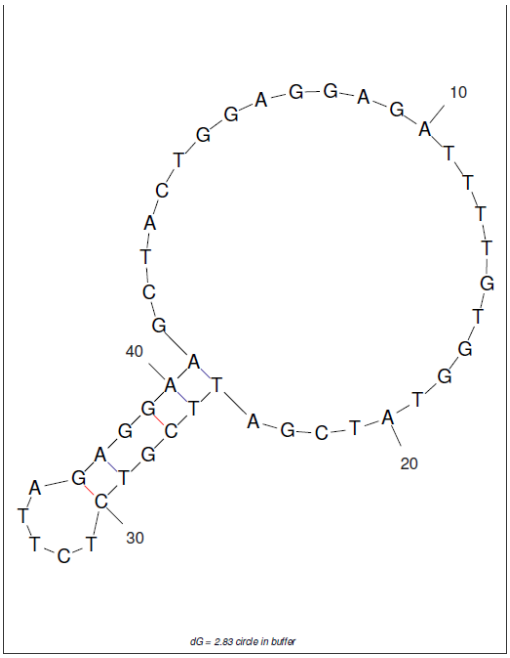
The only difference between the two is the addition of the Cy3 molecule between bases 20 and 21. Below is a scale representation from Spartan 08 version 1.2.0 that shows the T7 RNAP (left) and the circular DNA (right). The size of the DNA circle is similar in size to the T7 RNAP.



Appendix B

Secondary Structure of 45nt DNA circle

Using Mfold free software, the most energy favorable secondary structure for the DNA circle in buffer is shown below. About two thirds of the DNA is a circle, but one-third of the bases form a hairpin. The Cy3 molecule is not shown in the image below, but it would be between bases 20 and 21. Because the Cy3 would be in the circular part of the DNA, it would not change the secondary structure much from what is shown here.



Appendix C

Ethanol Precipitation Protocol

1. Aliquot DNA so that no more than 100µL of DNA is in a 1.5mL microcentrifuge tube
2. Add 1mL 99.9% ethanol and 15µL of 2mM NaCl to each tube with DNA
3. Place in a -80ºC freezer for 45 minutes
4. Centrifuge at 12,500 rpm at 4ºC for 15 minutes
5. Decant ethanol solution
6. Wash the pellet to remove salt by adding 500µL of 70% ethanol to the tubes, briefly vortex and then centrifuge at 4°C for 10 minutes
7. Decant ethanol
8. Remove remaining ethanol with vacuum centrifuge
9. A DNA pellet should remain

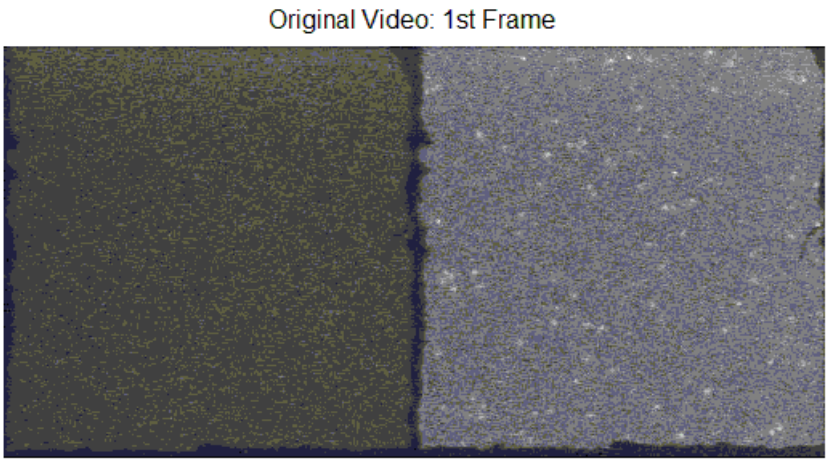
Appendix D

Summary of Spot Select 0.1 Script

There are 14 steps in the script from uploading the Avi file of the movie that will be analyzed to plotting the intensity of the spots.

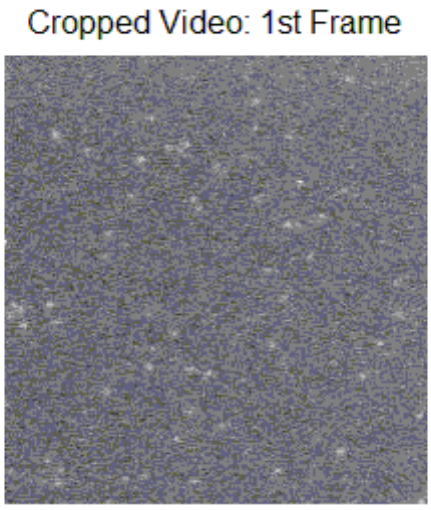
1. Upload original Avi file

   The script will show the 1st frame of the AVI



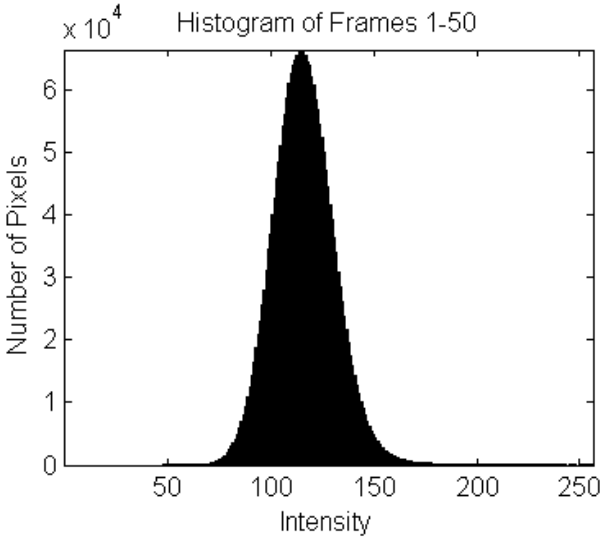
2. Crop Video

   The rough edges around the video from the optical splitter and the camera are cropped. Also, one channel of the optical splitter image is cropped out, leaving only the image with the fluorescent spots.



3. Histogram of the intensity values of the pixels

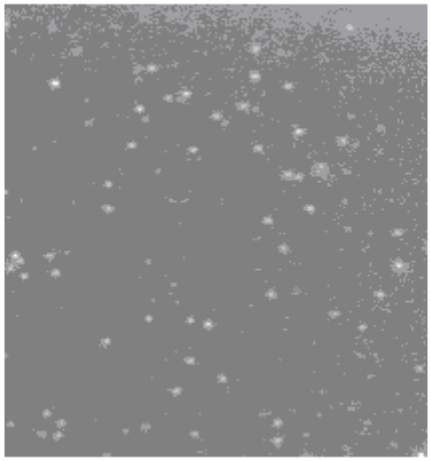
   This gives a histogram of all of the pixel values in the 1st 50 frames of the movie



4. Maxima of each pixel

A 10 frame moving average filter is applied to the pixels of the 1st 50 frames of the movie. An image is formed that shows the maximum intensity value of each pixel in the 1st 50 frames.

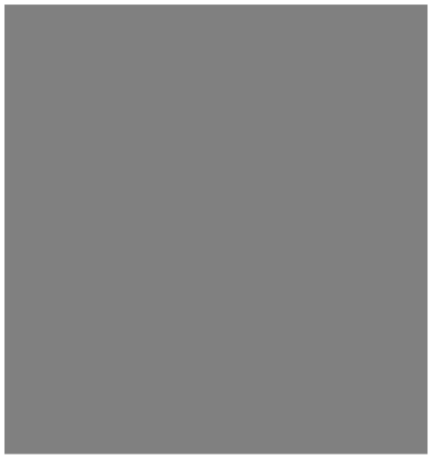
Pixel Maxima



5. Background calculated from the maxima

The background of the movie is assumed to be smooth. The image of the maxima of each pixel is shrunk to 1/10 its size. The shrunken image is then recreated by substituting each pixel with the lowest pixel value in a 3x3 pixel region around the pixel of interest. The image is blurred to smooth out the background, and then the image is increased back to normal size. The resulting image is the background calculated from the maxima.

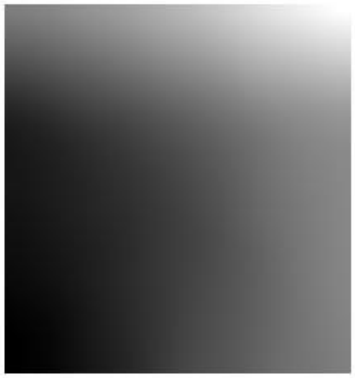
Background from Maxima



6. Contrast stretched background calculated from the maximum

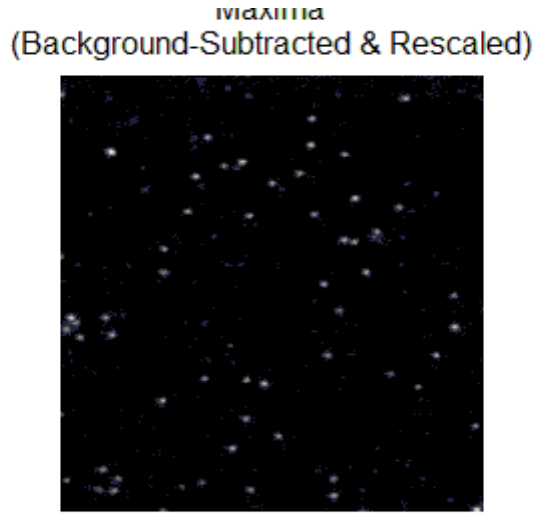
The contrast is stretched from the background that was calculated from the maximum of each pixel. The lowest pixel is shown in black and the highest value pixel is shown in white. The stretching of the contrast clearly shows the difference in the background in different places in the movie.

Contrast-Stretched Background from Maxima



7. Background corrected maximum

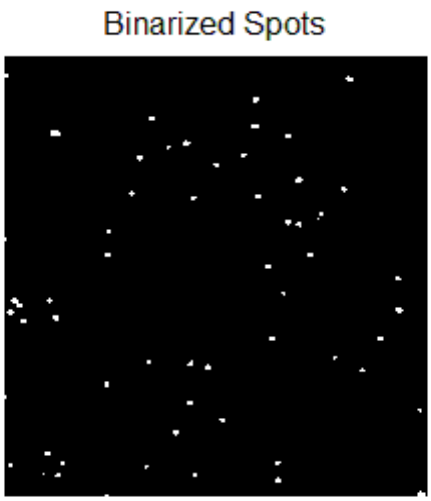
The background image is subtracted from the maxima image and any negatives are changed to 0 since intensity cannot be negative. The pixel values are normalized, so, instead of the range being from 0-255, it is now 0-1.



8. Binarized spots

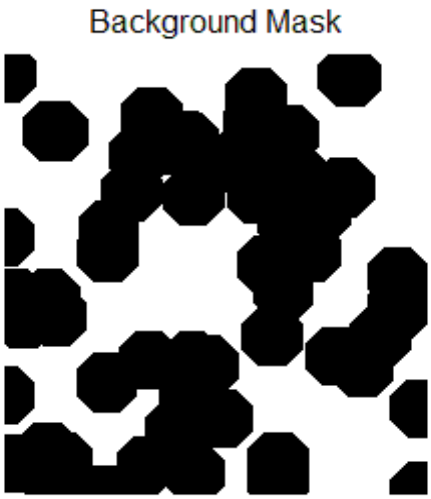
From the background corrected maxima image, any pixel that is greater than the threshold value is considered a potential spot and any pixel less than the threshold value is considered background. The threshold is chosen by the user; it is a percentage of pixel intensity value. For these experiments I used a pixel intensity of 0.3, so any pixels that are more intense than 30% of the pixels in the movie are potential spots. Any grouping of 8 or more pixels above the threshold is considered a spot and is given a value of 1. Any groupings of less than 8 pixels above the

threshold or any pixels below the threshold are given a value of 0 and are considered background. To use this algorithm in Matlab there is a choice between 4 or 8 pixels. 8 was chosen because it was assumed that the light from any fluorphore would illuminate at least 7 surrounding pixels.



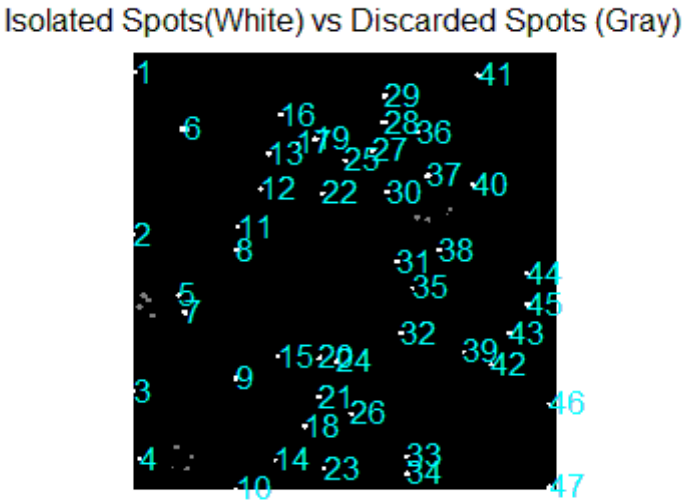
9. Background mask

This image is made from the binarized image. Each spot from the last image is given a value of 0. A 15 pixel circle around each spot is also given a value of 0. Every other pixel in the image is given a value of 1. This creates a mask of only the background pixels. This background is used later in the script for the illuminant correction.



10. Numbered spots

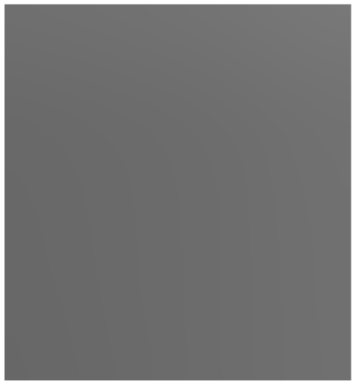
Removes spots from the binarized image that are closer than 7 pixels. The remaining spots are numbered. The centroid of each spot is found. A look up table is made for the position of spots and a location of an 11x11 area of pixels around centroid of spot. From this point on in the script, a spot refers to the centroid of the potential spot and the 11x11 area of pixels around it.



11. Mean Background

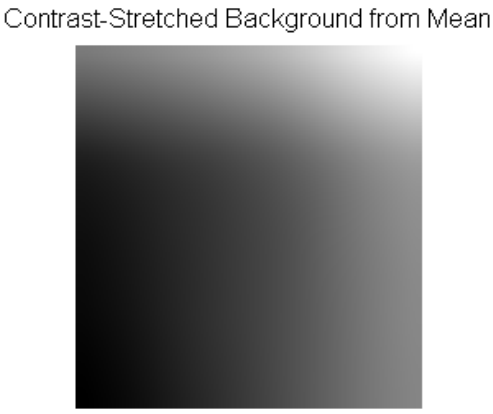
The mean value of all pixels over all frames is calculated and an image is formed. The background of the movie is again assumed to be smooth. The image of the mean of each pixel is shrunk to 1/10 its size. The shrunken image is then recreated by substituting each pixel with the lowest pixel value in a 3x3 pixel region around the pixel of interest. The image is blurred to smooth out the background, and then the image is increased back to normal size. This image is the calculated background from the mean. The background from the mean is used as the background that is subtracted when looking at the intensities of individual spots.

Background from Mean



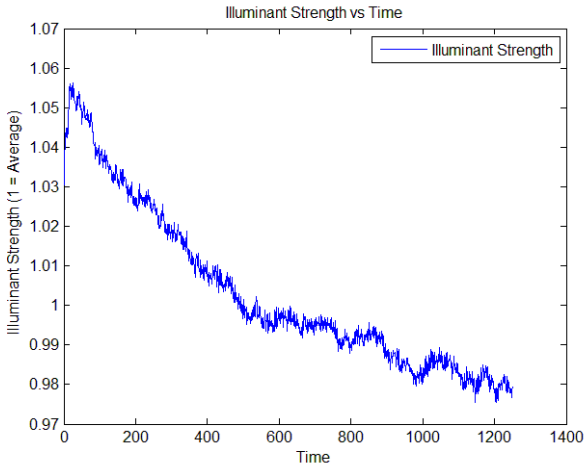
12. Mean contrast background

The contrast is stretched from the background that was calculated from the mean of each pixel. The lowest pixel is shown in black and the highest value pixel is shown in white. The stretching of the contrast shows clearly shows the difference in the background in different places in the movie.



13. Graph of illuminant strength

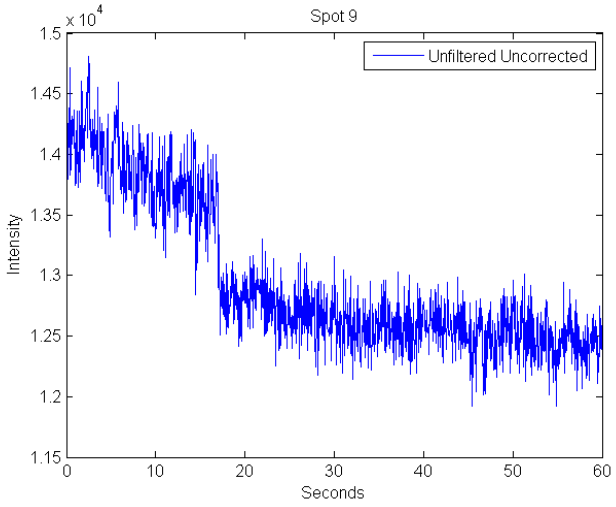
Shows the change of the illuminance over time during the movie. The illuminance is calculated by taking the average of all of the pixels in the background for each frame. The background mask was used to determine which pixels were part of the background. This number is graphed for each frame of the movie.



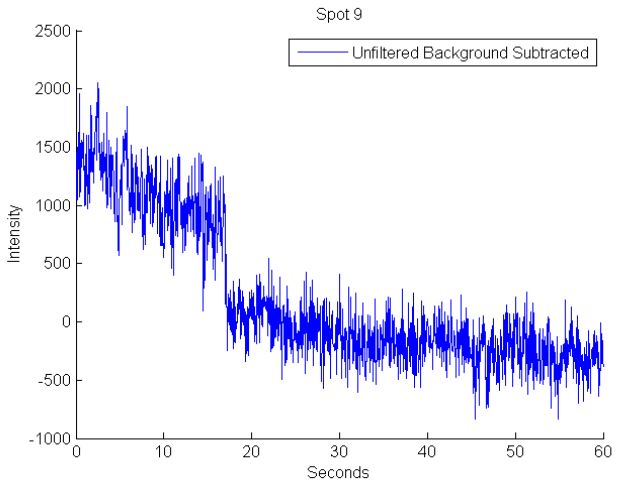
14. Plot intensity data of the spots

Intensity data for each spot is plotted over time.

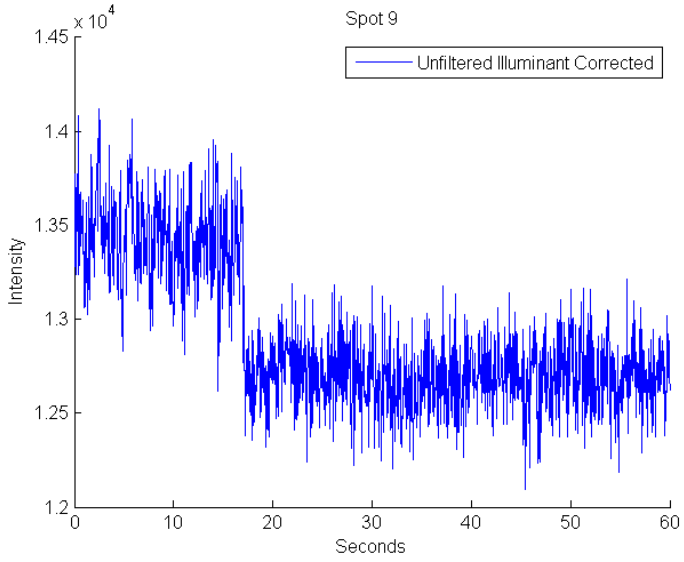
i. Original- The intensities of each spot (11x11 pixel area) are summed and these are the intensity value for each spot at each frame. The intensity values are from the original cropped video. These values are graphed over time either in frames or seconds.



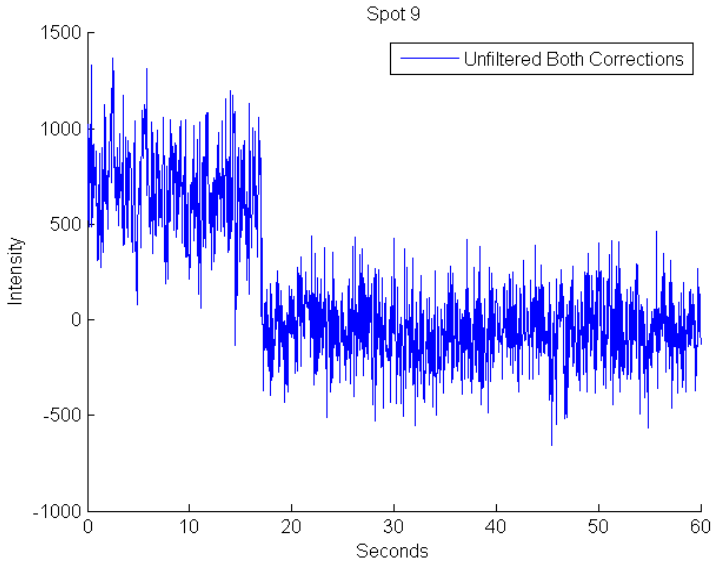
ii. Background corrected- The mean background is subtracted from each frame of the cropped movie giving the background corrected video. The intensities of each spot (11x11 pixel area) are summed and these are the intensity values for each spot at each frame. These values are graphed over time either in frames or seconds.



iii. Illuminant corrected- Background pixels from the mean background image are averaged creating the mean average background intensity. The background mask was used to determine which pixels are considered background. The illuminance of each frame, calculated in the illuminance graph step, is divided by the mean average background intensity to get the illuminance ratio. All of the pixel values of the current frame are divided by the illuminance ratio, which is done for each frame. The resulting values are graphed over time either in frames or seconds.



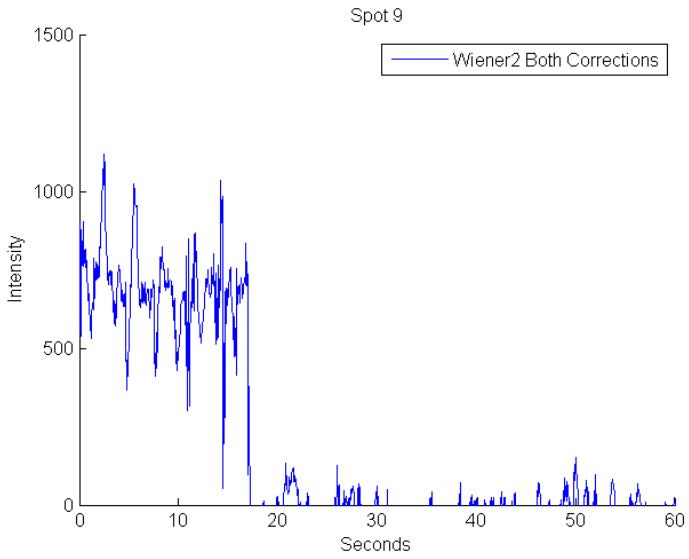
iv. Background and illuminant corrected- The illuminance is corrected 1st as described above, and then the background is corrected for each frame. These values are graphed over time either in frames or seconds.



v. Wiener filter- A 10 frame wiener filter can be applied to any of the above plot data. The wiener filter smoothes out some of the noise from the data using the following algorithm

$$b(n_1, n_2) = \mu + \frac{\sigma^2 - v^2}{\sigma^2(a(n_1, n_2) - \mu)}$$ [Equation 3]

Where v^2 is the average of all of the local estimated variances, μ is the local mean around each frame, and σ^2 is the local variance around each frame, a is the value of the frame of interest. These values are graphed over time either in frames or seconds.



Appendix E

SpotSelect 0.1 code

```
classdef StateNav < hgsetget
    properties
        CurrentStep = Step()
        CurrentState
        FunctionsBack
        FunctionsForward
        StateNames = ...
        {
            'Start', 'Video Loaded', 'Video Cropped', 'Histogram',
'Maxima', 'Maxima Background', ...
            'Maxima Contrast Background', 'Maxima Corrected',
'Binarized Spots', 'Background Mask', ...
            'Labeled Spots', 'Mean Background', 'Mean Contrast
Background', 'Illuminant Strength', ...
            'Plots'
        }
        StatesCount
    end
    methods
        function Obj = StateNav()
            Obj.FunctionsForward = ...
            {
                @Obj.LoadVideo, @Obj.CropVideo, @Obj.CreateHistogram,
@Obj.FindMaxima, @Obj.FindMaximaBackground, ...
                @Obj.ShowMaximaContrastBackground, @Obj.CorrectMaxima,
@Obj.Binarize, ...
                @Obj.CreateBackgroundMask, @Obj.FilterSpots,
@Obj.CreateSpotsLUT, ...
                @Obj.ShowMeanContrastBackground, @Obj.CreatePlotData
@Obj.ShowPlots
            };
            Obj.FunctionsBack = ...
            {
                @Obj.UnLoadVideo, @Obj.UnCropVideo,
@Obj.UnCreateHistogram, @Obj.UnFindMaxima, @Obj.UnFindMaximaBackground,
...
                @Obj.UnShowMaximaContrastBackground,
@Obj.UnCorrectMaxima, @Obj.UnBinarize, ...
                @Obj.UnCreateBackgroundMask, @Obj.UnFilterSpots,
@Obj.UnCreateSpotsLUT, ...
                @Obj.UnShowMeanContrastBackground,
@Obj.UnCreatePlotData @Obj.UnShowPlots
            };
            Obj.StatesCount = length(Obj.StateNames);
            Obj.CurrentState = State(1, Obj.StateNames{1});
            Obj.CurrentStep.StateStart = Obj.CurrentState;
```

```
            Obj.CurrentStep.StateCurrent = Obj.CurrentState;
            Obj.CurrentStep.StateEnd = Obj.CurrentState;
        end

        function [Video Plot] = Forward(Obj, Video, Plot, States)
            % Set States for Current Step
            Obj.CurrentStep.StateStart = Obj.CurrentState;
            Obj.CurrentStep.StateCurrent = Obj.CurrentStep.StateStart;
            Obj.CurrentStep.StateEnd.Number =
Obj.CurrentStep.StateStart.Number + States;

            % Coerce End State to Be In Range
            if Obj.CurrentStep.StateEnd.Number > Obj.StatesCount;
                Obj.CurrentStep.StateEnd.Number = Obj.StatesCount;
            end
            Obj.CurrentStep.StateEnd.Name =
Obj.StateNames{Obj.CurrentStep.StateEnd.Number};


            % If State Change
            if Obj.CurrentStep.StateStart.Number <
Obj.CurrentStep.StateEnd.Number
                % For Each State Change
                for StateCurrent = Obj.CurrentStep.StateStart.Number :
Obj.CurrentStep.StateEnd.Number - 1
                    Obj.CurrentStep.Function =
Obj.FunctionsForward{StateCurrent};
                    [Video Plot] = Obj.CurrentStep.Function(Video,
Plot);
                    Obj.CurrentStep.StateCurrent.Number = StateCurrent
+ 1;
                    Obj.CurrentStep.StateCurrent.Name =
Obj.StateNames{Obj.CurrentStep.StateCurrent.Number};
                end
                Obj.CurrentState = Obj.CurrentStep.StateEnd;
            end
        end

        function [Video Plot] = Back(Obj, Video, Plot, States)
            % Set States for Current Step
            Obj.CurrentStep.StateStart = Obj.CurrentState;
            Obj.CurrentStep.StateCurrent = Obj.CurrentStep.StateStart;
            Obj.CurrentStep.StateEnd.Number =
Obj.CurrentStep.StateStart.Number - States;

            % Coerce End State to Be In Range
            if Obj.CurrentStep.StateEnd.Number < 1;
                Obj.CurrentStep.StateEnd.Number = 1;
            end
            Obj.CurrentStep.StateEnd.Name =
Obj.StateNames{Obj.CurrentStep.StateEnd.Number};


            % If State Change
```

```
            if Obj.CurrentStep.StateStart.Number >
Obj.CurrentStep.StateEnd.Number
                % For Each State Change
                for StateCurrent = Obj.CurrentStep.StateStart.Number :
-1 : Obj.CurrentStep.StateEnd.Number + 1
                    Obj.CurrentStep.Function =
Obj.FunctionsBack{StateCurrent - 1};
                    [Video Plot] = Obj.CurrentStep.Function(Video,
Plot);
                    Obj.CurrentStep.StateCurrent.Number = StateCurrent
- 1;
                    Obj.CurrentStep.StateCurrent.Name =
Obj.StateNames{Obj.CurrentStep.StateCurrent.Number};
                end

                Obj.CurrentState = Obj.CurrentStep.StateEnd;
            end
        end

        function Change(stateName)

        end

        % 2 Video Loaded -> 1 Start
        function [Video Plot] = UnLoadVideo(Obj, Video, Plot)
            close;
            Video = rmfield(Video, 'Video');
        end

        function ShowOriginalVideo1stFrame(Obj, Video)
            imshow(Video.Video(:, :, 1));
            title('Original Video: 1st Frame');
        end

        function [Video Plot] = LoadVideo(Obj, Video, Plot)
            aviDesc = 'AVI Video Files (*.avi)';
            [file, path, ~] = uigetfile({'*.avi', aviDesc},'Load
Video');
            mmObj = mmreader([path file]);
            avi = read(mmObj);
            Video.Video = squeeze(avi(:, :, 1, :));
            Obj.ShowOriginalVideo1stFrame(Video);
        end

        function [Video Plot] = UnCropVideo(Obj, Video, Plot)
            Video = rmfield(Video, 'VideoCropped');
            Obj.ShowOriginalVideo1stFrame(Video);
        end

        function ShowCroppedVideo(Obj, Video)
            imshow(Video.VideoCropped(:, :, 1));
            title('Cropped Video: 1st Frame');
        end

        function [Video Plot] = CropVideo(Obj, Video, Plot)
```

```
            % Need update for uniform crop
            if Video.Crop.Uniform == true
                Video.Crop.Left = Video.Crop.Top;
                Video.Crop.Right = Video.Crop.Top;
                Video.Crop.Bottom = Video.Crop.Top;
            end
            vidWid = size(Video.Video, 2);
            vidHt = size(Video.Video, 1);
            vidTop = Video.Crop.Top + 1;
            vidBot = vidHt - Video.Crop.Bottom;
            vidRight = vidWid - Video.Crop.Right;
            if Video.Optosplit == false
                vidLeft = Video.Crop.Left + 1;
            else
                vidLeft = vidWid / 2 + Video.Crop.Left + 1;
            end
            Video.VideoCropped = Video.Video(vidTop:vidBot,
vidLeft:vidRight, :);
            Obj.ShowCroppedVideo(Video);
        end

        function [Video Plot] = UnCreateHistogram(Obj, Video, Plot)
            Video = rmfield(Video, 'Histogram');
            Obj.ShowCroppedVideo(Video);
        end

        function ShowHistogram(Obj, Video)
            bar(Video.Histogram);
            axis tight;
            title('Histogram of Frames 1-50');
            xlabel('Intensity');
            ylabel('Number of Pixels');
        end

        function [Video Plot] = CreateHistogram(Obj, Video, Plot)
            Video.Histogram = zeros(256, 50);
            for FrameNum = 1:50
                Video.Histogram(:, FrameNum) =
imhist(Video.VideoCropped(:,:,FrameNum));
            end
            Video.Histogram = sum(Video.Histogram, 2);
            %Video.Histogram = Video.Histogram;
            Obj.ShowHistogram(Video);
        end

        function [Video Plot] = UnFindMaxima(Obj, Video, Plot)
            Video = rmfield(Video, 'Maxima');
            Obj.ShowHistogram(Video);
        end

        function ShowMaxima(Obj, Video)
            imgCur = Video.Maxima ./ max(Video.Maxima(:));
            imshow(imgCur);
            title('Pixel Maxima');
        end
```

```
        function [Video Plot] = FindMaxima(Obj, Video, Plot)
            tempAvg = convn(Video.VideoCropped(:, :, 1:50),
ones(1,1,10)/10, 'valid');
            Video.Maxima = max(tempAvg,[],3);
            Obj.ShowMaxima(Video);
        end

        function [Video Plot] = UnFindMaximaBackground(Obj, Video,
Plot)
            Video = rmfield(Video, 'MaximaBackground');
            Obj.ShowMaxima(Video);
        end

        function ShowMaximaBackground(Obj, Video)
            % background with 0 = black, 255 = white
            imshow(Video.MaximaBackground,[0 255]);
            title('Background from Maxima');
        end

        function [Video Plot] = FindMaximaBackground(Obj, Video, Plot)
            bgs = imopen(imresize(Video.Maxima, 0.1), strel('square',
3));
            filter = imfilter(bgs, fspecial('disk', 5), 'replicate');
            Video.MaximaBackground = imresize(filter,
size(Video.Maxima));
            Obj.ShowMaximaBackground(Video);
        end

        function [Video Plot] = UnShowMaximaContrastBackground(Obj,
Video, Plot)
            Obj.ShowMaximaBackground(Video);
        end

        function [Video Plot] = ShowMaximaContrastBackground(Obj,
Video, Plot)
            % background with minval = black, maxval = white
            imshow(Video.MaximaBackground,[]);
            title('Contrast-Stretched Background from Maxima');
        end

        function [Video Plot] = UnCorrectMaxima(Obj, Video, Plot)
            Video = rmfield(Video, 'MaximaCorrected');
            [Video Plot] = Obj.ShowMaximaContrastBackground(Video,
Plot);
        end

        function ShowCorrectedMaxima(Obj, Video)
            imshow(Video.MaximaCorrected);
            title({'Maxima', '(Background-Subtracted & Rescaled)'});
        end

        function [Video Plot] = CorrectMaxima(Obj, Video, Plot)
            Video.MaximaCorrected = Video.Maxima -
Video.MaximaBackground;
            Video.MaximaCorrected(Video.MaximaCorrected < 0) = 0;
```

```
            Video.MaximaCorrected = Video.MaximaCorrected ./
max(Video.MaximaCorrected(:));
            Obj.ShowCorrectedMaxima(Video);
        end

        function [Video Plot] = UnBinarize(Obj, Video, Plot)
            Video = rmfield(Video, 'SpotsBinarized');
            Obj.ShowCorrectedMaxima(Video);
        end

        function ShowBinarizedSpots(Obj, Video)
            imshow(Video.SpotsBinarized);
            title('Binarized Spots');
        end

        function [Video Plot] = Binarize(Obj, Video, Plot)
            Video.SpotsBinarized = Video.MaximaCorrected >
Video.PixelThreshold;
            Video.SpotsBinarizedLabeled = bwlabel(Video.SpotsBinarized,
8);
            Obj.ShowBinarizedSpots(Video);
        end

        function [Video Plot] = UnCreateBackgroundMask(Obj, Video,
Plot)
            Video = rmfield(Video, 'BackgroundMask');
            Obj.ShowBinarizedSpots(Video);
        end

        function ShowBackgroundMask(Obj, Video)
            imshow(Video.BackgroundMask);
            title('Background Mask');
        end

        function [Video Plot] = CreateBackgroundMask(Obj, Video, Plot)
            Video.BackgroundMask = Video.MaximaCorrected <
Video.PixelThreshold;
            % leave a very large 15 pixel margin around spots
            Video.BackgroundMask = imerode(Video.BackgroundMask,
strel('disk', 15));
            Obj.ShowBackgroundMask(Video);
        end

        function [Video Plot] = UnFilterSpots(Obj, Video, Plot)
            Video = rmfield(Video, 'SpotProperties');
            Obj.ShowBackgroundMask(Video);
        end

        function ShowLabeledSpots(Obj, Video)
            imshow(double(Video.SpotsBinarizedLabeled > 0) +
double(Video.L2 > 0),[]);
            for i = 1 : length(Video.SpotProperties)
                LabelX = Video.SpotProperties(i).Centroid(1);
                LabelY = Video.SpotProperties(i).Centroid(2);
                text(LabelX, LabelY, num2str(i), 'Color', 'cyan');
```

```
            end
            title('Isolated Spots(White) vs Discarded Spots (Gray)');
        end

        function [Video Plot] = FilterSpots(Obj, Video, Plot)
            SpotDistanceMinimum = 7;
            Video.L2 =
uint16(zeros(size(Video.SpotsBinarizedLabeled)));
            for i = 1 : max(Video.SpotsBinarizedLabeled(:))
                SpotsDilated = imdilate(Video.SpotsBinarizedLabeled ==
i, strel('disk', SpotDistanceMinimum));
                if ~any(SpotsDilated & Video.SpotsBinarizedLabeled ~= 0
& Video.SpotsBinarizedLabeled ~= i)
                    Video.L2(Video.SpotsBinarizedLabeled == i) = i;
                end
            end
            Video.SpotProperties = regionprops(Video.L2, 'Centroid',
'Area');
            Video.SpotProperties([Video.SpotProperties.Area] == 0) =
[];
            Obj.ShowLabeledSpots(Video);
        end

        function [Video Plot] = UnCreateSpotsLUT(Obj, Video, Plot)
            Video = rmfield(Video, {'SpotsLUT' 'Mean'
'MeanBackground'});
            Obj.ShowLabeledSpots(Video);
        end

        function ShowMeanBackground(Obj, Video)
            imshow(Video.MeanBackground, [0 255]);
            title('Background from Mean');
        end

        function [Video Plot] = CreateSpotsLUT(Obj, Video, Plot)
            % first prepare lookup table to copy frame pixels to
appropriate spot ...
            ssize = 11;   % insert an odd number here
            Video.SpotsLUT = zeros(ssize, ssize,
length(Video.SpotProperties));
            for i = 1 : length(Video.SpotProperties)
                % create array of ssize rows around the row of each
spot
                r = round(Video.SpotProperties(i).Centroid(2)) - (ssize
- 1) / 2 : round(Video.SpotProperties(i).Centroid(2)) + (ssize-1) / 2;
                c = round(Video.SpotProperties(i).Centroid(1)) - (ssize
- 1) / 2 : round(Video.SpotProperties(i).Centroid(1)) + (ssize-1) / 2;
                r(r < 1) = 1;
                r(r > size(Video.VideoCropped, 1)) =
size(Video.VideoCropped, 1); %avoid rows outside of image
                c(c < 1) = 1;
                c(c > size(Video.VideoCropped, 2)) =
size(Video.VideoCropped, 2); %avoid cols outside of image
                [C R] = meshgrid(c, r);
                Video.SpotsLUT(:,:,i) =
sub2ind(size(Video.VideoCropped), R, C);
```

```
            end

            Video.Mean = mean(Video.VideoCropped, 3);
            bgs = imopen(imresize(Video.Mean, 0.1), strel('square',
3));
            Video.MeanBackground = imresize(imfilter(bgs,
fspecial('disk',5), 'replicate'), size(Video.Mean));
            Obj.ShowMeanBackground(Video);
        end

        function [Video Plot] = UnShowMeanContrastBackground(Obj,
Video, Plot)
            Obj.ShowMeanBackground(Video);
        end

        function [Video Plot] = ShowMeanContrastBackground(Obj, Video,
Plot)
            imshow(Video.MeanBackground, []);
            title('Contrast-Stretched Background from Mean');
        end

        function [Video Plot] = UnCreatePlotData(Obj, Video, Plot)
            Video = rmfield(Video, 'Frames');
            Plot = rmfield(Plot, {'Illuminant', 'Data'});
            [Video Plot] = Obj.ShowMeanContrastBackground(Video, Plot);
        end

        function ShowIlluminantStrength(Obj, Plot)
            plot(Plot.Illuminant);
            axis auto;
            %axis([0 500 -0.1 1.2]);
            legend('Illuminant Strength');
            title('Illuminant Strength vs Time');
            ylabel('Illuminant Strength (1 = Average)');
            xlabel('Time');
        end

        function [Video Plot] = CreatePlotData(Obj, Video, Plot)
            Video.Frames = size(Video.VideoCropped, 3);
            BGIll = mean(Video.MeanBackground(Video.BackgroundMask));
            Plot.Illuminant = zeros(1, Video.Frames);
            SpotsQty = size(Video.SpotsLUT, 3);
            Spots = zeros(11, 11, SpotsQty, Video.Frames, 4);

            for FrameCurrent = 1:Video.Frames
                Frame(:, :, 1) = double(Video.VideoCropped(:, :,
FrameCurrent));
                FrameThis = Frame(:, :, 1);
                Frame(:, :, 2) = FrameThis - Video.MeanBackground;
                FrameIlluminance =
mean(FrameThis(Video.BackgroundMask));
                Plot.Illuminant(FrameCurrent) = FrameIlluminance /
BGIll;
                Frame(:, :, 3) = FrameThis /
Plot.Illuminant(FrameCurrent);
```

```
                Frame(:, :, 4) = Frame(:, :, 3) - Video.MeanBackground;

                for PlotType = 1:4
                    PlotTypeFrame = Frame(:, :, PlotType);
                    Spots(:, :, :, FrameCurrent, PlotType) =
PlotTypeFrame(Video.SpotsLUT);
                end
            end

            SpotsRowSum = sum(Spots, 1);
            clear Spots;
            SpotsColSum = sum(SpotsRowSum, 2);
            clear SpotsRowSum;
            Plot.Data = zeros(SpotsQty, Video.Frames, 8);
            Plot.Data(:, :, 1:4) = squeeze(SpotsColSum);
            clear SpotsColSum;

            % Wiener2
            for PlotType = 1:4
                PlotOrig = Plot.Data(:, :, PlotType);
                [PlotW2 ~] = wiener2(PlotOrig, [1 10]);
                Plot.Data(:, :, PlotType + 4) = PlotW2;
            end

            % Plot graph of computed illuminant strength
            Obj.ShowIlluminantStrength(Plot);
        end

        function [Video Plot] = UnShowPlots(Obj, Video, Plot)
            Obj.ShowIlluminantStrength(Plot);
        end

        function [Video Plot] = ShowPlots(Obj, Video, Plot)
            Plot = UpdatePlots(Plot);
        end

        function [Video Plot] = UnDoSomething(Obj, Video, Plot)

        end

        function ShowSomething(Obj, Video)

        end

        function [Video Plot] = DoSomething(Obj, Video, Plot)

        end

    end
end
```

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