Design and configuration of laser tweezers microscopy for force measurements on single DNA molecules

Carlos Jeziel Gonzalez Banuelos

The University of Texas Rio Grande Valley

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Design and Configuration of Laser Tweezers Microscopy for Force Measurements on Single DNA Molecules

by

Carlos Jeziel Gonzalez Banuelos

A Thesis Presented to the Graduate Faculty of the College of Science, Mathematics and Technology in Partial Fulfillment of the Requirements for the Degree of

Masters of Science

In the Field of Physics

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November 2013
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We have to recognize those who support us on our way. That's why today I finish my graduate studies. I want to express my gratitude to those who I have had close and unconditionally supporting me in different ways. I want to acknowledge and thank Dr. Ahmed Touhami, for all the time and concrete support given to me. Not only since the Masters, but since the first day of class I had with him during my bachelors studies. I appreciate all the suggestions and advice given to me, and everything he has done for me. To Dr. Joe Romano and Dr. Andreas Hanke for their support along all these years during my stay in the university, as well for the time they devoted to accept part of the thesis committee. I will always acknowledge Dr. Boris Ermolinsky, I want to thank him for all the times he was there whenever I needed him, for his patience, and for all the time he spent teaching me in his lab, and even for scolding at me, curiously I learned a lot that way. I learned a lot from him and I will always carry this with me. Much of what we are today is because of our foundations, and these foundations were built by many other professors, professors like Dr. Yingchen Yang who has been a guide in the professional and academic aspect, that's why I also thank him for all this time. In a like manner again to Dr. Natalia Guevara and Dr. Juan Guevara for their time and teachings during class and in their laboratory. In the same way to Mr. Robert Stone who was the first person who taught me physics at this university. I want to thank him for not scaring me during my first physics course. I thank him for being an excellent professor. To Adrienne Zermeno, for always being the support and help of everyone who needed it. In a very special way to all the professors, staff and students from the Physics and Astronomy Department, the Center for Gravitational Waves and Astronomy and the Engineering Department for their teachings and friendship they have given me throughout my time at this university. To the CGWA for its funding and support to carry out my research, without it, none of this would have been possible. Thanks again for all the support. To my Mom and Dad, Thanks for believing in me and supporting my life plan until the last moment. I love you so much; I will continue growing, trying to make you proud of me. To my sisters, I want to say today how important they are to me, and how they have also been part of my inspiration and my improvement as the professional that I am. To my friends, I want to thank, because although like my family, without having worked on my thesis or my research, have helped keep me inspired and focused on my goals. I give thanks to thee, my God, to appear every day of my life as I seek the mysteries of science. I express my gratitude once again to all of you. Consider all of you important in my life, and know that my achievements are achievements shared with you. I send you a hug.
Abstract

In this study, a detailed description of the optical tweezers microscopy technique is presented, as well as the methodologies used to prepare the DNA molecule for mechanical measurements at the nanoscale. The main objective is to initiate and extend the experimental biophysical studies on the DNA-proteins interactions at the University of Texas at Brownsville (UTB). DNA-binding proteins control almost all aspects of cellular function, such as transcription; chromosome maintenance, replication and DNA repair depend on the interaction of proteins with DNA. In view of such an important role played by DNA–protein interactions, various techniques have evolved over the years to elucidate them. Each technique, with its own advantages and drawbacks, serves a very specific purpose. The optical tweezers has evolved as one of the powerful tools for studying the DNA–Protein complexes at a single molecule level. It allows to characterize the mechanisms involved in DNA–protein complex formation in different conditions with high resolution. It quantitatively identifies protein position along DNA molecules, DNA flexibility, curvature and conformational change after protein binding. This thesis describes the design and calibration of the optical tweezers. We measure relative displacements with nanometer accuracy and forces with an accuracy of 10%. The capability of the instrument is demonstrated by stretching a single molecule of DNA because of the elasticity of DNA has previously been well characterized. Every DNA sample used in this study has been engineered biochemically in order to accomplish proper linkage between the biological system and its supports.

Breaking down the main problem leads us to four different aspects, optical tweezers, engineered molecules, coupling molecules/supports system, and the gathering of data. There are a variety of methods used to approach these problems. For the optical tweezers we will be mainly dealing with the calibration of objectives, lasers, stage control, trapping a bead and tracking the bead. Sample preparation involves polymerase chain reaction (PCR), spectrophotometer analysis, DNA electrophoresis, DNA purification process, DNA binding tests, Dot Blot Analysis, measuring of size of particles, zeta potentials, and multimode reader. We are able to confirm visually through the microscope a complete bond system by engaging all results from the experiments.
We consider that our study will open up new and exciting research opportunities at UTB to study biological interactions at the level of single molecules. Also our system will be a very useful equipment to demonstrate to local students the physical principals of optics applied to biological systems.
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-Stranded Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-Stranded Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer Ribonucleic Acid</td>
</tr>
<tr>
<td>P</td>
<td>Persistence Length</td>
</tr>
<tr>
<td>L</td>
<td>Contour Length</td>
</tr>
<tr>
<td>T</td>
<td>Torsional Persistence Length</td>
</tr>
<tr>
<td>S</td>
<td>Elastic Stretch Modulus</td>
</tr>
<tr>
<td>E</td>
<td>Young’s Modulus</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>MT</td>
<td>Magnetic Tweezers</td>
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<tr>
<td>OT</td>
<td>Optical Tweezers</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>N.A.</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>MO</td>
<td>Microscope Objective</td>
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<tr>
<td>TIRF</td>
<td>Total Internal Reflection Fluorescence</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>Neodymium-Doped Yttrium Aluminum Garnet; Nd:Y$_3$Al$<em>5$O$</em>{12}$</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-Coupled Device</td>
</tr>
<tr>
<td>QPD</td>
<td>Quadrant Photo Diode</td>
</tr>
<tr>
<td>PSD</td>
<td>Power Spectral Density</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>NaN3</td>
<td>Sodium Azide</td>
</tr>
<tr>
<td>pH</td>
<td>Power of Hydrogen</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>Sulfo-NHS</td>
<td>N-hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>WLC</td>
<td>Worm-Like Chain</td>
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<tr>
<td>FJC</td>
<td>Freely Jointed Chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>R.M.</td>
<td>Reaction Mixture</td>
</tr>
<tr>
<td>S.T.</td>
<td>Sample Tube</td>
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<tr>
<td>TS#</td>
<td>Test Tube #</td>
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<tr>
<td>R.T.</td>
<td>Room Temperature</td>
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<tr>
<td>3D</td>
<td>Three Dimensional</td>
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<tr>
<td>fc</td>
<td>Corner Frequency</td>
</tr>
<tr>
<td>c</td>
<td>Speed of Light</td>
</tr>
<tr>
<td>rms</td>
<td>Root-Mean-Square</td>
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<tr>
<td>bp</td>
<td>Base Pairs</td>
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<tr>
<td>rad</td>
<td>Radian</td>
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<tr>
<td>nm</td>
<td>Nanometers</td>
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<tr>
<td>pN</td>
<td>pico-Newton</td>
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<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascals</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
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<tr>
<td>ng</td>
<td>Nanograms</td>
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<tr>
<td>o/u</td>
<td>Optical Units</td>
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<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>mol</td>
<td>Moles</td>
</tr>
<tr>
<td>µL</td>
<td>Microliters</td>
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<tr>
<td>µm</td>
<td>Micrometers</td>
</tr>
<tr>
<td>mW</td>
<td>Milliwatts</td>
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<td>mM</td>
<td>Milli Molar</td>
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1. Introduction

The discovery of DNA structure 60 years ago (1953) [1] marked the beginning of a process that has transformed the foundations of biology and medicine, and accelerated the development of new fields, such as molecular biology and genetic engineering. Today, we know much about DNA, its properties, and function. We can determine the structure of short DNA fragments with picometer precision, find majority of the genes encoded in DNA, and we can manipulate, stretch and twist individual DNA molecules [1]. Yet, there are aspects of DNA function that defy our understanding, mostly because the molecule is just one, essential component of a complex cellular machinery [1].

DNA is made up of two polymeric strands composed of monomers that include a nitrogenous base (A-adenine, C-cytosine, G-guanine, and T-thymine), deoxyribose sugar, and a phosphate group. The sugar and phosphate groups, which form the backbone of each strand, are located on the surface of DNA while the bases are on the inside of the structure (see figure 24, 25). Weak hydrogen bonds between complementary bases of each strand (i.e., between A and T and between C and G) give rise to pairing of bases that holds the two strands together. The base pairs (bp) are flat and stack on top of each other like dominoes with centers separated by approximately 0.34 nm. In normal conditions each base pair is rotated relative to its predecessor by approximately 34°, giving rise to the familiar right-handed Watson-Crick double helix [1].

The chemical nature of the backbone gives each strand an orientation- one end is called the 5'-end and the other the 3'-end. In duplex DNA the two strands run antiparallel to each other. A closed DNA (also called a plasmid or ring) is formed when the ends of each strand are joined by a covalent bond.

The DNA of any organism must be folded and packed in a complicated fashion in order to fit inside a cell. This is complicated by the fact that DNA resists bending and twisting deformations and also has a tendency to repel itself electrostatically [2]. In addition to being compacted, portions of DNA must be accessible at various moments during the lifetime of the cell, so that the genes encoded in the DNA can be expressed and proteins produced when necessary. The effort to understand how DNA is packed and unpacked in cells, and how its mechanical properties influence the processes of transcription, replication and recombination, is one of the driving forces behind the development of mathematical models of DNA [2].
1. DNA Mechanics

The discovery of the double-helical structure of DNA (Watson & Crick 1953) emphasized the role of the polymer as a carrier of genetic information encoded by a precise sequence of base pairs (bp). However, the regulation of the expression of this biological information depends strongly on the physicochemical properties of the DNA molecule. Of these the mechanics of bending and twisting are especially important [3].

Four kinds of DNA deformation are considered here: curvature, bending, torsion, and stretching. DNA curvature and DNA bending refer to lateral deviations of the helix axis from a linear trajectory [4, 5]. Curvature refers to nonlinear geometries adopted by double-helical DNA fragments in a specified solvent and ion environment, but in the absence of bound proteins.

Local curvature and bending deformations of DNA are critical to three aspects of DNA function in cells. First, the extreme length of genomic DNA (∼2 m in the case of diploid human DNA) necessitates remarkable but reversible compaction for storage. The detailed basis of DNA compaction in nuclei and viruses is not fully understood but involves DNA wrapping or spooling on protein scaffolds or condensation with other oligocationic substances [6]. Second, deformed or deformable DNA sequences may provide cues allowing the proper positioning of machinery that detects punctuation signals in genomes [7]. Third, DNA deformation is required for the integration of DNA sequence information encoded at remote sites.

1.1. Measures of DNA Stiffness

1.1. a. Persistence Length

DNA stiffness can be described in terms of several measurable parameters [8]. One measure of DNA stiffness is longitudinal persistence length \( P \) (∼150 bp at 25 °C, 0.2 M ionic strength [9, 10]), formally defined as the average projection of the molecular end-to-end distance vector on its initial path vector, in the limit of infinite chain length [9, 11]. Persistence length is thus a measure of the resistance of a polymer to lateral bending. As DNA length decreases below the persistence length, the molecular behavior approaches that of a rigid rod with elastic resilience [11]. This rigid-rod approximation becomes particularly applicable to DNA fragments of ∼\( P/2 \) (∼75 bp). Another useful interpretation of persistence length is the distance over which
the root-mean-square (rms) bend angle in any particular direction is 1 rad (∼60°). The helix axis of an average DNA molecule changes direction by ∼60° over every 150-bp segment. Very long DNA molecules are globally flexible such that the average end-to-end molecular distance approximates (PL)0.5, where L is the molecular contour length.

1. 1. b. Torsional Rigidity

DNA also displays local stiffness in torsion. This property can be expressed in terms of a “torsional” persistence length, that is, the length over which DNA tends to resist twisting about the helix axis. By analogy to longitudinal persistence length, the DNA length required to give an rms twist deviation of 1 rad (∼60°) from the initial reference frame can be defined as the torsional persistence length \( T \), which has a calculated value of ∼180 bp, a value similar to the longitudinal persistence length \( P \). Remarkably, the DNA distance required for two sites to become insensitive to torsional phasing (i.e. to obtain an rms torsional deviation of 180°) is ∼2000 bp. Thus, over shorter distances, strong face-of-the-helix (phasing) effects can be reasonably expected for bound proteins. The combination of lateral and torsional DNA stiffness has profound implications for three-dimensional nucleoprotein structures involving DNA [12].

1. 1. c. Young’s Modulus

Besides resisting longitudinal and torsional deformation, DNA resists stretching beyond the contour length associated with its canonical B form (figure 1). Resistance to such stretching is characterized by an elastic stretch modulus, \( S \), which, when divided by the cross-sectional area of the molecule, gives the familiar Young’s modulus, \( E \). Interestingly, the measured longitudinal persistence length \( P \) was observed to be roughly constant between 10 mM and 600 mM ionic strength, whereas the elastic stretch modulus \( S \) increased dramatically: overstretching DNA was easier at low ionic strength. Interpreting the observed relationship between \( P \) and \( S \) will require additional studies [13].

1. 1. d. Contributions of Interphosphate Repulsions to DNA Stiffness

What accounts for the stiffness of DNA? Surprisingly, the answer to this fundamental question remains unresolved [14]. Various possibilities can be considered. In one view, the
tendency to maximize base stacking is the dominant force resisting DNA deformations such as lateral bending, torsion, and stretching. On the other hand, mutual interphosphate repulsions (and their interactions with the local base-pair dipoles) might cause DNA rigidity by resisting deformed conformations, which crowd phosphate groups. In yet another view, DNA stiffness reflects equilibrium between forces that tend to compress the DNA (such as base pair stacking) and interphosphate repulsions (which tend to stretch it) [14]. Estimating the relative contributions of base stacking and electrostatic repulsions to DNA stiffness and deformation remains an important and active research area.

When DNA is laterally bent, phosphates on the inner face of the bend experience crowding. However, such a deformation simultaneously increases interphosphate distances on the opposite face of the site of bending, such that the costs associated with phosphate crowding on one face and the favorable energy of interphosphate stretching on the opposite face might tend to cancel [15].

Manning theory [16] proposes that the stable double-helix structure of DNA represents equilibrium between stretching forces (caused by interphosphate repulsions) and compressive forces (caused by attractive interactions between nucleotides such as, but not necessarily limited to, stacking forces between base pairs).

The mechanical flexibility of DNA plays a key role in all of its cellular functions, including folding, packaging, regulation, recombination, replication, and transcription. The intimate connections between DNA's molecular interactions and its physical properties are therefore of considerable interest to biologists [17]. Moreover, free molecules of DNA in solution are relatively well behaved, and therefore afford an excellent experimental system for studies of polymer physics [18-21].
Figure 1. Primary and Auxiliary Interactions on a DNA molecule. The primary interactions act perpendicular to the long axis of the molecule as well as between the base pairs along the axial direction of the molecule. They are the covalent bond interactions between the phosphate groups in the phosphate backbone running along the long axis of the molecule; base stacking interactions between the bases in the stack along the axis of the molecule; hydrogen bond interactions between complementary bases perpendicular to the axis of the molecule. There are other interactions that further stabilize it and adjust all the important details of the structure. These auxiliary interactions are composed of the water-DNA interactions surface hydrogen bonding interactions between the polar moieties of the DNA surface and the water molecules as well as the Counterion-mediated electrostatic interactions along the charged phosphate groups along the DNA. The auxiliary interactions are connected with the biological environment of DNA; i.e. an aqueous solution with cationic counterions that ensure the overall electroneutrality of the system.

2. DNA Protein Interactions

Specific protein-DNA interactions are necessary for the proper function of the cellular machinery. Gene regulation depends on transcription factors being able to find particular DNA sequences, and a restriction enzyme must only cut the correct sequence.
These unique features are most apparent when one compares the study of protein-DNA interactions to two other fields where molecular interactions play an important role: protein folding (self-interactions along a polymer chain) and protein-protein binding (interactions between two macromolecules of the same type). In protein fold prediction, the goal is to discriminate the native structure from an enormous number of very different misfolded structures. For protein-protein binding, a model needs to predict not only which proteins interact but also find the correct orientation of the interaction out of the large number of possible misinteracting structures. Because of the linearity and limited alphabet of DNA, protein-DNA interactions are much simpler to study.

The problem of protein-DNA specificity can roughly be framed by dividing the interaction into two energetic components: direct and indirect readout. When a protein is bound to DNA, the DNA is often bent away from the lower energy unbound conformation.

2.1. Classes of DNA-Bending Proteins

In class 1 of DNA-bending proteins (figure 2a), the protein contacts bent DNA on its convex surface, inducing the helical axis to curve away from the bound protein [22]. In contrast, in the Class-2 of DNA-bending proteins (figure 2b), the protein contacts bend DNA on its concave surface, curving the helical axis toward the bound protein. Engaged surfaces of class-2 proteins typically present cationic amino acids to the DNA, suggesting that electrostatic interactions are important for DNA binding by these proteins. Proteins that bend DNA do so because their binding free energy is sufficient to pay the energetic cost of deforming the relatively stiff double helix.

Figure 2. Classes of DNA-bending proteins. Class-1 bending proteins (TBP, red) bind the DNA minor groove, unwind DNA, and induce bending away from the protein-DNA interface by intercalation of hydrophobic-amino-acid side chains between base pairs. Class-2 bending proteins (CAP, red) form complexes in which DNA bends toward the protein-DNA interface.
3. Techniques used to Study DNA mechanics

The elastic behavior of DNA molecule has been investigated in various laboratories using a variety of techniques such as, hydrodynamic drag, magnetic beads, glass needles and optical traps [23]. Magnetic beads attached to the ends of DNA via biotin-Avidin molecules can be pulled by external magnets. These magnetic tweezers are a useful tool, particularly in the range between 0.01 and 10 pN. Slightly higher force regimes can be probed with optical tweezers, which allow one to apply and sense forces on micron-sized dielectric particles, such as glass microspheres, in an aqueous environment [24]. Here we will briefly describe the two main techniques used in this field.

3. 1. Atomic Force Microscopy technique

The atomic force microscopy (AFM) is a nondestructive scanning probe technique in which a tiny tip is used to scan the surface of a sample. AFM probe tips are extremely sharp (typically on the order of tens of nanometers), so AFMs can routinely image extremely small features (few nanometers) on flat surfaces. Another major application of AFM is force spectroscopy in which forces of the order of a few piconewtons can be routinely measured with a vertical distance resolution of better than 0.1 nanometers. For this method, the AFM tip is extended towards and retracted from the surface as the deflection of the tip is monitored as a function of the sample displacement.

AFM based force spectroscopy has emerged as a powerful tool to study the rupture of molecular bonds, ranging from covalent bonds to the unfolding of proteins and nucleic acids [25]. The resolution of this method is about 5 pN, but it can also be used to measure forces in the nanonewton range. This wide range in force measurements has been used to study several regimes of DNA stretching behavior in detail and has also been extended to study DNA–protein and DNA–drug interactions [26].

3. 2. Optical Tweezers

Optical Tweezers have a long history in theoretical and experimental physics. In the 17\textsuperscript{th} century, Johannes Kepler speculated about the radiation pressure of light from the tail of comets, and 150 years later Maxwell predicted the pressure from electromagnetic radiation. In the early
20th century, Ledebew, Nichols and Hull performed the first experiments observing this pressure [27]. But not until 1969 did Arthur Ashkin realize that this pressure, though a small force, could be useful to trap small particles. He trapped dielectric spheres with radiation and gravitational forces [28], and in 1986, he trapped a particle with gradient force of a single laser beam [29]. By selecting a wavelength in the near-infrared region of 800–1,100 nm, where light is poorly absorbed by most living matter, optical tweezers can be used to grasp, capture and manipulate micrometer-scale objects non-invasively and with exquisite precision. The loads exerted by optical traps fall conveniently into the piconewton range, and are therefore perfectly suited to studying the forces between and within biomolecules. Combined with light sensors that can monitor the displacements of trapped objects accurately down to the sub-nanometer level, optical tweezers have revolutionized the nascent field of single-molecule biophysics, making it now possible to study the processes of life at the level of individual molecules.

Laser tweezers have been applied to a wide variety of biological problems, for example, measurements of the forces generated during DNA transcription, the properties of neuronal membranes, and the forces generated by the molecular motors dynein, kinesin and myosin [30, 31].

The field of optical trapping continues to grow tremendously. As the technique becomes increasingly accessible, more and more biophysicists are using it to tackle fundamental questions in the life sciences down to the single-molecule level. In this thesis we will describe in details the construction and the manipulation of the first optical tweezers on our campus.

4. Thesis Objective

The goal of this research is to study the thermodynamics and dynamics of conformational changes in double-stranded DNA (dsDNA) by single molecule stretching experiments using optical tweezers microscopy. Individual dsDNA molecules are attached to a chemically functionalized coverslip and the force applied to the DNA, as a function of the extension, is measured via the motion of a trapped microsized bead. Optical tweezers technique is introduced, as well as the methodology used to set up proper linkage between biological molecules and their supports. Every DNA sample used has been engineered biochemically in order to accomplish proper linkage between the biological molecules and their supporting surfaces. The main objective is to initiate and extend the experimental biophysical studies on the DNA-proteins interactions and other biomolecules at UTB.
For controlling the DNA molecule, it requires to modify both ends of its structure. One of the ends of the molecule is fixed to a modified coverslip, while the other end is attached to a modified polystyrene bead being trapped (controlled) by the optical tweezers. The PCR technique was used to amplify the DNA molecule. Primers or oligonucleotides with biotin and amino groups are used to modify the DNA structure during the PCR process (detailed procedure is described in the materials and methods section). The 5’ end of the DNA molecule is then biotinylated while the 3’ end carries an amino group. The biotinylated dsDNA molecule binds to a streptavidin-coated glass coverslip, and the other end with an amino group binds to a coated bead with carboxyl groups. This thesis is thus composed of two sections: the design and calibration of the optical tweezers and the calibration of the elasticity of the DNA by stretching single molecules of DNA.
2. Materials and Methods

1. Optical Tweezers

1.1. Physical Principles of the Optical Tweezers

An Optical Tweezers is capable of manipulating really small particles in a range of nanometers to micrometers through a highly focused laser beam. A laser beam is focused by a high-quality microscope objective to a spot in the specimen plane. This spot creates an "optical trap" which is able to hold a small particle at its center [32] (Figure 3). The narrowest point of the focused beam, known as the beam waist, contains a very strong electric field gradient. [33] The forces felt by this particle consist of the light scattering and gradient forces due to the interaction of the particle with the light. Recently, optical tweezers are built by modifying standard optical microscopes. [33]. The forces that such an instrument is capable of measuring are of the order of 1 to 200 piconewtons (pN).

Figure 3: Optical Tweezers Trapping Principle [32]. \( F_{\text{Gradient}} \) is the force generated from opposing the refracted photons through the bead, while \( F_{\text{scattering}} \) is the force from opposing the reflected photons.
In order to understand better the principle of how to trap molecules/particles we need to go to the lowest level of the light constituents, photons. Let's imagine a flashlight being turned on emitting light. A photon with momentum “p” is directed straight to a flat mirror. The photon will be reflected having a final momentum of negative “p". The change in momentum is then “Δp” = \( p_f - p_i = p - (-p) = 2p \). While the photon was reflected on the flat mirror a force was exerted on the mirror as well, we called this force the radiation pressure force \( F_R \) (figure 4). You could say then that somehow you were pushing on the mirror with a small force due to that photon. Having that on mind in order to try to move something bigger or heavier we just need a powerful source of photons in order to get a stronger radiation pressure force. Our solution for a powerful source of photons is in using a Laser light.

![Figure 4. Scheme of how in theory we could balance objects and make them levitate using photons.](image)

In the previous figure, (figure 4), we used a laser reflected on a inclined mirror then the laser hits a flat mirror which makes the laser being reflected generating a radiation pressure force on it. Assume the flat mirror is in the air and that the radiation pressure force is balanced with the force of gravity then we will accomplish a balanced system having the flat mirror levitated in the air. Let’s assume as well that the flat mirror is levitating, we will still have a big problem. The only reason the mirror will be stable is because the photons from the laser are supposed to hit right on the center to balance the mirror, but they are not. The laser photons are not stable and they won’t hit right on the center. This is because there are fluctuations. Eventually the flat mirror will fall down. Theory is right, but geometry not. The only reason it will not work is because of the geometry used here. The best geometry we could use here is a sphere. This is why we will use polystyrene beads (spheres) in order to accomplish our objective of trapping small particles with the laser. The next figure, (figure 5), will illustrate better the geometry of how the forces will get balanced.
The laser hitting the polystyrene bead has two options, it gets reflected or refracted. Let's consider each case. When the laser gets reflected it will create a scatter force on the opposite side of the sphere due to the radiation pressure ($F_{\text{Scattering}}$). If the laser is refracted it will be refracted twice, first when going into the bead and second when it comes out of the bead. This will generate a gradient force due to the refraction ($F_{\text{Gradient}}$). We consider the cosine component from the gradient force ($F_G \cos \theta_i$) in order to balance the forces acting on the bead.
Radiation pressure is a force per unit area on an object due to the change in momentum of light. All light consists of photons that each has momentum $p$. For light of specific wavelength $\lambda$, the magnitude of the momentum of a single photon is given by:

$$|p| = \frac{h}{\lambda}$$

The intensity of the light is determined by the number of photons passing through a given area per unit time. The momentum flux of photons from light of intensity given by the pointing vector $S$ is:

$$d\left(\frac{dp}{dt}\right) = \frac{n}{c} S dA$$

Where $n$ is the index of refraction, $c$ is the speed of light, $p$ is the total momentum of the photons, and $dA$ is an element of area normal to $S$. Thus, in principle one can directly calculate the force on a given area due to the light momentum flux on that area [34].

Here we trace only two rays of light, denoted rays 1 and 2, which are focused by a lens (or series of lenses that make up a microscope objective). Each ray is refracted at the surface of the bead so that its direction of propagation changes according to Snell’s law, which states that $n_1 \sin \theta_1 = n_2 \sin \theta_2$, where $n_1$ is the index of refraction of the medium surrounding the sphere (usually water) and $n_2$ is the index of refraction of the sphere. Here $\theta_1$ is the angle of incidence of the ray with respect to a line perpendicular to the surface of the sphere and $\theta_2$ is the angle with respect to the same line at which the ray propagates within the sphere. Snell’s law indicates that the change in direction of the light at the interface of the bead with its surroundings depends strongly on the index of refraction of each medium. Since these experiments are usually done in water, $n_1$ is 1.33, while $n_2$ is 1.55 if the sphere is made of polystyrene [34].

Figure 6. Schematic diagram showing the force on a dielectric sphere due to refraction of two rays of light, 1 and 2. The resultant force on the bead due to refraction is towards the focus.
1.2. Experimental Details of the Optical Tweezers

1.2.1. List of Equipment:

Our optical setup is designed to bring a 1070-nm continuous-wave laser beam to a diffraction-limited spot near the focus of a microscope objective (Nikon 100× oil-immersion plan Apochromatic with NA=1.49). The main parts of our optical tweezers are as follow:

- Inverted Microscope Nikon Eclipse Ti
- Computer/NIS Elements-D Software
- iXon Andor Camera
- E3500 Multiple Spot Optical Tweezers System Optics
- Mad City Labs Nanodrive stage-control
- Tracking Laser OZ-1000 50 mW MAX (λ = 600 – 700 nm)
- Microscope light Nikon TI-PS100W output 12V 8.4 Amps
- Trapping Laser IPG Laser 6W max (λ = 1060 – 1100 nm)
- E4100 QPD Force Measurement
- 100x Plan Apo TIRF 1.49 N.A. Oil Immersion Nikon M.O.
Figure 9. Complete Setup of our Optical Tweezers and its components.

1. Inverted Microscope Nikon Eclipse Ti
2. Computer/NIS Elements-D Software
3. iXon Andor Camera
4. E3500 Multiple Spot OT System Optics
5. Mad City Labs Nanodrive stage-control
6. Tracking Laser OZ-1000
7. Microscope light Nikon
8. Trapping Laser IPG Laser
9. E4100 QPD Force Measurement
10. 100x Microscope Objective

Figure 10. Our Optical Tweezers and its components.
It all starts with turning on the computer and microscope light, adjusting the light coming through the microscope condenser. An enlarged-extension sample-holder is being used to get a closer look at the sample from the 100x objective. The sample used is being placed between a glass slide and a coverslip (thickness #1, 0.13-0.16mm). The sample-holder is used inverted so we may have the sample with the coverslip facing directly the microscope objective. The correct cover glass thickness is important to achieve the best resolution with a given objective. Generally speaking, the higher the numeric aperture of the objective, the more serious the loss in resolution if the wrong cover glass thickness is used.

The optimum cover glass thickness of many objectives is 0.17mm. Now, why is it that the most commonly available cover glasses are of category 1 (0.13-0.16mm), which is thinner than the calculated optimum? The answer is a bit more complex: The thickness of the cover glass is not the only parameter that is important. The specimen is embedded in mounting medium. The thickness of this medium must be added to the thickness of the cover glass in order to reach the optimum thickness. In between the coverslip and the objective we add half a drop of immerse oil in order to produce the suitable refractive index to achieve an accurate resolution. We used the iXon camera to look visually through the computer at our molecule.

The iXon Ultra platform takes the popular back-illuminated 512 x 512 frame transfer sensor and overclocks readout to 17 MHz, pushing speed performance to an outstanding 56 fps (full frame), whilst maintaining quantitative stability throughout. The iXon Camera offers a thermoelectric cooling down system that goes to -100°C and has the industry-lowest clock induced charge noise. Additional unique features of the iXon Ultra include USB connectivity and direct raw data access for on the fly processing. All these allowed us to see live the current status of our specimen in many different situations, low light, different mediums, etc.

1.2.2. Optical Setup

The optics from our optical tweezers is pretty much as simple as this diagram (figure 11). Our lenses and beam splitters are enclosed in an optical cage that connects the laser to the sample plane of the microscope. The most basic optical tweezers setup will likely include the following components: a laser (usually Nd:YAG), a beam expander, some optics used to steer the beam location in the sample plane, a microscope objective and condenser to create the trap in the sample plane, a position detector quadrant photodiode to measure beam displacements and a microscope illumination source coupled to a CCD camera.
An Nd:YAG laser (1070 nm wavelength) is a common choice of laser for working with biological specimens. This is because such specimens (being mostly water) have a low absorption coefficient at this wavelength [35]. A low absorption is advisable so as to minimize damage to the biological material, sometimes referred to as opticutio. Perhaps the most important consideration in optical tweezers design is the choice of the objective [36].

1.2.3. Calibration of Objectives

The idea is to be able to match a certain length in our plane of view with a known measurement. We will measure through the software accurately reliable information from the samples to calibrate each objective used. Calibration of the different objectives is imperative in order to accomplish this. The idea behind the calibration is like resetting all given parameters of length controls and provide new ones. We choose as our control parameter a polystyrene bead with known diameter of 2μm. We check which objective is currently being used and measure the length of our particle through the software to verify if it matches the right length. If not, we still know the right length, but we need to change those fixed length values. We add a new calibration in the software; by clicking in “quick calibration” we are able to draw a line of certain length and
give it a value (like 3μm for our beads). In order to create a new and permanent button with a predetermined calibration we click on calibration, new, create your button and give right click to rename it and edit the length parameters. A total of six different calibration buttons were created in order to have all the three objectives (20X, 40X, and 100X), ready to use at any time having an accurate measure on any experiment performed.

The diagram to modify, edit and correct calibrations is shown below:

1. 2.4. Nanopositioning Stage

The Nano-LP, piezo-controlled nano-precision stage, was purchased from Mad City Labs, Madison, WI (figure 12). The Nano-LP is ultra-low profile, three axis nanopositioning system with 100 micron range of motion in all three axes (x, y, and z). The low height of the Nano-LP allows it to be easily integrated into existing inverted optical microscopes, LabView programs to control stage movement are integrated in the optical tweezers software. Once the desired bead is trapped the stage is used via the software to move precisely the bead with nanometer accuracy in the desired direction.
1.2.5. Trapping a single polystyrene bead

A sample is prepared by squeezing a small drop of a dilute concentration of 2 μm polystyrene beads between a cover slip and a glass slide. A typical sample concentration is made by diluting a concentrated beads solution (1% solid) to 1:1000 dilution using a phosphate buffer solution. Too many particles in the sample can lead to undesirable effects when aligning and calibrating the single-beam optical trap, for example, simultaneous trapping of multiple particles or difficulty in moving a single trapped particle within the sample chamber often occurred. Then the sample is mounted on the nanopositioning stage on the inverted optical microscope.

At the focal plane, set the power of the trapping laser approximately between 10 and 100 mW (higher power is not required for experimental purposes and might damage the lenses). Then adjust the focus such that the trap center is approximately 5–10 μm from the bottom surface of the chamber (on the z axis); you may still be able to observe defocused Airy disks, which you can use to locate the lateral position of the single-beam optical trap. Move the stage until a polystyrene sphere is in the center of the field of view. The polystyrene sphere will be influenced by the presence of the laser; the response may be characterized by the following observations:

(i) The polystyrene sphere will ‘hop’ toward the beam and become localized at the beam focus (Brownian fluctuations will be suppressed). This indicates that the laser is successfully trapping.

(ii) The sphere is trapped in the beam but appears out of focus. This indicates that the trapping (tweezing) plane and the imaging plane are not coincident.

(iii) Polystyrene spheres are pushed out of the focus toward the top surface of the chamber.

Figure 13. Trapped bead having the tracking laser on top while surrounded by other beads moving with Brownian motion.
A simple way to test the quality of the trap before using the QPD force detection system is the Q values test. In this test, the sample is moved at a different constant velocity using a motorized stage. In theory this creates a Stokes drag force (laminar flow) on the trapped 2 μm polystyrene microsphere. At a certain velocity, the trapped particle escapes from the trap. At this instance, the drag force just surpasses the applied optical force. That velocity is also known as the escape velocity. The applied drag creates an external force $F_{\text{drag}}$ that is approximately equal to the applied optical force $F_{\text{escape}}$ upon reaching escape velocity. The Q values provide an estimate of the quality of the trap.

1.2.6. The Tracking Laser

The OZ-1000 50mW pigtail laser ($\lambda=635$nm) is a red laser, which you will align on top of your trapped molecule/particle. The mirror knobs (x, y) from the optics cage (figures 13, 14) are used to move the laser spot to any place on the specimen plane. Nonetheless there is another pair of mirror knobs in the optic cage designed to move the trapped laser along with the trapped molecule/particle. The tracking laser will remain fixed on top of our molecule/particle on the specimen plane and the variations from its positions will be recorded on the photodetector.

Figure 14. The tracking laser is focusing on top of the trapped bead ready to start recording information through the QPD. The yellow lines show the distance being measured by the software through the calibrated objective.
1.2.7. Quadrant Photodiode (QPD)

The QPD consists of 4 photodiodes in a quadrant formation to allow X and Y position calculation. Within a certain range of light intensities, the output voltage of a photodiode scales linearly with the intensity of light incident upon the diode. The light incident upon each quadrant in the QPD generates a voltage. The analog circuitry then outputs a voltage $V_x$ and $V_y$ which are proportional to the actual X and Y position of the incident beam. As the light scatters in a predictable way off of the spherical beads, this information can be used to recover actual bead position within a narrow range around the center of the trap.

The QPD software supplied with Elliot Scientific Force Measurement Optical Tweezers Systems offers control over system settings, data acquisition, display and export of the captured data. The screen is divided into a number of sections that control the various features of the program as described.

![Figure 15. E4100 QPD](image)

![Figure 16. Schematic of the Quadrant Photo Diode position detection system. The signal from the diodes are amplified by low-noise preamplifiers and then networked to calculate the X and Y position of the incident light beam [37].](image)
Top of the screen is the Data acquisition controls and setting section. This is divided into 3 control panels as follows:

i) Sample Setting

The left hand panel contains two fields with clickable buttons to increase or decrease the number of Samples per Channel and the Sampling Rate in Hertz. These parameters set the amount of Sampling Time, Samples per Channel divided by Sampling Rate shown in seconds in an adjacent display, which the QPD will use to collect data.

ii) Input Voltage

The center panel also contains two fields with clickable buttons to increase or decrease the default Minimum Value and the default Maximum Value of the input voltage range that the QPD is able to read.

iii) Capture Controls

The right hand panel features two clickable buttons adjacent to corresponding colored indicators.

- **Continuous Capture** is the default. In this case both buttons will be unlit, displaying their ‘off” status, and the upper indicator will be green.

- **Single capture** is set by clicking on the upper button after the program is paused. It will illuminate and set the program to record a single data value.

- **Multiple capture** is set by clicking on the lower button. It will illuminate and set the program to record a multiple data values, but only after reloading the program, making sure it is in Pause mode and then enabling multiple capture.
A useful reminder of what each color indicates is shown above the Capture Controls panel and in the table below.

<table>
<thead>
<tr>
<th>Color</th>
<th>Legend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>Continuous Capture</td>
</tr>
<tr>
<td>Yellow</td>
<td>Paused</td>
</tr>
<tr>
<td>Red</td>
<td>Stopped</td>
</tr>
<tr>
<td>Dark Red</td>
<td>Undo Stop</td>
</tr>
</tbody>
</table>

1.2.9. Export Setting Control Panel

The program saves captured data to disk in a variety of formats dependent on the choice of capture mode and whether the data is processed or not.

Single capture mode allows for a choice of up to five unprocessed datasets to be saved to the default data directory or a user selected directory. These are: Histogram, Raw Power Spec, Raw Time Series Data, Var(iance) and Pos(ition). Ticking an adjacent checkbox before clicking on the Save Single Data button can choose one or more datasets.

If Capture Multiple Data has been ticked in Export Multiple Data Set, then the program will automatically save all unprocessed datasets to the chosen data directory when the multiple capture routine has completed. Entering a value into Number of Data sets the number of iterations and the delay between each capture period is set in seconds by the value entered into Delay Time.
1.2.10. Trap Stiffness

To determine the direct trap stiffness data, data for Viscosity of Medium, Height Above Coverslip and Diameter of Particle are required. Viscosity is entered as Pascal seconds (Pa.s), particle height above the coverslip and particle diameter are entered in microns (μm).

- **Stiffness Measurements** results are presented in the Stiffness Measurements panel via six tabbed displays (right).

  Three tabs, X, Y and Z respectively, show the Histogram and Raw Power Spectrum data. The latter is the unprocessed data.

  If data processing has been activated, then the **Fitting Power Spectrum** tab (below right) will show three graphs of the nonlinear fit of the processed power spectrum. The roll off frequency is displayed to the right for each axis.
• **Calculation of Trap Stiffness/Optical Potential** tab again shows three graphs (below right), one for each axis. These detail the optical potential taken from the trap position histogram and show the mean trap energy level and the calculated trap stiffness values for X, Y and Z.

The power spectrum for Brownian motion in a harmonic potential is Lorentzian, with a corner frequency \( f_c = \frac{\kappa_x}{2\pi\beta} \) where \( \kappa_x \) is the trap stiffness in the x direction and \( \beta \) is the viscous drag coefficient. Very near the coverglass, this frequency becomes exceedingly sensitive to the proximity of the surface to the bead, because of the strong dependence of drag on height, \( h \) [38]. For example, drag doubles at \( h/r \approx 1.2 \), where \( r \) is the bead radius: the bead is therefore about a radius away from the surface when the corner frequency drops by a factor of \( \sim 2 \). This method was used to set the trap center to one radius over the specimen plane, with an accuracy of ±50 nm for a 0.52-μm-diameter bead.

**Trap Height determination**

Once we focused at the bead we are trapping, we used the fine knob to focus at the bottom of the coverslip, very close to its surface. From there we used the nanopositioning stage control from Labview, which allowed us to move in the X, Y, and Z directions with nm precision. We move in the Z direction one nm at the time in order to get back at the focus where our bead will be moving with Brownian motion. The change in distance from the nanopositioning stage control software (Labview) will determine the height at which our bead is being trapped.

Three complementary methods of trap stiffness calibration have been described [38]:

1. Power spectral estimation. This involves determining the corner frequency of the Brownian motion of the bead, \( f_c \), which depends only on the ratio of the stiffness to the viscous damping.

2. Equipartition theorem. The stiffness is obtained directly from \( \frac{1}{2} \kappa_x \langle x^2_{\text{bead}} \rangle = \frac{1}{2} k_B T \), where \( k_B T \) is Boltzmann's constant times the absolute temperature. This involves measurement of the mean-square displacement of the thermal motion of a trapped bead, \( \langle x^2_{\text{bead}} \rangle \), using a position-calibrated detector with sufficiently high bandwidth and low noise.
3. Periodic (e.g., sine wave or triangle wave) oscillation.

Determination of the F-x relationship requires measurements of both force and extension in the direction of the applied stretch, which lies at a variable angle $\theta$ in the x-z plane and changes during the course of an experiment (figure 17). These parameters were derived through their geometrical relationship to the interferometer signals in a series of four steps, as follows:

1. Determine $z_{\text{bead}}$ using:

$$z_{\text{bead}} = \frac{z_{\text{trap}}}{\kappa_z} \left( \frac{x_{\text{biot}} - x_{\text{bead}}}{x_{\text{bead}}} \right) + 1$$

where $x_x$ and $x_z$ are trap stiffness in the x and z directions, respectively. $x_x/x_z$ was taken as 5.9, based on the asymmetry of the light distribution near the focus [39]. $x_{\text{bead}}$ was derived from the interferometer signal at the trap height, according to $x_{\text{bead}} = V_d/s (z_{\text{trap}}, 0)$. $z_{\text{trap}}$ was set before the stretch, and $x_{\text{biot}}$ was measured during the course of an experiment.

2. Use $z_{\text{bead}}$ to obtained a revised estimate of $x_{\text{bead}}$, according to:

$$x_{\text{bead}} = V_d/s (h, z_{\text{bead}})$$

3. Compute the DNA extension, $L_{\text{DNA}}$, from geometry using:

$$L_{\text{DNA}} = \frac{z_{\text{trap}} - z_{\text{bead}}}{\sin \left[ \tan^{-1} \left( \frac{z_{\text{trap}} - z_{\text{bead}}}{x_{\text{biot}} - x_{\text{bead}}} \right) \right] - r}$$

4. Compute total force exerted by the bead on the DNA from:

$$F = \frac{k_x (z_{\text{trap}}, 0) x_{\text{bead}}}{\cos \left[ \tan^{-1} \left( \frac{z_{\text{trap}} - z_{\text{bead}}}{x_{\text{biot}} - x_{\text{bead}}} \right) \right]}$$
Figure 17. Experimental geometry. One end of the DNA is anchored to the coverglass by streptavidin-biotin conjugation. The other end is attached to a bead held in the optical trap, making an angle $\theta$ with the coverglass. Forces on the bead cause it to be displaced from equilibrium: note that the bead position (small black dot) does not coincide, in general, with the position of the trap center (small blue dot) or the laser beam waist (horizontal arrows). Various distances, defined in the text, are indicated.

Figure 18. (Left) Mechanical analog of motion of $x_{\text{DNA}}$ is not coupled 1:1 to $x_{\text{bd}}$ because of the compliance of the DNA ($k_{\text{DNA}}$) and the trap ($k_{\text{trap}}$).

Figure 19. (Right) Elasticity measurement of the 2051-nm long dsDNA tether (green) stretched up to 12 pN is well described by a wormlike chain model [40-42] (black). The elasticity of single-stranded DNA (ssDNA, purple) is significantly different than dsDNA. When held at constant force, this difference in extension can be used as the basis of a biophysical measurement, which monitors the fraction of ssDNA in real time to deduce enzymatic motion [43].
• **Results**

Additional graphs (below right) show the trap position as 2D (X and Y) or 3D traces. Three more graphs display the trap position relative to time and a further two graphs chart the variance of the trap position in X and Y – which is useful for making sure that only one particle remains in the trap.

Other plots (far right) show the amplitude of voltage signal on the QPD in a schematic format relative to the acquisition time. For best result, ensure X and Y are set to zero and that Z is the highest possible value.
Switch on the QPD control box and QPD software. We use our own Labview software to capture the position \((x, y \text{ and } z)\) time series of the trapped polystyrene fluorescent microsphere (see figure 20) and analyze the power spectrum to obtain the roll-off frequency or corner frequency, \(f_c\), that is, the frequency response of the particle motion (see figure 21).

![Red fluorescence signal from optical trap.](image)

**Figure 20 (Left).** Red fluorescence signal from optical trap.

**Figure 21 (Right).** Power spectrum for 1 \(\mu\text{m}\) polystyrene fluorescent sphere trapped at 5 \(\mu\text{m}\) away from the coverslip at an applied power of 15 mW.

![Power spectrum for 1 \(\mu\text{m}\) polystyrene fluorescent sphere trapped at 5 \(\mu\text{m}\) away from the coverslip at an applied power of 15 mW.](image)

**Figure 22 (Left).** Linear dependence of trap stiffness determined from the roll-off frequency with optical power of the trap. The lateral trap stiffness varies with the applied optical power in a linear fashion (1 \(\mu\text{m}\) fluorescent polystyrene sphere trapped at an axial depth of 5 \(\mu\text{m}\) away from the coverslip).

![Linear dependence of trap stiffness determined from the roll-off frequency with optical power of the trap.](image)

**Figure 23 (Right).** We show the typical position histogram of a trapped particle with trapping (tweezing) power of 15 mW at 5 \(\mu\text{m}\) above the coverslip.
2. Preparation of DNA Sample

2.1. Basic Information and History of DNA Molecule

The nucleic acids are polymeric macromolecules, or large biological molecules, essential for all known forms of life. Nucleic acids, which include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), are made from nucleotide monomers. Together with proteins, nucleic acids are the most important biological macromolecules; each is found in abundance in all living things, where they function in encoding, transmitting and expressing genetic information—in other words, information is conveyed through the nucleic acid sequence, or the order of nucleotides within a DNA or RNA molecule [44].

Deoxyribonucleic acid (DNA) is a molecule that encodes the genetic instructions used in the development and functioning of all known living organisms and many viruses. DNA is a nucleic acid; alongside proteins and carbohydrates, nucleic acids compose the three major macromolecules essential for all known forms of life. Most DNA molecules are double-stranded helices, consisting of two long biopolymers made of simpler units called nucleotides—each nucleotide is composed of a nucleobase (guanine, adenine, thymine, and cytosine), recorded using the letters G, A, T, and C, as well as a backbone made of alternating sugars (deoxyribose) and phosphate groups (related to phosphoric acid), with the nucleobases (G, A, T, C) attached to the sugars.

DNA is well suited for biological information storage, since the DNA backbone is resistant to cleavage and the double-stranded structure provides the molecule with a built-in duplicate of the encoded information.
The two strands of DNA run in opposite directions to each other and are therefore antiparallel, one backbone being 3' (three prime) and the other 5' (five prime). This refers to the direction the 3rd and 5th carbon on the sugar molecule is facing. Attached to each sugar is one of four types of molecules called nucleobases (informally, bases). It is the sequence of these four nucleobases along the backbone that encodes genetic information. This information is read using the genetic code, which specifies the sequence of the amino acids within proteins. The code is read by copying stretches of DNA into the related nucleic acid RNA in a process called transcription.

Within cells, DNA is organized into long structures called chromosomes. During cell division these chromosomes are duplicated in the process of DNA replication, providing each cell its own complete set of chromosomes. Eukaryotic organisms (animals, plants, fungi, and protists) store most of their DNA inside the cell nucleus and some of their DNA in organelles, such as mitochondria or chloroplasts [45].

DNA is a long polymer made from repeating units called nucleotides [46-48]. DNA was first identified and isolated by Friedrich Miescher and James Watson and Francis Crick first discovered the double helix structure of DNA. The structure of DNA of all species comprises two helical chains each coiled round the same axis, and each with a pitch of 34 angstroms (3.4 nanometers) and a radius of 10 angstroms (1.0 nanometers) [49].

2.1.1. Base Paring

In a DNA double helix, each type of nucleobase on one strand bonds with just one type of nucleobase on the other strand. This is called complementary base pairing. Here, purines form hydrogen bonds to pyrimidines, with adenine bonding only to thymine in two hydrogen bonds, and cytosine bonding only to guanine in three hydrogen bonds. This arrangement of two nucleotides binding together across the double helix is called a base pair. As hydrogen bonds are not covalent, they can be broken and rejoined relatively easily.

Figure 26. Nitrogenous bases bonding with their complementary bases A-T, C-G.
The two strands of DNA in a double helix can therefore be pulled apart like a zipper, either by a mechanical force or high temperature [50]. As a result of this complementarity, all the information in the double-stranded sequence of a DNA helix is duplicated on each strand, which is vital in DNA replication. Indeed, this reversible and specific interaction between complementary base pairs is critical for all the functions of DNA in living organisms [47].

Genetic Code

The genetic code is the set of rules by which information encoded within genetic material (DNA or mRNA sequences) is translated into proteins by living cells. Biological decoding is accomplished by the ribosome, which links amino acids in an order specified by mRNA, using transfer RNA (tRNA) molecules to carry amino acids and to read the mRNA three nucleotides at a time. The genetic code is highly similar among all organisms and can be expressed in a simple table with 64 entries.

The code defines how sequences of these nucleotide triplets, called codons, specify which amino acid will be added next during protein synthesis. With some exceptions [51], a three-nucleotide codon in a nucleic acid sequence specifies a single amino acid. Because the vast majority of genes are encoded with exactly the same code (see the RNA codon table), this particular code is often referred to as the canonical or standard genetic code, or simply the genetic code, though in fact some variant codes have evolved.
A DNA Cholera template of approximately 420 bp is used.

Primers (Oligonucleotides):
VspDF (Forward): /5BiosG/ATCGGTCGACCTTCCCGCTTTTGATG: ➔ Biotin
VspDR (Reverse): /5AmMC12/ATCGGTCGACCTTCCCGCTTTTGATG: ➔ Amino Group

We used a 420 bp DNA cholera template because we know how well it will behave under PCR conditions (ID: AM_06_21_13_10uM).

2.2. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary Mullis [53], PCR is now a common and often-indispensable technique used in medical and biological research labs for a variety of applications [54, 55]. These include DNA cloning for sequencing. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short
DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium Thermus Aquaticus. This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis.
3. Results and Discussions

1. Design of a dsDNA to be measured and used by Optical Tweezers

![Diagram showing PCR scheme from DNA template to dsDNA.]

Figure 29. PCR scheme from DNA template to dsDNA.

The DNA cholera template was analyzed with the spectrophotometer NanoDrop 2000 and NanoDrop 1000. The NanoDrop is a microvolume spectrophotometer for measuring DNA, RNA, and protein. Using the patented sample retention system the NanoDrop accurately measures samples as small as 0.5 µL, and reports sample concentration, purity ratios, and full spectral data.

![Spectrophotometer plot for purity ratios of our cholera template.]

Figure 30. Spectrophotometer plot for purity ratios of our cholera template.
260/280

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

260/230

This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants, which absorb at 230 nm.

- **PCR Experiment**

<table>
<thead>
<tr>
<th>Component</th>
<th>μl Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers</td>
<td></td>
</tr>
<tr>
<td>10 μM VspDR (NH2)</td>
<td>20 μl</td>
</tr>
<tr>
<td>10 μM VspDF(Biotin)</td>
<td>20 μl</td>
</tr>
<tr>
<td>Master Mixture</td>
<td>100 μl</td>
</tr>
<tr>
<td>(Taq 2X MeanGreen Master Mix)</td>
<td></td>
</tr>
<tr>
<td>Water H2O (Milli-Q 18.2 MΩ)</td>
<td>59 μl</td>
</tr>
<tr>
<td>DNA Cholera Template (06.21.13)</td>
<td>1.5 μl</td>
</tr>
</tbody>
</table>

Table 2. PCR Experiment elements and volumes.
• Procedure

**Initialization step:** This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

**Denaturation step:** This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

**Annealing step:** The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

**Extension/elongation step:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand.

**Final elongation:** This single step is occasionally performed at a temperature of 70–74 °C for 5 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Figure 31. Scheme from PCR process.
Equipment: Eppendorf II

The 200 μl from our final solution is divided in four different PCR tubes, each one with 50 μl.

\[ T_m = 2^\circ (A-T) + 4^\circ (G-C) \] = Melting Temperature

<table>
<thead>
<tr>
<th>STEP</th>
<th>TEMP</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94 °C</td>
<td>5 min</td>
</tr>
<tr>
<td><strong>30 Cycles:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>60 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>1 min</td>
</tr>
<tr>
<td><strong>Final Extension</strong></td>
<td>72 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Table 3. PCR cycling process.

PCR experiment and cycling process takes approximately 2 hrs.

Theoretically after PCR we should have dsDNA molecules with modified terminals of Biotin and Amino Groups.

![Engineered dsDNA scheme](image)

Figure 32. Engineered dsDNA scheme.

We checked through gel electrophoresis to determine the length of our molecule.

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge [56]. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an Agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving [57]. Proteins are separated by charge in Agarose because the pores of the gel are too large to sieve proteins. Gel electrophoresis can also be used for separation of nanoparticles.
Preparing Gel Electrophoresis

Preparation for 1X TAE buffer:

\[
\frac{50\times TAE}{1} \rightarrow \frac{1\; ml}{50\; ml \; water} = 1X\; TAE
\]

\[
\frac{20\; ml \; 50\times TAE}{1000\; ml \; H2O + 20\; ml \; 50\times TAE} = \frac{20\; ml}{1000\; ml + 20\; ml} = \frac{20}{1020} = 1X\; TAE
\]

For our electrophoresis experiment we need to prepare 1.5% of Agarose gel in 40 ml of 1X TAE buffer.

\[
\frac{40\; ml \; TAE}{0.6\; grams\; Agarose} = 1.5\%\; Agarose\; gel
\]

We used the microwave in order to heat the 40 ml of Agarose gel [1.5%] for around 30 sec. Afterwards, we run the microwave every 4 sec to check for boiling and caring to prevent spilling. Then, we let the flask to cool down for around 5 min. Check if the flask is suitable to hold in hands (should be hot! around 50 °C – 60 °C).

It is added 5 μl of ethidium bromide to the mixture being careful of it due that it is carcinogenic. Add the Agarose gel liquid into the base from the electrophoresis apparatus to sit and become jelly. Just after you add the gel to the base, add the spacer to make the wells in the gel. After 20 to 30 min, once the Agarose has become jelly, remove the spacer. Switch the base aligned along the tray. Add 1X TAE liquid buffer until you have covered slightly above the gel and both small side chambers from the electrophoresis apparatus. Add in one of the slots from the gel 5 μl of our PCR DNA stock solution. In an adjacent slot add 5 μl of the DNA marker for 100 bp. Add 2 μl of ethidium bromide on the chamber of the positive terminal. Set the voltage to 100 V and let it run for around an hour.
We proved through Electrophoresis that the PCR experiment was successful. We got a length between 400 and 500 bp.

After corroborating we have the right length from the gel electrophoresis, Ethanol Precipitation for nucleic acids is performed for DNA purification.

- **DNA Purification Protocol**

To our 400 μl of reaction mixture (R.M.) is added 44 μl of 3M NaOAc (Sodium Acetate pH=5.2). Twice the volume is added of ethanol 95% ≈ 880 μl in ethanol.

Incubate 20 min at – 20 °C. Total volume at this point ≈ 1340 μl (should look green). Centrifuge at max speed (15,000 rpm) for 20 min at room temperature [Micromax RF Thermo IEC].
Add 500μl of 70% ethanol, and wash gently by hand for about 5 to 8 min. Centrifuge for 5 min at max speed at room temperature. Remove the liquid and add 500 μl of 70% ethanol and wash again during 5 min. Centrifuge during 5 min at max speed at room temperature, remove liquid. Let it sit for around 10 min so that ethanol will evaporate. After time is out, check by odor to see if there are still signs of ethanol. Once there is no signs of ethanol odor with may continue. Add 20 μl of water and check if DNA dissolves after shaking by hand. DNA should dissolve very fast and viscosity should increase. Shake by hand for 5 min.

**Physics Behind Ethanol Precipitation**

Ethanol precipitation of nucleic acids is all about solubility…

First we need to know why nucleic acids are soluble in water. Water is a polar molecule, it has a partial negative charge near the oxygen atom due the unshared pairs of electrons, and partial positive charges near the hydrogen atoms (see figure #).

Because of these charges, polar molecules, like DNA or RNA, can interact electrostatically with the water molecules, allowing them to easily dissolve in water. Polar molecules can therefore be described, as hydrophilic and non-polar molecules, which can’t easily interact with water molecules, are hydrophobic. Nucleic acids are hydrophilic due to the negatively charged phosphate (PO3-) groups along the sugar phosphate backbone.

The role of the salt in ethanol precipitation…

The role of the salt in the protocol is to neutralize the charges on the sugar phosphate backbone. A commonly used salt is sodium acetate. In solution, sodium acetate breaks up into Na+ and [CH3COO]-. The positively charged sodium ions neutralize the negative charge on the
PO3- groups on the nucleic acids, making the molecule far less hydrophilic, and therefore much less soluble in water.

The role of the ethanol…

The electrostatic attraction between the Na+ ions in solution and the PO3- ions are dictated by Coulomb’s Law, which is affected by the dielectric constant of the solution. Water has a high dielectric constant, which makes it fairly difficult for the Na+ and PO3- to come together. Ethanol on the other hand has a much lower dielectric constant, making it much easier for Na+ to interact with the PO3-, shield it’s charge and make the nucleic acid less hydrophilic, causing it to drop out of solution.

The role of temperature in ethanol precipitation…

Incubation of the nucleic acid/salt/ethanol mixture at low temperatures (e.g. -20 or -80C) is commonly cited in protocols as necessary in protocols. However, according to Maniatis et al, this is not required, as nucleic acids at concentrations as low as 20 ng/mL will precipitate at 0 -4C so incubation for 15-30 minutes on ice is sufficient.

The wash step with 70% ethanol…

This step is to wash any residual salt away from the pelleted DNA.
A second run using the spectrophotometer is used to check the purity of our final solution.

Figure 37. Spectrophotometer results from DNA purity test.

A second gel electrophoresis is performed in order to check the length of the resultant solution with DNA free from salts.

The protocol is the same as before, except on the samples used.

The sample preparation for this electrophoresis is the following:

DNA sample:
- 8 \mu l of water
- 1 - 1.5 \mu l of loading buffer
- 1 \mu l of DNA stock solution

Ladder:
- 7 \mu l of 100 bp Ladder

Set the voltage at 80 V and let it run for approximately 2 hrs.

Figure 38. Result 2. Purified DNA sample from stock solution showing a length of around 420 bp. The small concentration close to 100 bp could be accounted for non specific bind of residuals of nucleotides.
• Discussion-I

Having demonstrated that the PCR experiment was successful and having checked that now our DNA sample is clean we need to test for the binding of the biotin and amino-group sites.

Having a concentration of 1874.7 ng/μl we know that 1 bp = 650 Da (Daltons) and 1 Da = 1.66 exp(-24) grams.

Our DNA molecule is ≈ 420 bp, so

\[ \approx 420 \text{ bp} \times \frac{650 \text{ Da}}{1 \text{ bp}} \times \frac{1.66 \times 10^{-24} \text{ grams}}{1 \text{ Da}} = 4.531 \times 10^{-19} \text{ grams or } 273,000 \text{ Da} \]

So if we have 1874.7 ng/μl then

\[ \frac{N}{\mu l} = \frac{1.874 \times 10^{-6}}{273,000 \text{ Da}} \times 6.023 \times 10^{23} = 4.12 \times 10^{12} \text{ mol/μl} \]

Experiment

First we will test the binding properties directly to the slide and coverslip coated with streptavidin. According to Xenopore, the company at which we bought the slides and coverslips, they revealed through AFM studies that the streptavidin is present as a monolayer, with few uncoated spots and few multilayer attachment points. There are approximately 10 exp (13) biotin-binding sites per square centimeter. We will dilute to different concentrations the stock solutions from DNA we have. We will incubate different concentrations in the coverslips and slides on top of them to test later through the spectrophotometer if there is still presence of DNA.

In theory DNA should bind the coverslip and slide and when we take the solution from the slide we shouldn’t get any or much less concentration in the spectrophotometer.

For incubation, a chamber with 100% of humidity is to be created in order to prevent evaporation.
Take 18 μl of PBS buffer 1X and add 2 μl of DNA to dilute it 20 times. From the dilutions done we took 5 μl of the resultant solution of each tube, (tubes 1 – 7). We placed them on top of the slide and coverslip coated with streptavidin and placed inside the humidity chamber to incubate for 1 hr.

<table>
<thead>
<tr>
<th>S.T.</th>
<th>Concentration (mol/μl)</th>
<th>O/U</th>
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</thead>
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<td>S.T.</td>
<td>4.12 × 10^12</td>
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</tr>
<tr>
<td>TS1</td>
<td>2.06 × 10^10</td>
<td>2.5</td>
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<tr>
<td>TS2</td>
<td>1.06 × 10^10</td>
<td>1.25</td>
</tr>
<tr>
<td>TS3</td>
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<td>0.525</td>
</tr>
<tr>
<td>TS4</td>
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</tr>
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<td>TS6</td>
<td>6.25 × 10^8</td>
<td>0.075</td>
</tr>
<tr>
<td>TS7</td>
<td>3.1 × 10^8</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Table 4. Dilutions for the DNA Stock Solution.

Figure 39. Incubation of DNA Sample Tubes 1-7.

Figure 40. Humidity Chamber
After one hour of incubation, we tested using the spectrophotometer to check for any concentration of DNA. Our first assumption is that if there is none or little concentration of DNA means that we had binding on the coverslip.

Figure 41. Sample Tube 2 spectrophotometer results from DNA purity test.

Figure 42. Sample Tube 3 spectrophotometer results from DNA purity test.
<table>
<thead>
<tr>
<th>#</th>
<th>Sample ID</th>
<th>User name</th>
<th>Date and Time</th>
<th>Nucleic Acid Conc.</th>
<th>Unit</th>
<th>260/280</th>
<th>260/230</th>
<th>Sample Type</th>
<th>Factor</th>
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<td>0.65</td>
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<td>3</td>
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<td>747-052275</td>
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<td>ng/µl</td>
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<td>7/29/13 13:25</td>
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Table 5. Additional Spectrophotometer Data using NanoDrop 2000
Discussion-II

In table 5 we compare the control samples and the tested samples. The control samples are the samples obtained from the DNA purification through ethanol precipitation process. Those samples should display certain concentrations while the incubated dilutions should display much smaller concentrations. The fact is that the concentrations were about the same after the incubation. This only demonstrates that the approach was not the proper one. Further experiments are performed in order to prove binding for biotin and the streptavidin sites.

Dot Blot Analysis

Enhanced Chemiluminescence was used to check for binding of the DNA stock solution with streptavidin on the nitrocellulose membrane.

Figure 43. Structure for the Dot Blot Analysis Technique.

Streptavidin Peroxidase polymer is used on the nitrocellulose membrane. The nitrocellulose membrane is used to immobilize proteins in Western blots and Atomic Force Microscopy for its non-specific affinity for amino acids.
We take 10 μl of every tube from DNA PCR stock solution and put them on the nitrocellulose membrane. The vacuum is used to filtrate and get it to stick in the membrane, and then we dry it on the vacuum oven (vacuum oven 280a). Afterwards, cross linker UV light is used for 1 min at 6 energy units (ultra LUM UVC-508). Block the membrane with 1% casein (protein from milk) PBS and incubate for 15 min. Incubate once again with Avidin HRP in a 1:10000 dilution.

\[
\begin{array}{ll}
2 \mu l & \text{Avidin HRP} \\
20 ml & \text{Casein PBS}
\end{array}
\]

Incubate in Avidin HRP for 1 hr. The enzyme horseradish peroxidase (HRP) amplifies a weak signal and increase detectability of a target molecule.

Purpose: To prove in a general way that we have biotin and that is binding to the nitrocellulose membrane.

![Figure 44. Nitrocellulose membrane results with streptavidin-biotin binding evidence.](image)

The plus control (+) are nucleotides with biotin, that’s why the luminescence at high concentrations (first slot, figure 44). Minus control (-) is zero. The sample DNA with biotin at high concentrations is way higher than the binding sites for the biotin-streptavidin conjugation that is the reason for no luminescence; at low concentrations all DNA molecules bind to the nitrocellulose membrane enabling luminescence.

Conclusion: Regardless that the membrane was old, we were able to prove that we have biotin binding on the membrane.

Now, we need to attest for binding of the beads coated with carboxylic groups with the amino group site from the DNA.
The process is the following:

Take 19 μl of water and 1 μl of the DNA stock solution, vortex and centrifuge. Take 10 μl from tube 1 and add 10 μl of PBS.

This process is repeated 9 more times.

![Figure 45. Dilution of the DNA stock solution sample.](image)

Take 200 μl of the beads coated with carboxyl groups being completely dissolved, vortex or ultrasonic to disrupt agglomeration. Centrifuge for a short time at max speed. It should look like this.

![Figure 46. Centrifuged Polystyrene beads coated with carboxylic groups.](image)

Remove NaN3 (water plus sodium azide) from the beads.
Wash 3 times with water:
Add water, vortex, and centrifuge; remove water with a transfer pipette.
Wash with MES buffer (pH=4.7).
**(There would be agglomeration due to the fact that we removed some kind of detergent within the bead’s solution in order to prevent agglomeration)**

Ultrasonic and add 1 μl of detergent 0.1% tween-20 diluted 1000 times (1 μl/ 1 ml). Incubate for 10 min to check if the beads agglomerate (tween-20 shouldn’t interfere with our reaction).
NHS and Sulfo-NHS are used to prepare amine-reactive esters of carboxylate groups for chemical labeling, crosslinking and solid-phase immobilization applications. Carboxylates (-COOH) may be reacted to NHS or Sulfo-NHS in the presence of a carbodiimide such as EDC, resulting in a semi-stable NHS or Sulfo-NHS ester, which may then be reacted with primary amines (-NH2) to form amide crosslinks. Although NHS or Sulfo-NHS is not required for carbodiimide reactions, their use greatly enhances coupling efficiency.

EDC is a carboxyl and amine-reactive zero-length cross linker.

We took two tubes, one with 3.5 mg of NHS and the other with 1.5 mg of EDC. For the conjugation we added 60 μl of water to the tube with the 3.5 mg of NHS, and vortex. Add 1 ml of beads to the 1.5 mg of EDC, and shake a little. Take 33 μl of the NHS solution (60 μl + 3.5 mg) and add it to the beads with EDC, and vortex for a while. Wash the final solution in 1 ml of PBS buffer, vortex and centrifuge. Wash with 1 ml of PBS with 0.005% of 10%-Tween-20.

Wash 3 times the tubes with100 μl of PBS. Prepare 0.005% of PBS with tween-20:

50 ml PBS + 25 μl tween-20 = 0.005%

Take 1 ml of 0.005% solution plus 2 μl of –COOH beads (directly from bottle).

As a random, but consistent choice we picked sample tubes (S.T.) numbers 2, 4, 6, 8, 10 to experiment with.

![Figure 47. Samples used for the multimode reader.](image)

Step 1: 4 μl of suspension of nanoparticles in 1 ml PBS/BSA at room temperature (R.T.) for 30 min in the compact rocker CR95 (shaker).

Step 2: Centrifuge for 10 min and replace with Avidin-HRP 1ml 1:5000.

Step 3: Vortex and biosonic.

Step 4: Shake at R.T. for 1 hr.

Step 5: Wash in 1 ml 4 times:
- Centrifuge
- Remove
- Add PBS
- Vortex and gently shake

We mixed the activated beads with different concentrations of DNA from our stock solution. All beads will have bind to a DNA molecule and by washing the first time we get rid of the excess of DNA molecules. Same procedure is done with Avidin-HRP, which will amplify a weak signal and will increase detectability of the target molecule. We mixed with Avidin-HRP and then we wash once again to get rid of the excess of HRP.

After washing suspend once again in 20 μl of PBS

Note: In the suspension on PBS there was a mismatch on volume, some of the tubes (2, 4, 6, 8, 10) ended up with 23 μl, 21 μl, 24 μl, etc. not all the volumes were consistent with a final volume of 20 μl.

The samples 2, 4, 6, 8, 10 are placed on the micro-plate wells plus a (+) control.

Our (-) control will be sample 10 containing beads in PBS.

The (+) control is only HRP with the TMB.

Samples contain beads coated with carboxyl groups attached to the amino groups from the DNA molecules, we washed few time through the previous processes to get rid of excess of DNA molecules that will not be binding to the beads. We tested for the presence of biotin being reacted to Avidin-HRP and TMB. If we happened to get a reading from the spectrophotometer wavelengths close to 650 nm we will be attesting the presence of DNA molecules, which are bound to the beads.
The following figure (figure 48) explains how was arranged the micro-plate wells:

On row 1 is placed the original samples from the tubes 2, 4, 6, 8, 10. In row 2 we dilute twice, take 10 μl from row 1 and added into row 2, which contains 10 μl of PBS, the dilution keeps going until it reaches row 6.

TMB (1mg/ml) is added to the array cells. 3,3’,5,5’-Tetramethylbenzidine or TMB is a chromogenic substrate used in staining procedures in immunohistochemistry as well as being a visualizing reagent used in enzyme-linked immunosorbent assays [58]. TMB is a white crystal powder that forms a pale blue-green liquid in solution with ethyl acetate. TMB can act as a hydrogen donor for the reduction of hydrogen peroxide to water by peroxidase enzymes such as horseradish peroxidase.
Figure 49. Shows the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) to 3,3',5,5'-tetramethylbenzidine diimine.

The resulting diimine causes the solution to take on a blue color, and this color change can be read on a spectrophotometer at a wavelength of 650 nm.

The reaction can be halted by addition of acid or another stop reagent. Using sulfuric acid turns TMB yellow. The color may be read at 450 nm [59].

TECAN 200 PRO is a multimode reader used to scan for absorbance of wavelength close to 650 nm to identify conjugation between Avidin-HRP carboxylic groups binding to amino groups.

Figure 50. Multimode reader results from diluted samples.
Figure 51. Multimode reader Results from cell A6 and C6.

Figure 52. Multimode reader results from cell H1.

Figure 53. Spectrum Data from microplate well A6 containing HRP and TMB.
Figure 54. Spectrum Data from microplate well H1 demonstrating presence of DNA molecules on the beads.

Absorption is observed in well A6, which is the (+) control sample used. In well H1 we have absorption at 650 nm, and regardless that the absorption is low there still is presence of biotin being reacted to Avidin-HRP and TMB. The spectrophotometer reads 650 nm, which attest for the presence of DNA molecules bound to the polystyrene beads.

Discussion-III

Having proved all binding sites from our DNA molecule we will be able to trap our DNA molecule through laser tweezers. The approach is simple, from the H1 well concentration we will add around 10 μl to the coverslip and put it against the slide and it will incubate for around 15 min. We will wash away using water and if we are able to see –COOH beads on the surface of the coverslip using the CCD camera this will mean that the conjugation of the whole system is a success. Afterwards, we just need to trap the bead and use the tracking laser in order to collect the data from it.
Nonlinear Elasticity properties of DNA and the Worm-Like-Chain Model

Two models from statistical mechanics are often used to describe quantitatively force distance relationships during polymer extension: the freely jointed chain (FJC) and the wormlike chain (WLC) models. The WLC model was first treated numerically by Fixman and Kovac (1973), after which a preliminary analytical approach was performed by Kovac and Crabb (1982). Its complete treatment was achieved by Marko and Siggia and reported by Bustamante et al. (1994). In particular, Bustamante et al. (1994) have shown that the force-extension diagram of a DNA molecule is well described by a worm-like chain (WLC) model.

The WLC model describes the DNA molecule as a homogenous spring of constant bending elasticity. The polymer molecule is represented as an irregular curved filament, a wormlike chain, which is linear within the persistence length p, a parameter that represents the stiffness of the molecule (figure 55).

![Figure 55. Irregular curved filament, wormlike chain.](image)

An interpolation formula that approximates the force-distance relationship in the WLC model can be expressed as:

\[ F = \frac{k_B T}{p} \left[ \frac{1}{4(1 - \frac{x}{L})^2} + \frac{x}{L} - \frac{1}{4} \right] \]

where “p” is the persistence length (estimated to 53 nm for dsDNA). The contour length is written as “L”, “k_B” is the Boltzmann constant and “T” is the absolute temperature. In our measurement on DNA stretching we have used the same equation for the WLC without any further modification.
Experimental Measurements of the DNA stretching

Since the DNA molecule is flexible, we must extend it to measure its length. We attach one end of the molecule to a movable cover glass and the other end to a bead held in an optical trap. We stretch the molecule by moving the cover glass while monitoring the tension in the DNA (equal to the force exerted by the optical trap). The DNA was subsequently stretched by moving the cover glass with respect to the trap using the nanopositioning stage, while the position of the bead was recorded at nanometer scale resolution. Stretching experiments were performed at room temperature in phosphate buffer. Biotin-streptavidin linkages can withstand more than 70 pN of force over the period of our experiments. Three data channels were recorded: position detector output, laser light intensity, and piezo drive voltage. These data were then used to compute single-molecule Force-Extension relationships, an example of which is shown in figure 56. DNA was typically stretched by 60 pN; a force of this magnitude is in the range of those required to convert DNA to its overstretched form, which has been shown to develop with forces in excess of 65 pN. As a single molecule of dsDNA is stretched beyond its B-form contour length, the force required to stretch the molecule increases dramatically, as shown in figure 56 (black curve). If one end of the DNA molecule is allowed to rotate freely, at about 68 pN, a cooperative overstretching transition occurs, in which very little additional force is required to stretch the molecule (plateau in the black curve). This result is in very good agreement with similar measurements performed using the optical tweezers or other techniques such as the AFM and the magnetic tweezers. In addition, we have used the WLC equation above to fit the linear part of the force-extension curve. As shown in figure 56 (red curve), the WLC model fits very well a small part of the curve (under 25 pN) using persistence length p 46 nm. This finding is also in a good agreement with what has been reported by others.

We were hoping to perform more experiments under various conditions and using different sizes of the DNA molecule. Unfortunately, we were unable to use the microscope because we had to move it to a new location. We definitely need to perform several more experiments to test all the aspects and the capabilities of our system.
Figure 56: The force-extension relationship for a single DNA molecule in phosphate buffer. The red curve is the WLC fit performed using a persistence length of 46 nm.

The slope represents the stretching of our DNA molecule (420 bp). The change in position gives us the contour length of the molecule. The initial and end values are 650 nm and 810 nm; and its difference is 160 nm. 1 bp is 0.34 nm times 420 bp (the length of our molecule) is 143 nm. The difference from 160 nm and 143 nm accounts for the linker’s length.
Conclusion and Future Work

In summary, in this study we have designed and configured a state-of-the-art optical tweezers technique to study dynamics and mechanics of biological molecules at the nanoscale. The microscope is well calibrated and can be used to manipulate not just biological molecules but also any micro/nanoparticles under various conditions. Also in this study we have presented detailed procedures for biomolecule modifications and manipulations necessary to complete the optical tweezers measurements of single molecules.

The mechanical properties of DNA measured in this study could be used as a standard calibration procedure prior to any optical tweezers measurements. We check the calibration by stretching DNA molecules and comparing the fit elasticity parameters of previously published data. Due to its speed, noninvasive (except perhaps local heating) nature, and convenience in the manipulation of microscopic objects, the optical trap is an excellent tool for single-molecule studies, but only those that can accommodate its limitations, which in our setup include:

- imprecision (200 nm) in the initial manual positioning of tethers;
- a useful lifetime of one hour or less for single samples of DNA tethers;
- inaccuracy in the stretching or geometry conversion for short (400-nm or less) tethers.

As a future work, our optical tweezers will be used intensively to measure the DNA-protein interaction as the main objective. Also other studies on protein-protein interaction will be performed.
References


