DEVELOPMENT OF A MAGNETIC MANIPULATOR FOR MICROBIOLOGICAL AND SINGLE MOLECULE INVESTIGATIONS

Jay Kenneth Fisher

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Approved by:
Richard Superfine
Sean Washburn
Kerry Bloom
Tim Elston
Henry Hsiao
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Forces play a critical role in a wide range of biological phenomena from single protein conformational dynamics, cell division, and mucociliary clearance, to name a few. The majority of existing instruments for microbiological force application can be divided into two categories; those that can apply relatively high forces through the use of a physical connection to a probe, and those that apply significantly smaller forces with a detached probe. Magnetic manipulators utilizing high fields and high field gradients have been able to reduce this gap in maximum applicable force, but the size of such devices has limited their use in applications where high force and high numerical aperture (NA) microscopy must be combined. This dissertation focuses on the development of a magnetic manipulation system that is capable of applying forces in excess of 700 pN on a 1 micron paramagnetic particle and 13 nN on a 4.5 micron paramagnetic particle, forces over the full $4\pi$ steradians, and a bandwidth in excess of 3 kHz while remaining compatible with a commercially available high NA microscope objective. This device
has been combined with a feedback enhanced, high resolution (2.4nm), high bandwidth (10 kHz), long range (100 micron xyz range) laser tracking system. The design and testing of this instrument is discussed, as well as biological investigations that take full advantage of the instrument’s capabilities. This dissertation concludes with the presentation of a technique that utilizes polymer dynamics to determine tension and polymer specific properties in both in vitro and in vivo settings. Future investigations involving this analysis technique will benefit greatly from the application of the manipulation device described in this dissertation.
ACKNOWLEDGEMENTS

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<tr>
<td>3DFM</td>
<td>Three Dimensional Force Microscope</td>
</tr>
<tr>
<td>A</td>
<td>Amp</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating Current</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscope</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge Coupled Device</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Drag Coefficient</td>
</tr>
<tr>
<td>FCC</td>
<td>Face-Centered-Cubic</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>$k_b$</td>
<td>Boltzmann’s constant</td>
</tr>
<tr>
<td>kHz</td>
<td>kilohertz</td>
</tr>
<tr>
<td>$L_c$</td>
<td>Contour length</td>
</tr>
<tr>
<td>MMF</td>
<td>Magnetomotive Force</td>
</tr>
<tr>
<td>$\mu$m</td>
<td>Micron</td>
</tr>
<tr>
<td>mm</td>
<td>Milimeter</td>
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<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nN</td>
<td>nanoNewton</td>
</tr>
<tr>
<td>$p$</td>
<td>Persistence length</td>
</tr>
<tr>
<td>PSD</td>
<td>Power Spectral Density</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>---------------------</td>
</tr>
<tr>
<td>pN</td>
<td>picoNewton</td>
</tr>
<tr>
<td>RMS</td>
<td>Root Mean Squared</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
</tr>
<tr>
<td>T</td>
<td>Tesla</td>
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<tr>
<td>UNC-CH</td>
<td>University of North Carolina at Chapel Hill</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
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Chapter 1: Introduction to Microbiological Force Application Techniques

As our understanding of the complexity of biological systems has grown, so too has the desire to investigate the underlying small scale properties responsible for macroscopic behavior. Whether it is a measurement of the forces exerted by cilia to propel healthy mucus inside the lungs, assessment of the response of protein-molecule complexes to tension, or the determination of the mechanical properties of single DNA molecules, experiments at this scale require not only the ability to detect position changes with high sensitivity, but the ability to apply forces with high sensitivity as well.

Recently developed nano-scale manipulation devices have allowed such investigations to be carried out, but in most cases the range of forces that may be applied, force directionality, probe invasiveness and manipulation bandwidths limit the applications for a specific manipulation modality. In this context, we set out to develop a versatile instrument that would allow for the non-invasive application of forces that are large enough to be relevant for cellular level investigations while remaining sensitive enough for single molecule experiments. Although many of the principles behind the workings of this instrument have been utilized in diverse applications for more than half a century, the specific device represents a significant advancement in the field of biologically-focused instrumentation with application to cellular and sub-cellular manipulation. The magnet based manipulation device presented in this manuscript, together with its particle
tracking subsystem form the device we call the Three Dimensional Force Microscope (3DFM).

Existing cellular and sub-cellular microbiological force application methods may be divided into two general categories; those that can apply relatively high forces through the use of a physical connection to a probe, and those that apply smaller forces with a detached probe. Among the attached-probe techniques, glass fibers or microneedles have been used to measure the effects of forces on the movement of chromosomes[1-3] and the force exerted by myosin on actin[4]. More recently atomic force microscopy, using techniques relying on the deformation of a cantilever spring element[5], has emerged as a suitable method to obtain sub-nanometer spatial resolution[6] with picoNewton force sensitivity[7]. In a method similar to typical atomic force microscopy, Evans[8] has developed a method using a deformable vesicle attached to a pipette where deformations of the vesicle are used to measure the forces between membrane-bound molecules and target specimens such as other vesicles or flat substrates. While providing important insights within their domains, these attached-probe methods suffer from the invasiveness of the attached fiber, cantilever or pipette, as well as an inherent limitation in the directional flexibility of the probe.

To address these shortcomings, laser tweezing (trapping) methods have been developed that use a refractive microbead, often below 1 micron in diameter, as a mechanical probe. The bead can be free to move throughout the accessible volume within a specimen, or can be functionalized to be attached to specific molecular groups or proteins. In this method a “trap” is formed using the power gradient of a focused laser
beam to attract refractive materials toward the waist of the focus\[9, 10\]. The force generated on standard polymer microbeads by the optical trap can be varied by changing the intensity of the laser, and may be accurately calibrated\[11\] as a function of laser power. Laser tweezers have been applied to a wide variety of biological investigations, for example, measurements of the forces generated during DNA transcription\[12\], the properties of neuronal membranes\[13\], and the forces generated by the molecular motors dynein, kinesin and myosin\[14-18\]. Its limitations are in the achievable force (generally less than 200 pN), specimen heating at higher forces\[19, 20\] (approximately 5.6°C/Watt of laser power at 975nm\[21\] and 15°C/Watt of laser power at 1064nm laser wavelength\[22, 23\] in water) which can cause convection forces in the pN range\[21\], and the non-specificity of forces which act on all particles having a refractive index that differs from the surrounding media within the range of the optical trap. Conversely, the ability to apply force selectively to a single particle within a set of other particles in a specimen is possible with optical tweezers.

As with laser tweezers, magnetic methods apply a force to a particle through field gradients. In this case the field is magnetic and specific to a magnetically permeable probe because typical biological materials are at most only weakly magnetically active. Moreover, high magnetic fields at the frequencies of interest do not generate significant heat, so high forces can be applied to the bead without the generation of specimen damaging heat. The sensitivity of this force application method is limited by the detection system, the viscosity of the specimen, and remnant magnetization of the magnetic materials. Typically magnetic systems can measure forces down to approx .01 pN\[24\].
Beginning with Crick’s[25] in vitro studies of the viscoelastic properties of cytoplasm in 1949, magnetic forces have been used to investigate a wide range of...
biophysical properties and phenomena. Many of the systems that have been reported have applied forces in a single direction, often with one pole tip[26-29]. Among these single tip systems, Bausch developed a device capable of applying up to 10 nN on a 4.5 um paramagnetic bead[29]. Valberg introduced a magnetic system designed for applying torques[30]. Strick applied a multipole geometry to apply forces upward while applying a torque to a ferromagnetic bead[31]. Amblard[32], introduced an eight pole instrument whose construction was designed to apply torques as well as forces within a specific z-plane (the specimen plane). Haber[33] and Huang[34] have both constructed systems designed to be compatible with a high numerical aperture (NA) objective that produce a uniform field gradient. Huang implemented a full octapole design utilizing a backiron to complete the magnetic flux circuit, resulting in increased field efficiency. Gosse presented a six pole design where the poles were located above the specimen, allowing forces in the upward direction but relying on gravity for downward movement[35]. This system also included optical tracking of the bead through processing of images acquired by a camera. Our previously described magnetic force prototype[36] consisted of a tetrapole design, capable of applying forces in many directions except those opposite the pole tips, as well as forces in the nanoNewton range. This system used a stage-feedback-laser tracking system to provide bead position information over a 100x100x20 μm volume.

The magnet-based manipulation system presented here represents several advances over existing designs. First, we have separated the pole tips from the flux-generating current coils, allowing easy reconfiguration of the field geometry at the sample. The poletips are fabricated from commercially available thin foils, flattening the
system geometry and allowing the use of high NA objectives. Second, the bandwidth of the
magnetics as been extended into the kHz range through the use of appropriate
magnetic materials and drive electronics. Material considerations of prior designs were
limited by the use of iron to bandwidths below 40 Hz due to eddy current generation.
Third, we demonstrate a system that can apply forces over the full $4\pi$ steradians. With the
bead near the pole tips, we have measured forces of over 700 pN on a 1 micron diameter bead, and over 13 nN on a 4.5 micron diameter bead.

A thorough discussion of the instrument we have developed, as well as biological investigations utilizing this device follow in the subsequent sections. In the next chapter, Chapter 2, the design considerations/specifications for our magnetic system will be addressed. We will then discuss the as-built design, and, where appropriate, the effectiveness of what has been implemented. This will include maximum achievable forces, force directionality, and force bandwidth. In Chapter 3, processing of the poles responsible for coupling flux generated by our electromagnets down to the specimen will be discussed. The advantages and disadvantages of the three different methods we have investigated for the processing of raw materials into finished poles will be presented, with the majority of the chapter spent on our ultimate method of choice, acid etching. This section will conclude with a discussion of the limitations of the current pole production technique and possible ways to make improvements. Chapter 4 will focus on the detection and imaging subsystems of the instrument. Laser interferometry, the basis of the high-bandwidth position detection system, and the optics necessary for its incorporation into a commercially available inverted microscope will be presented. This discussion will also include the electronics associated with the detection system, starting
with the photodetector and progressing to the data acquisition boards in the computer. In Chapter 5, force calibration, including the Stokes Law method and a unique white-noise based method that takes advantage of our high-bandwidth manipulation capabilities will be presented. The white-noise section represents a partially completed experiment where initial results have been obtained but the technique has yet to be validated against an existing accepted method for calibration.

In the remaining chapters, experiments involving biological specimen will be discussed. In Chapter 6, cilia manipulations completed using our previous (1st generation) magnetic system shall be detailed. These experiments demonstrate our ability to track a bead attached to a cilium (or multiple cilia) in three dimensions and manipulate it towards each pole in this tetrapole system. This is part of a series of experiments that are currently being carried out by other researchers in our lab in order to determine the force applied by beating cilia. Chapter 7 first discusses the theory behind investigations of the properties of DNA using the freely-jointed and worm-like chain models. This discussion is extended to the relaxation of polymers under tension post-rupture, a phenomenon utilized in Chapter 8 for in vitro and in vivo investigations into single molecule properties and tension forces. This chapter also contains experimental results from manipulations of DNA and chromatin in vitro. Finally, Chapter 8 discusses the development of an in vitro system to be used in parallel with the in vivo system developed by the Bloom lab for the investigation of DNA/Chromatin properties, intracellular viscosities, and the tension force exerted by the mitotic spindle on the chromosome during cell division. While initial experiments involving these two parallel
systems do not involve the use of magnetic forces, subsequent *in vitro* experiments would greatly benefit from the application of known forces.

Before continuing on to Chapter 2, it is important to note that the work presented in this Dissertation is part of a collaborative effort with members of the Computer Science, Physics, Biology and Biomedical Engineering departments at the University of North Carolina at Chapel Hill. In an effort to acknowledge the contributions of individual members of these departments, a section will be included at the end of each of the following chapters recognize the contributor and their addition.
1.1 References


Chapter 2: Magnetic System Design

Our desire to create a magnet-based manipulation system for use in biological and biomedical experiments has motivated the development of the system described in this chapter. Here we begin with a discussion of existing magnet-based manipulation systems of varying complexity developed by other groups. From these existing systems and our desired applications we draw motivation for the design goals for our magnet-based manipulation system, including a specific target for maximum achievable force. Discussion of these design goals will be followed by the presentation of the relevant magnetic force equations. Next, we will introduce our as-built system, with unique components discussed in detail. This chapter will conclude with a report of the performance of our newly developed system and a comparison of these results to our design criteria.

2.1 Introduction

Force application from a single or dual pole tip magnetic system is the most easily accomplished, and as a result, the most commonly implemented form of a magnetic manipulation system[1-3]. These systems are usually designed for applications where directionality is sacrificed for the generation of high forces, with some of these systems capable of applying forces in excess of 10 nN on a 4.5 µm paramagnetic bead[4, 5]. While less complicated in design, the capabilities of these systems have evolved with the
use of software simulation packages such as Magneto, developed by Integrated engineering Software (Manitoba, Canada), and COMSOL Multiphysics (FEMLAB) (Göteborg, Sweden) for the optimization of material choices and design geometries and through the use of computer-aided design (CAD). For example, Haber constructed a 2 pole system for dsDNA manipulation generating relatively large, uniform field gradients with a tip spacing of 1.5 cm to maintain compatibility with most objectives[2]. This system represented a significant step in the generation of high field gradients from large pole gaps ($\approx 100$ T/m for a 1.5 cm gap compared to previously reported values of $\approx 20$ T/m for a 7.5 mm gap[6]). Other two pole systems using relatively large pole gaps include Ziemann’s oscillating magnetic bead micro-rheometer[7], and a similar device constructed by Keller[3] also used for the measurement of viscoelastic properties. Utilizing a significantly smaller pole gap (down to 150 um) in a system designed to generate constant field gradients, Hosu demonstrated forces on 1.28 um superparamagnetic beads of approximately 500 pN that remain constant over approximately 40 µm of specimen space[1]. Finally, Fischer et. al developed a sharp-tipped single pole system utilizing high field gradients in order to produce a force of 15 nN on a 4.5 µm bead approximately 10 um from the tip[4].

In addition to the one and two pole systems, multi-pole systems consisting of up to eight poles (four sets of two pole pieces) have been constructed for the purpose of multidirectional manipulation and the application of torques. Amblard constructed a 4 coil system with each coil driving two individual poles for the purpose of generation of a uniform magnetic field[8]. A feedback loop incorporated into this system used hall probes to measure field strength at each pole for comparison against a set point, with
differences between the actual field strength and desired field strengths compensated for by an integrator. Gosse’s six pole design with poles above the specimen plane also implemented a feedback loop, this time a proportional-integral loop, for the purpose of a position clamp[9]. This system adjusted coil currents in response to an error signal generated at 25 Hz by a 3D video tracking algorithm in order to hold the target bead at a constant location. However, due to this system’s pole geometry, true manipulation in the –Z direction was not possible. Instead, a characteristic current above which the bead is manipulated in the +Z direction, and below which it falls due to gravity is used for manipulation along the Z axis. A full octapole system integrated with a two-photon, three-dimensional imaging system capable of exerting over 200 pN on a 4.5 um bead with forces that vary within 10% over a 500 x 500 μm² work area was developed by Huang[10]. In addition, Huang presented simulation data reaffirming the importance of the backiron (yoke). Finally, in addition to the system discussed in this report, our research group developed a low-bandwidth first generation quadrapole system[11] for multidirectional manipulation when operated in a tetrapole geometry, and high force manipulations (up to 9 nN) when one pole was moved to within the specimen chamber.

Separate from the aforementioned single and multi-pole systems are a classes of devices using coaxial coils[12] for the generation of magnetic forces. While these systems produce smaller forces (usually on the order of pico-Newton), they do not suffer from the hysteretic effects associated with the use of soft iron magnetic cores or pole tips and, as a result, do not have the same bandwidth limitations. When used in combination with a permanently magnetized ferromagnetic bead (eliminating nonlinearities caused by
changes in the magnetization of the bead), the applied force will vary linearly and can be computed.

Twisting experiments where torques are applied to magnetic particle can be carried out with the use of a magnetic manipulation technique developed by Wang[13] know as Magnetic Twisting Cytometry (MTC). In this technique, magnetic moments of ferromagnetic beads are aligned using a brief strong magnetic field. Then a weaker, “twisting” field is applied perpendicular to the original field to induce bead rotation. The average bead rotation is measured using a magnetometer to monitor changes in the remanent magnetic field of the beads.

2.2 Design Considerations

While all of the systems described above have proven useful for their specific applications, no single system provides the range of forces, manipulation directionality, and compatibility with biological specimen and traditional high Numerical Aperture (N.A.) microscopy that we desire. Our ideal system would be variable enough to be used in the biological applications discussed in each of these experiments while remaining compatible with a commercially available microscope. As a result, our goal is to develop a system that can accomplish the following:

- coexist with a high NA liquid immersion microscope objective,
- exert forces comparable to the largest demonstrated to date (greater than 10 nN),
- provide $4\pi$ sr solid angle of force direction,
- produce force excitations in the kilohertz range,
be readily reconfigurable to match individual experimental needs.

- control specimen temperature.

In order to accomplish these goals there are many design considerations we must keep in mind, starting with the relevant magnetic force equations.

### 2.2.1 Magnetic Force Equations

Force on a magnetic bead is caused by an interaction between the bead’s magnetic dipole moment $m$ and the gradient $\nabla B$ of an incident magnetic field. For a soft, magnetically permeable bead, $m$ is entirely induced by the incident field. Subject to saturation properties of the magnetic material in the bead,

$$m = \frac{\pi d^3}{2 \mu_0} \left( \frac{\mu_r - 1}{\mu_r + 2} \right) B,$$

where $\mu_0$ is the permeability of free space in SI units, $\mu_r$ is the relative permeability of the bead, and $d$ is the diameter of the bead. The magnetic force is then,

$$F = \frac{\pi d^3}{4 \mu_0} \left( \frac{\mu_r - 1}{\mu_r + 2} \right) \nabla (B^2).$$

The field is produced by multiple electromagnet pole tips arranged in space to provide the necessary directional capability. Except very near a pole tip, the field’s behavior can be modeled by a monopole. According to this model, the magnitude of $B$ from a singly excited magnetic pole is proportional to $B_p/r^2$, where $B_p$ is the pole strength and $r$ is the distance from the pole. Correspondingly, $\nabla (B^2) = -4B_p^2/r^5$, and is directed toward the pole. Clearly, the distance between a pole tip and the bead is of primary importance in optimizing bead force.
2.2.2 General Design Considerations

The standard design for generating magnetic fields at a specimen is to couple the flux from a current carrying coil to the sample region through a permeable core that narrows at the specimen (a pole tip). The coil is typically wound around the core, with the end closest to the specimen tapered to concentrate the flux so that a large field and field gradient is created. The analogy between electric circuits and magnetic circuits (Figure 1) provides an immediate insight into magnetic system design. In a series electrical circuit it is obvious that, for a fixed voltage, the highest electric current will be produced when the circuit resistance is minimized. Using the magnetic-electrical circuit analogy, for a fixed magnetomotive force as generated by the current in the coils, the highest magnetic field will be produced when the circuit reluctance is minimized. This implies that the system should minimize air gaps and attempt to provide a high permeability path for the flux through a return loop.

a. Magnetic Circuit Topology

Schematic diagrams of electrical analogs for two magnetic circuits are shown in Figure 1. The magnetomotive forces (MMFs) are represented by voltage sources, and the reluctances of the various pieces of the magnetic path are represented by resistors. For simplicity in comparison, the number of coils for both system analogs has been made the same.
Figure 1: Electric Circuit Analog to the Magnetic Paths. The reluctances $R$ represent the return path reluctance $R_f$, the pole tip reluctance $R_p$, and the reluctance $R_a$ for the gap between the pole tip and the bead. The magnetomotive forces $M_i$ are generated by currents through coils wound around their respective legs. Labels of symmetrically defined reluctances have been omitted for the sake of clarity.

(A) Electric circuit analog for a typical systems with coils wrapped around the flux producing pole tip.

(B) Electric circuit analog for a modified system with coils wrapped around the flux return path segments.

Figure 1 (a) shows the topology of the typical magnetic system. A coil encircling each pole provides a MMF $M_i$ in series with pole reluctance $R_p$ and specimen reluctance $R_a$ due to the air/glass/specimen gap between the pole tip and the location of the bead. This is conceptually straightforward, where the $B_p$ of each pole is proportional to its coil current, with due regard to magnetic saturation of the pole.

Due to spatial constraints caused by large coils positioned close to the objective, it may be desirable to modify the topology of the typical system by moving the coils away from the pole pieces and instead encircle the return path pieces that connect the back ends of the pole cores in a ring (Figure 1 (B)). These encircled return path pieces are shown as MMFs $M_i$ in series with their respective return path circuit reluctances $R_f$. Pole reluctances $R_p$ connect the junctions between the $R_f$ to edges of the specimen space, and
specimen reluctances $R_a$ connect their respective pole tips to the bead. A simplified model of the magnetic path for each pole consists of the pole itself, a gap through the specimen space, and the return path.

It should be appreciated that both of the magnetic circuit analogs presented in Figure 1 are simplified models, and that parasitic reluctances actually exist between every pair of circuit nodes, some of them being significant. They are omitted here for clarity in understanding the behavior of the topology.

**b. Spatial Constraints**

Due to the desire to incorporate our magnetic manipulation system with a high numerical aperture (N.A.) imaging system, space directly above the objective lens (z height) is at a premium. Typical high N.A. water immersion objectives used in our lab have a working distance of 220-240 microns, meaning that in order to image at the specimen plane, the total height of the magnetic poles, their support structure, and the specimen chamber must not be greater than 320-340 microns (the extra 100 microns is a result of the objective manufactures allowance for coverglass thickness).
c. Force magnitude/directionality

The ability to apply forces at the sub picoNewton level is inherent to most magnetic manipulation devices (provided small currents may be sent to the coils). While this magnitude of this force is large enough for some single molecule investigations, it is insufficient for the majority of studies involving larger structures such as chromatin (the condensed form of DNA), cell membranes, and biological actuators (cilia, for example). Our desire to be able to use this device for a wide variety of experiments necessitates the development of a system that can apply forces ranging from sub picoNewton to nanoNewton, with a goal of exceeding the maximum published force applied by any other magnetic system to date (approximately 10 nN).
Existing multi-pole magnetic manipulation systems have demonstrated the ability to pull beads in multiple directions, but have yet to demonstrate the ability to manipulate in true 3D (over the full $4\pi$ steradians). In addition to our goal of producing large forces, we also aim to develop a system that can be configured for full 3D manipulations while maintaining compatibility with high NA microscopy. While the ideal system would have the ability to apply both high forces (> 1 nN) and manipulate in full 3D, we acknowledge that other constraints, specifically high NA microscopy compatibility and relatively low production expense will prevent this from being accomplished by a single device with a fixed pole geometry.

**d. Bandwidth**

Our desire to develop a system that offers many of the advantages of other manipulation modalities, including the ability to use this tool as a driven micro-bead rheometer, makes high bandwidth (> 100 Hz) manipulations a necessity. In addition to expanding the range of experiments for which this device may be used, with a magnetic system capable of achieving high bandwidths it will be possible to degauss the system (remove remnant magnetization of pole tip materials) rapidly, thus allowing us to apply much smaller minimum forces for a given tip-bead separation. The advantage to a rapid degauss routine is that high currents passed through the coils as part of the procedure will not cause large displacements of a magnetic probe since the currents will only be applied for very short times, thus limiting the effects of the degaussing routine as seen by the specimen.
e. Heating/Cooling System

Since our goal is to develop a magnetic manipulator for use in biological applications, maintaining biologically relevant temperatures (typically 37 °C) is of primary concern. This will need to be accomplished in a manner that spreads heat evenly across the specimen surface to prevent temperature gradients, without the use of components that will cause excess noise (vibrational, electrical, etc.) that may interfere with data collection. Additionally, it is desirable to have a system that can maintain a wide range of temperatures (say 20 - 50°C) so that temperature may be used as a variable in our investigations.

2.3 As built design

With these design goals in mind, we have developed the system that is discussed in the remaining sections of this chapter. In the following sections, we have pulled out specific components of the system for detailed description in an attempt to explain the motivation behind the design decisions that have been made.

As noted in section 2.1, the presence of a high permeability flux return path (back-iron/yoke in some systems) increases the efficiency of magnetic system. For this reason, our system uses a complete drive ring (Figure 3 A) upon which coils are mounted (Figure 3 B). The drive ring with mounted coils is approximately 25 mm in diameter, with appropriate space left in the center to provide access to the specimen chamber (or lower pole plate) by the objective (Figure 3 C). Vertically symmetric drive rings (complete with mounted coils) are then placed inside a frame that provides a carriage for x-y translation of the specimen and easy incorporation into our existing microscope. This
completed unit, shown in Figure 4, is what we call our Three Dimensional Force Microscope (3DFM) stage.

Figure 3: Drive ring build-up.
(A) Continuous drive ring upon which hand-wound coils are mounted.
(B) Drive ring with coils mounted, prior to attachment of wires and installation into the frame.
(C) Sketch showing how lower drive ring (with magnetic pole pieces mounted on top of coils) fits around the top of the system’s high N.A. objective (silver object at the bottom of (C)). The condenser (top of (C) is also visible in this figure.
Figure 4: 3DFM stage picture and diagram.

(a) Complete 3DFM stage with coils mounted in the open position. Note the presence of knobs used to translate the specimen carriage and alignment pins used to register the top drive ring in alignment with the lower drive ring with system is closed.

(b) Closed magnetic system.

(c) Side view sketch showing portions of the drive ring, coils, pole plates with mounted magnetic foils (discussed in chapter 3), and the specimen chamber.

**Drive Ring**

Our as designed continuous drive ring is machined from Metglas alloy 2417A (Honeywell, International Inc., Morriston, NJ) that is purchased from it’s manufacture in the form of tape wound toroidal cores with relative permeability of over 30,000 up to 30 kHz for field strengths above 0.01 T, a saturation induction of 0.57 T, and near zero magnetostriction; as a bonus, it is also corrosion resistant. With our early prototypes, this material proved to be superior in all respects. It is however difficult and expensive to machine. Consequently, subsequent systems have used a slightly different material,
Supermalloy (Magnetic Metals Corporation, Camden NJ), available as tape wound toroidal cores. With 2 mil tape, these cores have a relative permeability of over 10,000 up to 4 kHz for field strengths above 0.002 T, and a saturation induction of 0.6 T. With our current experiments, we have not noticed a significant difference between the behavior of systems utilizing these two materials.

Coils

A complex interplay between drive-ring castellation area, the dimensions of commercially available magnet wire, the geometry of the high NA objective lenses, and the width of the commercially available microscope coverslips chosen for pole plate substrates led us to the coil design described here. The coils have been wound with 6 x40 mil flat magnet wire, providing a high conductor fill ratio in the available space. Within the given constraints, this wire achieves optimal electrical efficiency. Nevertheless, these small coils coupled with the need for large magnetomotive forces (MMFs) result in the generation of more heat. We estimated that about 60 Ampere-turns of MMF should be adequate to saturate most pole plate configurations with pole tip gaps less than 0.5 mm. At 25 turns per coil, this requires 2.5 A. With a coil resistance of about 0.1 Ohm, a worst case power dissipation of the entire drive is 7.5 W with consequential undesirable temperature rise. Accordingly, we designed a provision for water cooling the drive and isolating it thermally from the specimen. We will discuss this in detail in a subsequent section.
**Poles**

The pole plates deliver magnetic flux from the drive rings to the specimen chamber. As shown in Figure 2, they are necessarily thin to fit into the tightly constrained space limited by the close working distance of a high NA microscope lens. A specimen can be placed directly on a pole plate, or in a separate coverslip sandwich that sits below one pole plate or in between two pole plates. The required range of forces for a given experiment are the main consideration when determining the location of the specimen with respect to the pole plates. The total thickness of the specimen + pole plate space can range from 150µm to 500µm, depending on the needs of a specific application. The upper drive ring is on a hinged mechanism allowing it to be lowered onto the specimen chamber such that all the magnetic components are properly aligned. This provides for easily changing between experiment-specific specimen chambers without the need to change magnetic drivers. Additionally, pole configurations may be selected from a standard library, or custom pole shapes can be fabricated for special purposes. Two example configurations we have studied are briefly described here, and results presented in detail later. Also, pole manufacturing techniques will be discussed in Chapter 3.

A hexapole design, with a face-centered-cubic (FCC) pole tip placement around the specimen chamber, provides for nearly uniform 3D force directionality over the full 4π sr of solid angle. Figure 5 shows how we implemented the FCC pole placement in two parallel planes, each containing 3 pole tips. In this configuration, a bead placed in the geometric center of the specimen chamber can be pulled over the full 4π steradians with modest force.
Figure 5: Hexapole configuration designed for the application of full 3D forces.

(A) Optical axis (dashed lines) is perpendicular to two planes, each plane containing three face centered cubic (fcc) points.

(B) The two plates form equilateral triangle with a cylindrical working volume between them.

(C) Magnetic flux is conducted from the electromagnets to the specimen using thin-foil poles in two parallel planes with tips having centers at the fcc locations.

(D) Top down view of the simulated field produced by an individual pole in the hexapole design.

(E) Simulated field as viewed from the side.

For cases where a force in only one direction is needed, a simple geometry is a single pole plate having one sharply pointed tip opposite a flat nosed tip, with the bead located quite close to the sharp tip. This configuration achieves high gradient near the sharply pointed tip, and high field strength at modest coil currents by a narrow gap between pole tips, augmented by close proximity of the bead to the sharp tip.
Stage Frame

Usability considerations require a mechanical stage to establish magnetic component alignment after a change of specimen chamber, and to allow manual x-y adjustment of the specimen slide relative to the pole plates during experiment set-up. This has been implemented using a semi-kinematic design shown in Figure 4. This design uses a hinged upper plate to allow the upper drive ring to be lifted clear off the specimen chamber to provide access to the specimen for adjustments or removal. In the closed position, the upper pole plate is constrained in z by adjustment screws (not shown) that allow the user to control the space between the specimen and the pole plates. The pole plate(s) are kinematically positioned in x and y by three dowel pins anchored in the lower platform. The lower pole plate is constrained in z by the castellations of the lower drive ring. A specimen slide holder allows manual xy adjustment of the slide which is held at its corners by four rectangular locators, two of which are visible in Figure 4 a.

Drive Electronics

The drive amplifier used to power the electromagnets is a six channel transconductance amplifier, such that its output current is proportional to its input voltage. Its transconductance gain is 0.5 A/V, with a maximum drive of ± 2.5 A per channel into a 5 µH inductive load typical of the 3DFM drive coil operation. The small signal bandwidth driving a nominal load is 30 kHz. While it is stable for loads up to 50 µH, its full power bandwidth of approximately 10 kHz cannot be maintained for loads above 5 µH. Measured large signal response to a triangle wave input signal retains good
linearity through 1 kHz with a 5 µH load. A more detailed description of the drive amplifier is available in Vicci[14].

Figure 6: Magnet drive schematic.

2.4 System Performance

The capabilities of the as built design are described in this section and are discussed in the context of our design goals. Our ability to image specimen and alter pole plate geometries for these different experiments represents the successful achievement of the compatibility with high NA objectives and the ability to readily reconfigure the pole and specimen geometries design goals.

Magnetic Forces
We have determined the maximum forces generated by the magnetic system by measuring the velocity of 1 µm super paramagnetic beads and 4.5 µm super paramagnetic beads (M-450; Dynal Biotech, Oslo, Norway) in a 1600 centipoise 25 °C sucrose solution. The viscosity of this solution was measured using a commercial viscometer (model No. 513; Cannon Fenske, State College, PA). Particle velocities were determined using Video Spot Tracker, a video tracking algorithm applied to brightfield images acquired using a 120 frames/second video camera. With the viscosity (\( \eta \)), the bead radius (\( a_b \)), and the bead velocity (\( v \)), we can use Stokes formula, \( F=6\pi \eta a_b v \), to calculate a magnetic force. Maximum force values of 700pN and 13nN were determined for the 1 and 4.5 micron beads respectively, using a point-flat geometry with a 550 µm gap (see Figure 7) made from 350 µm thick material with a saturation of approximately 20,000 Gauss and a permeability of 300 (MuShield, Mancheser, New Hampshire). This geometry was chosen for its simplicity and high field gradient near the pole tip. Force vs. Position data for 1 µm and 4.5 µm beads are displayed in Figure 8. Force calibration techniques will be discussed further in Chapter 5.

The results of our magnet force experiments indicate that we have successfully met our design goal of generating over 10 nN of force. To generate even larger forces, magnetic materials with higher saturation (results indicate saturation achieved below maximum applied current) and magnetic beads with a higher magnetic content than the 4.5 µm beads used here could be utilized. Additionally, sharper tips producing higher field gradients should be able to generate larger forces than seen here.
Figure 7: Example pole-flat pole piece.

(A) Wide area view of pole tip used for the calibration in Figure 8.

(B) Zoomed in view of the same pole tip showing 550 micron gap between tip and flat.
Figure 8: Force calibration for 1 and 4.5 micron beads using a pole-flat geometry with a 550 micron gap between the tip and the flat. The inset image shows the magnitude of the applied force mapped to height for a specific x-y plane.

**Magnetic Force Directionality**

A hexapole design with a face-centered-cubic (FCC) pole tip placement around the specimen chamber can be used to provide nearly uniform 3D force directionality over the full $4\pi$ sr of solid angle. Here we demonstrate the hexapole geometry’s ability to pull in any direction in three dimensions through simulation and experiments. For the simulations, monopole approximations of the pole tips in the FCC hexapole design were used to model the field and field gradient generated by a given pole tip excitation. This model was initially used to successfully verify full 3D force directionality by plotting 10000 randomly generated pole tip excitations (not shown). The monopole approximation was also used in the development of an analytical bead force model[14] that can be used to generate a pole tip excitations corresponding to a specified force vector. These calculated excitations were used to determine the relative values of the coil currents in the experimental verification of simulated results.

To verify the ability to pull in all directions, we first demonstrate large-scale magnetic symmetry by pulling a 2.8µm superparamagnetic bead (M-280; Dynal Biotech, Oslo, Norway) towards each magnetic axis of symmetry (Figure 9a.). This requires a total of 26 different excitations; towards each of the six pole tips individually, between two adjacent poles, and between each set of three adjacent poles. For each of the 26 excitations, the pole tip was energized for 3 seconds, with the excitation order arranged so that the bead returned to the geometrical center of the hexapole after every 2 excitations. In this experiment, movement in the expected direction is seen, but is off
from the expected location by 6 to 12 degrees (depending on the axis of rotation). The deviation of the lab coordinate system from its theoretical location is most likely the source of this difference. Additionally, the measured maximum percent difference for the average force generated by the one, two and three pole excitations was 31%, with an estimated average force of approximately 1.5 pN. It is estimated that this average force could be easily increased to 50 pN by increasing the coil currents by a factor of three before tip saturation and reducing the distance from the center of the FCC geometry to each pole tip by a factor of two.

Small-scale, fine control of bead position is demonstrated in Figure 9 (B). Here, force vectors have been generated to sample the angle space between three poles, filling one octant of the surface of the sphere. Forces were applied in each direction for 3 seconds, with the bead being returned back to the origin after each excitation via a force in the opposite direction. The small-scale bead control (filling of the octant) shown in Figure 9 (B), combined with the symmetry data of the first experiment (Figure 9 (A)) indicates that we would be able to fill all 8 octants on the surface of the sphere, and thus, pull the bead over the full $4\pi$ sr solid angle.
Figure 9: Experimentally obtained directional force data. Large blue rods indicate pole locations, light dots indicate force directions (directions in which the bead was pulled).

(A) Axis of symmetry data where forces were applied towards each pole, in between two poles, and in between 3 poles.

(B) One octant of pole excitations.

In addition to the magnetic force directionality shown above, by operating our system in an adjustable set-point position clamp (or “position command”) mode we can select the location for a probe to be held during the course of an experiment. In general, force measurements can be grouped into the canonical categories of force clamp mode and position clamp mode. In force clamp mode, the force is maintained constant (“clamped”) while the response of the specimen as measured by the change in the position of the probe is measured. In position clamp mode, the force response of the specimen is measured as the instrumentation system adjusts the force to keep the position constant. A spring based system can be thought of as a non-ideal, passive position clamp. As the position of the probe changes, the passive spring constant alters the force to bring the probe back to the set point. Therefore, a high spring constant characterizes a position clamp system, while a low spring constant characterizes a force clamp system. A passive position clamp system can be modified to be a force clamp system by implementing a feedback loop. This is now applied ubiquitously in atomic force microscopy systems, and more recently has been implemented in laser tweezers systems. Magnetic systems can be thought of as the “dual” to laser tweezers in that they have relatively low spring constants and therefore require feedback to establish the position clamp modality. The stiffness measurement discussed in Chapter 1, Table 1 refers to the restorative force per unit
distance exerted by the manipulation modality to maintain the probe’s position while operating in a passive mode.

In addition to the position clamp mode, the location of the setpoint position may be changed during an experiment to understand, for example, the force needed to translate a probe through an inhomogeneous region of a specimen. We refer to such a modality a “position command” mode. This system relies upon a theoretical model for the force as a function of position and coil current (described above). We have implemented this technique in a software control mode and as a freehand control where a haptic input device encodes a user’s hand motion as a motion of the probe particle. As seen in Figure 10, this can be accomplished over fairly large ranges with high precision.

Figure 10: Position command mode in the Three Dimensional Force Microscope (3DFM) combined with a haptic interface[15] allows precise control over the bead’s position. The blue line is a trace of the bead position as recorded by the laser tracking position detection subsystem. The manipulation starts at the upper left hand corner of the “C” and terminates in the lower right hand corner of the “a”.
The force directionality demonstrated in this section, specifically the ability to provide true 3D manipulations, represents a significant advancement over existing magnet-based manipulators. Coupled with the precise control of our haptic based positioning system and the ability to operate this device in a position clamp mode, we believe that this instrument offers unprecedented manipulation directionality capabilities.

**Magnetic System Bandwidth**

To determine the force bandwidth of the magnetic system, 1 micron superparamagnetic beads were oscillated using a six pole, planar geometry. Test frequencies were varied from 2 Hz to 4 kHz in a discrete manner. To account for the artifacts introduced by motion of the bead relative to the poles, a control-sinusoid was superimposed on each test frequency. Motion of the bead was followed using our laser tracking system with QPD signals recorded at sampling rate of approximately 10 kHz. These signals were then mapped into XYZ position errors, which when added to the sensed positions of 3-axis piezo stage, gives displacement of bead over time. Response to each test frequency was determined in four steps. First, we took PSD of the bead position during the time-window over which excitation at that test-frequency was applied. Second, we converted the amplitude of the peak at the test-frequency to bead response in terms of amplitude. Third, for the same time-window, we computed the response to the control frequency in the same manner that was used for the test-frequency response. Finally, we normalized the response to test-frequency by the response to the control frequency. This analysis revealed that the -3dB roll off in the response function is greater than 3 kHz.
Temperature Control:

Maintaining biological samples at stable and specific temperatures becomes difficult when higher magnetic forces are required for causing more power, up to 6 Watts to achieve pole saturation, to be dissipated by the magnetic coils. With air cooling, the aluminum lid varied in response to changing coil energetics with quick temperature variations of several degrees which translates to the biological slide. To reduce this fluctuation, one needs to either increase the thermal mass or more efficiently remove the heat. Since there is a strong desire to accurately control the (normally) elevated temperatures of biological tissues compared to that of the room a larger thermal mass would not completely solve this problem.

Figure 11: Stage temperature regulation.
(A) Air-cooled lid as viewed from coil side. Note approximately a 6 °C overall rise.
(B) Water-cooled lid with significantly less heating. Not a reduced peak of only 0.8 °C rise.

Use of a slightly larger thermal mass with more cooling fins was investigated but in the small confines of the slide area in a microscope, there is not much room for effective heat
sink fins, especially that to dissipate 6 Watts. The use of blowing air from an external source also was seen to cause some vibration problems. Therefore, we investigated low velocity fluid flow. Once thoroughly investigated, the advantage to having liquid-based external temperature regulation and variable flow allowed for much more controlled temperature setting and stability for biological samples became apparent. Using 6W maximum coil dissipation per side (upper or lower half) as the power to be controlled, we set a goal for 0.1 °C variation across the central slide area and an actual temperature rise of about 1 deg C. with water being used as the thermal transport fluid. Two tubes are drilled across the lid horizontally directly behind where the heat producing coils reside. These two internal cores are fed crosswise from the top flexible hoses at a variable rate of between 0.09kg to 0.25kg water/minute depending on temperature the slide should maintain and the power dissipated by the coils.

To test the effects of coil heating on an actual specimen without using the water cooling system, .957 micron polystyrene beads were evaporated on the surface of a 150 micron thick (#1.5) coverslip. This was then placed in the sample carriage in the stage, making sure that the sample was not resting on the bottom cores. The top cores were kept above the sample by using a 1mm glass spacer to support the lid of the magnet assembly. While tracking the position of a bead attached to the glass surface, current was sent to the magnets using a step function. The magnets were ‘on’ until the position of the bead seemed to stop changing, and were then turned off. Once the current was turned off, the position of the bead was tracked until the bead position appeared to remain constant.
Figure 12: Bead motion due to heating of the coverslip during high current magnet excitation.

Figure 13: Simulated coverslip deflection due to coil radiant heat.

The results of these experiments show that heat generated by the coils not only elevates the temperature inside the specimen chamber, but that localized heating of the coverslip results in a z-drift (perceived or actual) of the tracked probe. Simulations of the heating of the specimen chamber show that high temperatures generated by the coils can radiate directly to the thin specimen coverglass. Because of its low thermal conduction properties, tends to bend into a dome shape with a peak of up to 3 microns (seen in simulation in Figure 13). To reduce this thermal concentration from the center of the
coils, an insulation layer and a copper heat spreader is used between the pole slide and the coils which reduces the deflection by over 90%. The copper heat spreader carries very little heat flux (being convection coupled only to the coils) and, therefore, its temperature variations are greatly reduced across its surface to the upper lid heat sink. This copper spreader maintains the aluminum lid’s temperature to about 0.1°C across the entire surface as observed by the slides, and resulted in less than 200 nm coverslip bowing when driven at 2.5 amps for 1 minuet. This bowing was not detected when using the water cooling. The complete temperature regulation system is shown in Figure 13.

![Diagram](image)

Figure 14: Stack-up view of magnetic manipulator with temperature regulation modifications. For applications where high currents are required for extended periods of time, room temperature water may be used to remove heat from the system. Experiments requiring biological temperatures may use the same system for temperature elevation/regulation.

### 2.5 Conclusions
The overall goal when developing this magnet based manipulation device was to bridge the gap between existing low force, non-invasive manipulation methods and the high force, invasive methods described in Table 1.1. Additionally, the as built device had to remain compatible with high N.A. optics and be capable of maintaining biologically relevant temperatures. The successful accomplishment of our design goals has resulted in a system capable of producing forces as large or larger than those previously demonstrated from a magnet based manipulator, forces in the kHz range, and forces over full three dimensions for the first time. One of the main uniquenesses distinguishing this system from others is the use of thin foil pole plates. The magnetic properties of these materials as well as the processing techniques used in their production are discussed in the next chapter.

2.6 Acknowledgements

The overall design of this electromagnet based manipulation system was a product of the ideas presented in the weekly 3DFM meetings by multiple investigators, including Dr. Richard Superfine, Dr. Russell Taylor, and Leandra Vicci and Kurtis Keller. Leandra Vicci was responsible for the development of the drive ring, coils, and drive electronics, and Kurtis Keller for developing the specimen translation stage with temperature control that houses the coils. Computer control for the device has been designed and implemented by a number of members of the Computer Science department at UNC-CH, including Dr. Russell Taylor, David Marshburn, and Ben Wilde. System performance measurements were conducted by Jeremy Cribb, Kalpit Desai, and Ben Wilde.
2.7 References


Chapter 3: Pole Processing

3.1 Introduction

A significant difference between the design of our magnetic system and those developed by other groups is the use of thin foil magnetic poles. In this chapter, techniques used to generate these poles will be discussed. The chapter will begin with an introduction to material properties that are important to consider when selecting which foils to use. This will be followed by brief description of the three processing methods we have investigated, pulsed electrodeposition, laser cutting, and acid etching, and conclude with a detailed description of the capabilities of our preferred method, acid etching.

3.2 Magnetic Material Properties and Degaussing

When selecting magnetic materials to be used for both the pole plates and the magnetic drive ring, material properties, specifically the saturation magnetization, the permeability, and the coercivity, are of utmost importance. The saturation magnetization, the resulting magnetization when all magnetic dipoles in the material are mutually aligned with the external field, combined with the permeability, a measure of the extent with which a material can be magnetized, will determine the maximum flux that can be generated for a given number of amp-turns. Coercivity is related to the residual induced field in the material (remanence) once the inducing field has been removed. It is the H
field that must be applied opposite to that of the original field to reduce the B field to zero. These properties are indicated in the B-H curve shown in Figure 1. Material properties of secondary concern for magnetic manipulators, depending on the application, are the material’s bandwidth capabilities and magnetostriction. For high bandwidth drive signals, eddy currents caused in the material due to the AC magnetic fields generate a secondary field in opposition to the original magnetic field, reducing the efficiency of the manipulator. These currents can be greatly reduced through the use of laminations that break up the circuit generating the secondary fields, or by using one thin layer of material as we have done with our pole plates. Magnetostriction, the shifting of domain boundaries in the crystal structure of ferromagnetic materials when a field is applied, results in altered dimensions that may produce significant electromechanical noise in systems utilizing large amounts of the material.

The ideal material for our application would have high saturation and permeability, low remanence, low magnetostriction, and be available in relatively thin sheets or laminations. High saturation would allow for the production of larger fields, high permeability would make these large fields easier to obtain, and low remanence would allow for a force closer to zero to be applied without the need for a degaussing routine (discussed below).
The final topic to address in this section is a technique that may be used to reduce remanence in a magnetic material known as “degauss”. In this technique, the material in question is driven back and forth (in both the positive and negative directions) on hysteresis loops (see Figure 15) of smaller and smaller magnitudes. For each of these loops, the coercive force necessary to eliminate the remanence will decrease, until finally the remanence at zero current (zero H) is undetectable. In a high-bandwidth system such as the one we have developed, an entire degaussing routine may be accomplished in a fraction of a second.

3.3 Pole Materials and Methods

Pole plates that are used to couple the flux generated by the electro-magnets down towards the specimen region have been fabricated using three different methods. The first method, pulsed electrodeposition[1-3], deposits a magnetic material on the surface of
a coverslip in a pattern defined by a photolithography process. This method has the benefit of being able to form complex pole geometries at the expense of processing complexity. With pulsed electrodeposition, we have been able to produce poles lateral resolution to about 20 microns and Permalloy films of up to 30 microns thick. An example of a “comb” style pole plate (designed for uniform force generation over a large area) developed using this method is shown in Figure 16.

Our second method, laser machining, has been used to fabricate poles from commercially available thin magnetic foils. Laser machining is capable of cutting materials that exceeded 200 microns, and offers 10 micron lateral resolution. Example laser cut geometries are shown in Figures 17 and 18. This method may be problematic due to elevated temperatures at the cutting site resulting in an altered grain structure and the formation of slag (Figure 19). This altered material has higher remanence than the stock thin foil, and as a result is problematic in low force applications. Both the laser cut pieces and those developed using the process described below rely on CAD programs to define the desired geometries. An example 3-pole CAD drawing is shown in Figure 20.

Finally, acid etching of thin films has also been tested as a pole tip manufacturing process. This process is capable of producing lateral features on the order of the thickness of the material, and etch thicknesses that exceed the requirements of this application. As shown in Figure 21, this process is capable of making 3-pole structures with inner radii of approximately 50 microns. Below this specification, material between the pole tips will not be completely removed. We have also tested tip angle (Figure 22) and tip sharpness (Figure 23) to determine as-etched dimensional limitations. Unlike the laser cut pole pieces which are machined one at a time, etched poles are made in sheets of
40, reducing the processing cost significantly. An example sheet as received by the etching company is shown in Figure 24. Each pole plate is attached to the others populating the sheet with small metal tabs that are easily bent or cut for removal from the sheet.

 Preferential etching along grain boundaries shown in Figure 25 results in pole tips that are not smooth on the micro level, but the process does not affect the magnetic properties of the material being etched. However, the etched pole materials are prone to oxidization and must be treated to either prevent any oxidation or form a passivation layer in order to prevent the shape and/or magnetic properties of the tip from being altered over time.

<table>
<thead>
<tr>
<th>Process</th>
<th>Thickness</th>
<th>Resolution</th>
<th>Max Force</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photolithography</td>
<td>Sub-micron to 50 microns</td>
<td>20 microns</td>
<td>~ 1.2 ηN</td>
</tr>
<tr>
<td>Laser Cutting</td>
<td>50 + microns</td>
<td>10 microns</td>
<td>~ 14.0 ηN</td>
</tr>
<tr>
<td>Etching</td>
<td>50 + microns</td>
<td>10 microns</td>
<td>Same as Laser</td>
</tr>
</tbody>
</table>

Table 2: Pole tip processing methods

Different commercially available materials have been tested using the laser cutting and acid etching procedures to determine what properties (saturation, permeability) will generate the largest forces at material thickness that are compatible with our high N.A. objectives. Materials and their properties are below:
<table>
<thead>
<tr>
<th>Material</th>
<th>Saturation Induction (Tesla)</th>
<th>Permeability (Oersteds)</th>
<th>Coercivity (Oersteds)</th>
<th>Remanence (Tesla)</th>
</tr>
</thead>
<tbody>
<tr>
<td>METGLAS 2605C0</td>
<td>1.8</td>
<td>120000</td>
<td>0.05</td>
<td>1.65</td>
</tr>
<tr>
<td>METGLAS Alloy 2714A (Cobalt-based)</td>
<td>0.57</td>
<td>80000</td>
<td>0.005</td>
<td>.48</td>
</tr>
<tr>
<td>Silicon-iron</td>
<td>2</td>
<td>7000</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Permalloy 65</td>
<td>1.3</td>
<td>500000</td>
<td>0.03</td>
<td>1.3</td>
</tr>
<tr>
<td>Mumetal</td>
<td>0.8</td>
<td>100000</td>
<td>0.05</td>
<td>.6</td>
</tr>
<tr>
<td>NETIC</td>
<td>2.1</td>
<td>200</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>CO-Netic AA</td>
<td>0.8</td>
<td>30000</td>
<td>0.015</td>
<td>.37</td>
</tr>
</tbody>
</table>

Table 3: Magnetic properties for core/pole materials.

Figure 16: Photolithography “comb” plate made out of Permalloy.
Figure 17: Laser cut pole geometry.  
(A) Laser cut 3-pole geometry.  
(B) Zoomed-in view of (A) indicating pole inner radius of 209 microns.

Figure 18: Laser-cut comb geometry designed to generate uniform forces over a larger area.
Figure 19: Laser-cut 3 pole geometry showing slag at the corners caused by elevated temperatures. This temperature elevation alters the magnetic properties of the material in a way that increases the remnant force on magnetic particles.

Figure 20: Example CAD drawing used to generate a 3 pole structure.
Figure 21: Minimum tip separation test used to determine minimum achievable tip separation etching .004” thick material. For inner radii less than or equal to 25 microns, material in between the tips was not fully removed.

Figure 22: Tip cartridge drawing used to test a variety of etching parameters. Maximum tip sharpness was tested using designs for sharp tips, tips with varying radii, and tips drawn with blunt ends.
Figure 23: Tip sharpness test performed using different angles from base to tip. This test was performed to determine if the desired tip shape would remain for small angles. The results indicate that for angles as small as 27 degrees the tip remains intact.

Figure 24: Tip sharpness tests. Results indicate tip dimensions (either radius or blunt) smaller than 50 microns appear the same.
Figure 25: Etched tips as received from Fotofab (Fotofab, Chicago IL). Pole tips arrive from the manufacturer as individual units connected by tabs in a 12”x18” sheet yielding 40 parts per sheet.

Figure 26: SEM images of sample etched tips. Parts a and c are at 250x, b and d at 1000x. The top two images are of pole tips that have been etched using a dry hydrogen etch process. The bottom two, c and d, are untreated.
The results of the investigations described in this section and shown in Figures 19-23 may be summarized as follows:

- For our purposes, acid etched tips have the best post-processing material properties.
- Etched tips in the 3-pole geometry may be made that have a tip spacing with an inner diameter of approximately 50 microns.
- Etched tips can taper with an angle of less than 27 degrees.
- Specified tip radii of 50 microns or less appear the same, approximately 30-40 microns.

### 3.4 Post-Etch Pole Processing

Etched pole tips received from the manufacturer in most cases have an oxidation layer consisting of, Fe$_2$O$_3$ (rust) and Fe$_3$O$_4$ (black oxide) that has higher remanence than the bulk tip material alone. As a result, when using these tips in our magnetic system the force applied at zero current is greater than initially expected. In order to reduce this remanence, the oxide layer must be removed. We have investigated two different procedures for this purpose. In the first procedure, a ‘wet’ etch consisting of a phosphoric acid (10% H$_3$PO$_4$, 90% Ethanol) mixture is used. This removes both types of oxide and replaces them with a thin (~100nm-200nm) passivation layer of iron phosphate (“gun metal blue”). The iron phosphate has lower remanence than both of the iron oxides, but it is greater than the remanence of the non-oxidized material. The second method, dry etching, has been done with hydrogen plasma alone and with hydrogen/argon plasma. Both dry etches resulted in the removal of the Fe$_2$O$_3$ with some
of the Fe₃O₄ (~100nm) left behind. This passivation layer of black oxide is difficult to remove completely for any length of time because iron by itself is unstable and wants to be oxidized. In an attempt to prevent the oxidation with black oxide, a thick (up to 1µm) layer of parylene was evaporated on to the pole surface directly after this etching procedure. Initial results suggest that this is an effective procedure for preventing the formation of the black oxide. Long term studies are currently under way to validate this observation. Example sets of images from an investigation into the ability of parylene (10-100 nm thick) to prevent oxidation are shown in Figures 27 and 18. In these images, there is no obvious change in the physical appearance of the pole tip during the course of the experiment. The brightfield images of untreated pole tips shown in Figures 21 and 23 clearly have much more oxidation (after 5 days) then those that were passivated and then treaded with parylene.

Figure 27: Oxidation study for wet etched pole tips
In addition to being useful for reducing pole tip oxidation, the application of parylene will also help to prevent cellular toxicity that may result due to contact with high nickel content materials\cite{4} such as mumetals. Because parylene coatings are pinhole-free, relatively thin, and optically clear, they are a good choice for a pole-tip coating that will not affect the specimen.

These post-etch pole processing experiments have resulted in the formation of what we believe to be an acceptable method for removing the high remanence oxide layers and preventing their reformation over relatively long periods of time. Additionally, the use of parylene has the advantage that it should protect biological specimen from any potential damage that could be caused by contact with high nickel content materials.
3.5 Conclusions

The result of these investigations has been the development of a procedure for the generation of reproducible tip geometries made from commercially available thin magnetic foils. As a result of modern processing techniques, design geometries specified using a CAD program can be turned into completed parts in less than two weeks, with an approximate cost per part of $10 ($1 raw material, $8 etching, $1 parylene treatment). The ability to tailor pole geometry to a specific experiment for a relatively low cost again confirms the notion that this instrument will be useful for a wide variety of applications.

3.6 Acknowledgements

Development of the various pole tip geometries was aided by Leandra Vicci, with Brian Stoner and Christopher Bower (both with RTI International) responsible for the long term oxidation study and the coating of the poles with parylene. Jing Hao was responsible for production of the electrodeposited poles.
3.7 References


Chapter 4: Position Detection

Our desire to make quantitative assessments of a specimen’s response to an applied force necessitates the ability to track the location of a probe before and after force application. The sensitivity requirements for our position detection can be drawn from two sources with the highest sensitivity needs; force calibration, and single molecule experiments. For the force calibration experiments, our ability to accurately calibrate the forces over a wide range is directly related to our position detection system since we use Stokes Law \( F = \gamma v \) to determine the applied force. The result of using this technique is a strong dependence on the ability to track the position of the probe with high sensitivity for low force calibrations and at high bandwidths for large forces. Additionally, the high bandwidth capabilities of our manipulation system warrant a position detection system with similar bandwidth capabilities. For biological applications, the step size of a single molecular motor (approximately 8 nm[1]) sets a reasonable limit to our position sensitivity needs.

In order to determine the position of the manipulated probe, two different techniques are traditionally used. The first and most commonly used method, video tracking, captures an entire field of view allowing multiple probes to be tracked at one time. While this basic method is relatively easy to implement, it is bandwidth limited by camera frame rates (usually not over 120 Hz) and requires post-processing routines to get better than signal pixel resolution. The second method, laser tracking, uses light scattered
by a single probe to determine the probe’s location. While high-bandwidth, position sensitivity and real-time data acquisition capabilities of this technique makes it our method of choice, unassisted this technique has a working volume that is only on the order of the wavelength of the light used. To meet our long range tracking requirements, we have implemented a feedback loop that dynamically repositions the particle to be inside this trackable volume. This capability is provided by adding a close-loop, active positioning stage driven by a control computer using active feedback to keep the particle within the optical tracker’s working volume.

4.1 Introduction

The available analytical models describing the mapping of QPD signals into XYZ position relative to the beam waist[2, 3] puts stringent constraints on the shape, size and composition of the tracked bead. For biological experiments that involve application of force, beads of larger size are preferable for force maximization. Thus, we require more flexibility in probe characteristics than offered by an analytical model. As a result, we have developed a novel technique where instead of relying on a priori model of the light scatter, we estimate the mapping function before (and potentially during) each experiment. In the following paragraphs the basic principals behind the laser tracking system will be discussed first, with novel tracking system (developed by Dr. K.V. Desai) discussed at the end of this section.

The physical principle behind the laser tracking system is interferometry, which requires a sufficiently coherent light source, for which we use a near infrared (830 nm) laser diode. The tracker uses the interference between a reference beam and a
measurement beam to produce a diffraction pattern which represents the magnitude and phase of the measurement beam in a detection plane.

In Pralle’s method[2], the laser beam is focused by a condenser lens to a diffraction limited beam waist. An objective lens is placed on the opposite side and focused on the waist to capture the exiting beam. This reference beam is in the shape of two reentrant cones between the confocally arranged lenses. In the far field, the reference beam behaves as if it were emitted from a point source located at the beam waist.

If a small sphere (bead) with an index of refraction different from that of the surrounding medium is placed within the reference beam, it will scatter some fraction of the beam. The scattering pattern can be complex, but for beads less than half a wavelength in diameter, only the principal forward scattered lobe lies within the solid angle of the reference beam. The scattered light from the bead can be approximately regarded as originating from an isotropic point source located at the center of the bead. Larger beads also behave as nearly point sources, but with non-isotropic radiation patterns. In the far field, the interference of these two beams carries 3D position information in the form of a diffraction pattern. The diffraction pattern containing bead displacement information is collected at the back aperture of the condenser and imaged on the QPD.

A bead exactly at the beam waist will scatter a measurement beam in some fixed phase relative to the reference beam. In the far field, the phase difference between the beams is uniform over angle, so the interference pattern is featureless. If the bead is displaced in $x$, a fringe pattern with an $x$ dependence will occur; similarly for $y$. 
Measuring $z$ displacement relies on a phenomenon near the beam waist known as the Gouy phase shift in which light in the near-field about the beam waist propagates at apparently superluminal speed. Intuitively, this has to happen for the concave spherical wavefronts entering the beam waist zone to deform to planar at the waist, then to convex as they leave the zone.

If the bead is displaced from the beam waist in $z$, it will scatter a measurement beam in some fixed phase relative to the Gouy-shift dependent phase of the incident beam. This $z$-dependent phase variation is propagated to the far field where it interferes with the fixed phase of the reference beam. The resultant diffraction pattern has a “bull’s eye” appearance with rings that grow and shrink with $z$ displacement of the bead.

**Implementation**

Ideally the sum of all four QPD signals is a function of $z$ displacement, while the difference between the signals from the $-x$ and $+x$ sides of the QPD is a function of $x$ displacement, and similarly for $y$. Accordingly, from four measured QPD signals $q_i$, the position of the particle may be calculated using the three functions

\[
X = (q_0 + q_2) - (q_1 + q_3)
\]
\[
Y = (q_0 + q_1) - (q_2 + q_3)
\]
\[
Z = q_0 + q_1 + q_2 + q_3
\]

However, the dependence of $(X,Y,Z)$ on the bead displacement $(x,y,z)$ is nonlinear; indeed even non-monotonic. In addition, the scattering may change significantly if the bead size, or refractive index or the refractive index of the surrounding media is to change from that used to calibrate the system.
To account for these specimen specific variations, as well as changes to the alignment of the tracking system that may occur, a bead specific tracking calibration program has been developed. Before each experiment, small amplitude noise signals are injected into the 3-axis piezo driven stage (model Nano-LP 100; Mad City Labs Inc., Madison, WI), causing the bead position to change in a calibrated manner. These small perturbations in the bead position result in small changes in the QPD signals. Correlations of the injected noise with corresponding QPD signals are then analyzed to estimate the mapping function. This newly estimated mapping function is then used by the tracking program as a bead/specimen specific calibration.

### 4.2 Imaging Optics

The force and interferometric tracking subsystems have been added to an inverted optical microscope (model TE2000-E; Nikon Instruments, Melville, NY) that sits atop a 4’ x 5’ vibration isolation table (model 78-249; Technical Manufacturing Corp., Peabody, MA). The TE2000 uses an infinity-corrected optical system, allowing easy introduction of additional components such as cameras and illuminators and simplifying the addition of the laser tracking subsystem. In this system, spherical and chromatic aberrations are corrected for by the objective lens itself.

### 4.3 Laser Analysis

At the heart of the laser (interferometric) tracking system is the laser itself. Since fluctuations in the laser’s signal as seen by the photodector will be translated into position changes, it is critical to have a low noise, high stability device. For this reason, we have
collected intensity data as a function of time for a variety of commercially available lasers. The data presented below was collected using a Newport photodetector (model 818-ST, serial # 3582) and digital power meter (model 815, serial #2836). All lasers were allowed to warm up for more than 1 hour prior to data collection, with data collected at 100Hz.

Figure 29: Thorlabs laser test. The minimal drift in this laser makes it a possibility for use in the laser tracking system
Figure 30: Melles Griot laser 1 test. Low frequency intensity fluctuations in this laser are most likely a result of the device’s internal peltier cooler switching on and off.

Figure 31: Melles Griot laser 2 test. Large intensity fluctuations in this laser (approximately 10%) prohibit its use in our tracking system.
Point Source Laser
IFLEX1000-P-2-830-0.65-35-N
Part number: 011615
Serial number: 92953
Wavelength: 824.2
Power: 36mW

Figure 32: Point Source laser. The lack of intensity fluctuations with this laser make it a good fit for our application.

As apparent from Figures 29-32, the Point Source laser’s stability is superior to the Melles Griot models tested and slightly better than the Thorlabs laser. As a result, we have incorporated this laser into our tracking system.

4.4 Tracking Optics

The optical elements used in the tracking system can be divided into three main categories; (1) conditioning optics, (2) steering optics, (3) beam expansion. The conditioning optics are used to clean up the laser beam emitted from the fiber optic at the beginning of the system. The steering optics allow x-y positioning of the beam on the back aperture of the microscope’s objective as well as control over any angular component of the beam. The beam expansion optics are used to expand the beam so that
it just overfills the back aperture of the objective.

Figure 33: Hercules model laser tracking system diagram. A 830 nm, 36 mW fiber-coupled diode tracking laser is focused through a 15 micron spatial filter by lens L1. Lens L2 collimates the laser at the far side of the spatial filter before it passes through a neutral density filter (NDF 1) that decreases the optical power to 1.4 mW. The laser passes through the filter and on to mirror M1 which is responsible for axial translations of the laser beam through the rest of the lower optics system. Mirrors M2 and M3 steer the beam to BS1, which adjusts the angle of the beam entering the back of the objective. From BS1 the beam enters the back of the microscope where D1 (dichroic) directs the beam vertically through the objective. Lenses L3 and L4 expand the beam twofold to slightly overfill the back aperture of the objective lens (model Plan Apo 60x/1.20 WI; Nikon Instruments Inc., Melville, NY). Laser light passing through the specimen (.025 mW at specimen plane) is collected by a 100x, 0.7 NA air immersion Mitutoyo (model 378-806-2; Mitutoyo America Corporation, Aurora, IL) lens acting as the condenser. Hot mirror (H1) reflects longer wavelengths towards the QPD and allows the shorter wavelengths to pass. Lens L7 images the back focal plane of the objective on to the QPD.
(making the two conjugate pairs). Lens L8 forms a plane conjugate of the BFP of the condenser and the QPD for the purpose of imaging the diffraction pattern as seen by the QPD. BPF2 is an 830nm bandpass filter designed to only let the tracking laser through to the CCD. This CCD is used for the centering of the bead’s diffraction pattern on the QPD prior to the initiation of the tracking algorithm.

(1) Conditioning optics

Upon exiting a fiber optic, the tracking laser is focused using a short focal length lens on to a spatial filter to eliminate spatial varying intensity noise. In order to select the appropriate size for the spatial filter for a given input beam diameter and lens focal length, the following equations were used:

Angle of beam (\( \theta \))

\[
\theta = \tan^{-1}\left( \frac{R_{\text{beam}}}{FL_{\text{in}}} \right)
\]

Beam waist radius (\( R_{\text{beam}} \)) = \( \frac{\lambda}{\pi(\theta)} \)

The following parameters describe our system:

Laser wavelength (\( \lambda \)) = 835nm

Collimated beam size = .7mm

Focal length input lens = 8mm

As a result, our beam waist radius is calculated to be

\[
R_{\text{beam}} = \frac{835 \times 10^{-9}}{\pi(0.0437)} = 6.1 \times 10^{-6},
\]
resulting in a beam diameter of 12.2 microns.

By focusing the laser using an aspheric lens, the input beam is transformed into a central Gaussian spot with side fringes that are a result of the unwanted spatially varying intensity noise. By centering the Gaussian spot on the spatial filter, we can pass the clean portion of the beam and filter out the noisy portion. A spatial filter approximately 30% larger than the beam diameter calculated above is used to in order to eliminate this unwanted noise. The closest fit, and as a result the spatial filter used, has a pinhole size of 15 microns. Once the laser has passed through the pinhole, it expands until it reaches a collimating lens with a focal length of 35 mm.

(2) Steering optics

From this point, the collimated beam travels to a steering mirror that is responsible for lateral translations of the beam at the back aperture of the microscope objective (mirror M1 in Figure 5). Mirrors M2 and M3 are used to increase the path length so that the necessary beam expansion (discussed next) may be accomplished. The angle of the beam entering the back aperture of the objective is controlled using M4.

(3) Beam expansion

To completely overfill the back aperture of our most commonly used objective, a Nikon 1.2 NA 60x, the beam diameter must be larger 9mm. Anticipating the possibility that another objective used in this system may have a larger back aperture, the goal here will be to expand the beam to at least 10 mm. The reduction in laser power transmitted
through the objective due to this overfilling is not a major concern since we already plan to decrease this intensity using neutral density filters. To accomplish the necessary expansion we use a two section system. In the first section, both standard and Galilean beam expansion techniques are used. A 35 mm focal length lens placed one focal length (35 mm) away from the spatial filter collimates the beam. The beam expansion performed here is simply the focal length of the collimation lens (35 mm) divided by the focal length of the input lens (8 mm). The resulting 4.375x expansion is increased with the use of a Galilean telescope. Here, a lens with a negative focal length (-50 mm) creates a virtual focal point behind the lens location. From this virtual focal point the beam expands until it reaches a collimation lens placed one focal length (88 mm) away from the virtual focal point. This two lens system expands the beam another 1.76x, making the total magnification of this system 7.7x (see Figure 34). The resulting beam diameter after this section is 5.38 mm.

**7.7x Magnification**

Figure 34: Optics used for the first section of the beam expansion. These optics increase the beam diameter from the 0.7 mm to 5.38 mm.
The second set of lenses used to expand the beam is a simple two lens system with the first lens having a focal length of 150 mm and the second lens a focal length of 300 mm. Placing the second lens one focal length away from the first lens’s focal length results in a 2x beam expansion. The resulting beam diameter at the end of this section is 10.76 mm. This is large enough to meet our requirements for overfilling the back aperture of the objective lens.

4.5 Laser Tracking Electronics

4.5.1 Quadrant Photodiode Wiring

Quadrant photodiode (QPD) Wiring for Centrovision (Centrovision Inc., Newbury, CA) QD7 quadrant photodiode combined with position sensing circuitry. This QPD has been modified to have a 40 kHz cutoff freq (done by Centrovision), and the resistor network R1 has been replaced with a 470 kohm network to increase the gain. Also, the original photodetector has been replaced by QD-0.5-0 (lot #21191) because of its smaller gaps between the quadrants. The detector is set up to be differential, all quadrants are paired with the QPD Gnd channel (this is the detector of the can which shares a ground with the power supply and the QPD bias).

QD-0.5-0 (lot #21191).

Pin 1: QPD Out 0
Pin 2: QPD Out 1
Pin 3: QPD Out 2
Pin 4: QPD Out 3
Pin 5: QPD Gnd
Pin 6: V+ (12 Volts)
Pin 7: V- (-12 Volts)
Pin 8: Supply Common
Pin 9: Blank
Figure 35: Position sensing circuit supplied by OSI Optoelectronics (Newbury Park, CA) (formerly Centrovision). Individual quadrant signals are picked off prior to the difference amplifier.

Figure 36: Wiring diagram showing the back of the position sensing circuit board.
4.5.2 Three-Axis Closed Loop Piezo Stage

For the feedback enhanced tracking system, the tracked probe’s position inside the laser is maintained through the use of a closed loop three axis piezo stage (Mad City Labs, NanoLP-100, Madison, WI). This device has a full range of 100 microns in each axis, and is bandwidth limited to 200 Hz.

4.5.3 Computer Data Acquisition

The tracking computer uses an analog output board (model PCI-6733; National Instruments, Austin, TX) for stage positioning, and a multifunction I/O board for stage, QPD, and laser-intensity sensing (model PCI-6052E; National Instruments, Austin, TX).

4.6 System Performance
The position detection sensitivity of the completed laser tracking subsystem has been tested using 0.957 µm polystyrene beads (Polysciences, Inc., Warrington, PA) immobilized in agarose. To test the system, a single bead was placed at the beam waist of the tracking laser, resulting in a diffraction pattern on the QPD similar to the one shown in Figure 38. The bead was then moved by 4 nm square pulses in the positive x-direction using the 3-axis piezo driven stage, resulting in a lateral shift of the diffraction pattern in Figure 38. In Figure 39, the resultant QPD signal for a series of 4 nm displacement is shown for 3 different bandwidths of the measurement, i.e. 10 kHz, 1 kHz, and 100 Hz. Here, the steps are noticeable even at the full bandwidth rate (10 kHz), with the signal to noise ratio increased by filtering down to 100 Hz (approximately the bandwidth of a video tracking system). From these experiments we have determined that the lateral resolution of the system is 2.4 nm at 10 kHz. A similar experiment for the axial resolution of the system resulted in a value of 4.4 nm at 10 kHz.

Figure 38: QPD camera image showing bead diffraction pattern (dark rings). 4 nm input steps used to test the system performance result in horizontal and vertical shifts in the dark ring. Movements along the Z-axis cause the rings to grow larger and smaller.
Figure 39: Tracking system response to 4-nm displacement. QPD signal for 3 bandwidths of the measurement, i.e. 10 kHz, 1 kHz, 100 Hz are shown. DC offsets were added to the 1 kHz and 10 kHz datasets in the figure for clarity.

These results exceed our most stringent requirement of approximately 8 nm position sensitivity in all 3 axes. Additionally, the high bandwidth capabilities of our detection method ensure that we will be able to take full advantage of the high bandwidth manipulation system we’ve developed.

4.7 Conclusions

In this chapter we have discussed the development of a laser tracking system that offers high position sensitivity at a high bandwidth while remaining compatible with a commercially available inverted microscope. The main components of the system have been discussed in detail, with specific attention paid to the stability of the laser at the heart of the tracking system. Two sections related to the laser tracking system may be found in the appendix. The first section describes the initial setup and alignment of the optics necessary to implement the interferometric technique as well as image the bead’s
diffraction pattern on the photodetector. The second section discusses the daily alignment procedure necessary to insure optimal performance of the system.

4.8 Acknowledgements

Design of the optics system used to condition the laser beam and image the bead’s diffraction pattern on the QPD was assisted by Dr. Richard Superfine. Adaptors developed to allow the mounting of the QPD and the QPD camera to the upper optics assembly, as well as the hot mirror cube designed to allow mounting of the upper optics assembly to microscope, were designed by Kurtis Keller. The control system developed for probe position detection was developed by Dr. Kalpit Desai. Data acquisition hardware selection (and maintenance) and development of the user interface designed to display position data and force data was led by Dr. Russell Taylor, David Marshburn and Ben Wilde.
4.9 References


Chapter 5: Magnetic Force Calibration

5.1 Introduction

The relationship between the current sent to the electromagnet’s coils and the magnetic force applied to a probe is dependent upon the magnetic properties of the probe, the magnitude of the field at the probe, and the gradient of the field at the probe. Drastic changes in these quantities at different locations with respect to the pole tip necessitate the development of techniques (or application of existing techniques) in order to have a unique spatially varying calibration for each pole tip or probe location. In this chapter the most common approach used to calibrate magnet based manipulators, the use of Stokes Law, will be discussed. Additionally, a unique method relying on the high bandwidth capabilities of our 3DFM that will allow for the calibration for given pole (and pole location) as well as a specific probe and probe location to be developed.

5.2 Stokes Calibration

The traditional force calibration technique for electro-magnet manipulators is based upon Stokes formula \( F = 6 \pi \eta a_b v \), where \( \eta \) is the fluid viscosity, \( a_b \) is the bead radius, and \( v \) the bead velocity) and uses the velocity of magnetic beads pulled through a fluid of known viscosity to calculate the applied force. Particle velocity may be determined using either video or laser tracking techniques. A maximum intensity projection from an example data set is shown in Figure 40. Here the bead trajectory over
multiple frames can be seen, with the bead velocity (indicated by the space traveled by a bead in between frames) increasing significantly as the bead approaches the pole tip. In Figure 41, maximum achievable forces on two different bead types (1 µm and 2.8 µm) are plotted.

Figure 40: Maximum intensity projection showing paths of 4.5 µm beads during calibration experiment.
Figure 41: Force vs. Distance. Maximum forces obtained for 1µm and 2.8 µm superparamagnetic beads using Netic pole-flat geometry.

<table>
<thead>
<tr>
<th>Material</th>
<th>Bead Size</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Netic</td>
<td>1 µm</td>
<td>.8 nN</td>
</tr>
<tr>
<td>Netic</td>
<td>2.8 µm</td>
<td>2.6 nN</td>
</tr>
<tr>
<td>Netic</td>
<td>4.5 µm</td>
<td>14 nN</td>
</tr>
<tr>
<td>Co-Netic</td>
<td>1 µm</td>
<td>.3 nN</td>
</tr>
</tbody>
</table>

Table 4: Example maximum forces.

5.3 Force Ratio

Remnant magnetization of the poles at zero current (a result of hysteresis of the pole material) will result in a non-zero force when the current is removed. As a result, a more useful measurement of the range of applicable forces for various systems is a ratio of the maximum achievable force divided by the remnant force (assuming no Degauss
procedure). Typical Netic pole pieces with a tip radius of 10 um can produce maximum forces on a MyOne bead of 800 pN with a maximum remnant force of approximately 6 pN (resulting in a force ratio of 133). In similar experiments, a degaussing routine has been shown to decrease the remanence to a level where it is no longer detectable. For applications where small forces are required, the specimen may be positioned farther away from the pole tip, resulting in less maximum achievable force but maintaining the same force ratio.

Figure 42: Position vs. Time graph showing the effects of a degaussing routine at time t = .25

Variability in the magnetic material content inside the paramagnetic beads used for manipulations will ultimately result in errors in the quantity being measured with the magnetic system. In order to determine the error bars that are appropriate for a given magnetic bead experiment, the bead to bead variation in force for a specific bead type must be determined. This is accomplished by subjecting a population of beads to a
constant magnetic field and field gradient, and then determining the variation in bead velocity in order to ultimately determine the variation in bead force. An example of such an experiment is shown in Figure 43. The setup shown in Figure 43 A was used to determine the field and field gradient for the test permanent magnet, with the results shown in Figure 43 B.

Figure 43: Bead force variation experimental setup.

(A) Diagram showing the setup used to measure the field and field gradient for the magnet used in the bead to bead variability test.

(B) Resulting B-field (x-axis in mm). Over the length scale traveled by the beads in this experiment it is reasonable to assume the gradient is constant.

Knowing that the field and field gradient across a field of view using the 60x objective (approximately 100 microns) is relatively constant, we may assume that variations in bead force are caused solely by bead specific properties such as shape and magnetic properties. As shown in the SEM insert in Figure 44, the size of the beads used in this experiment do not vary significantly, leading us to believe that the main reason for the ± 10% difference in force is due to variations in the magnetic material content inside each bead.
Figure 44: Variation in forces applied to 2.8 micron beads due to differences in bead size and magnetic content. Here 21 Dynal 2.8 µm beads are pulled using a permanent magnet with a constant field (approximately 0.0057 T) and field gradient (0.5 T/m). The average force per bead is 0.087 pN with a bead to bead variation of ±10%. Determination of this variation is required for properly estimating errors in force measurements.

5.4 Driven (In Situ) Calibration

As seen in Figure 44, regardless of how well forces are known for a specific location with respect to the pole tip, variation in the magnetic content of the beads can cause rather large differences (±10%) in the applied force. In addition to this source of calibration error, the accuracy of a given calibration will be heavily dependent on the ability to determine if a probe in a subsequent experiment is in exactly the calibrated location. Laterally this can be accomplished with high precision, but difficulties selecting exactly the same focal plane in Z can cause position errors on the order of microns. Far away from the pole tip this positioning error may not be significant, but for high force applications this may not be the case. As a result, the ability to perform a bead specific
calibration, at the location of interest in a given experiment would represent a significant step in eliminating errors in calibration.

For a non-tethered bead free to diffuse in a Newtonian fluid, short bursts of magneto-motive force (in response to coil currents) may be used to perform a Stokes Law calibration for a specific bead in a given location. This task becomes more difficult for the case when the bead of interest is tethered to a structure such as a molecule of DNA or a cell membrane. To address this scenario, we have taken the first steps in developing an \textit{in situ} calibration technique that uses the 3DFM to probe potential wells in a method that is similar to techniques used for the calibration of an atomic force microscope (AFM) or laser trap. To begin discussion of this technique, we will use data collected from a laser trap calibration experiment and relate how it applies to a scenario where the magnetic calibration target is a bead tethered to DNA or a cell membrane.

A common approach for the calibration of a laser trap is to use the amplitude of a probe’s position fluctuations inside the trap to determine the trap stiffness or spring constant ($k$). This quantity describes the restorative force that is applied to the probe as it moves away from the center of the trap. The spring constant may be determined using techniques based on either time domain[1] or frequency domain[2] data. In the time domain, the position histogram shows a Boltzmann distribution. This histogram may then be converted to an energy profile (multiply the log of the counts in each bin by the temperature and Boltzmann’s constant) and fit to a quadratic ($F = \frac{1}{2}kx^2$) to determine the spring constant. In Figure 45 (A), the position histogram for a 1 µm polystyrene bead trapped in a laser trap incorporated in to one of our microscopes (see appendix 3) is shown. In Figure 45 (B), this histogram has been converted to energy (blue line) and fit
to a quadratic to determine the spring constant (red line). The resulting spring constant is in units of pN/nm.

Figure 45: Spring constant determination.

(A) Position histogram showing the step size for a 1 micron bead in a laser trap.

(B) Position histogram converted to energy (blue line). This data is fit to a quadratic to determine the spring constant \( k \) for the trap.

In the frequency domain, the cross-over frequency \( f_c \) of the power spectrum along with the 1 Hz intercept \( S_0 \) may be used to determine the spring constant using the following relation:

\[
k = \frac{2K_b T}{\pi^2 S_0 f_c^2}
\]

where \( K_b \) is Boltzmann’s constant, and \( T \) the temperature in Kelvin. In addition to the spring constant (but unrelated to this discussion) the viscosity \( \eta \) of the media may be determined from the following expression:

\[
\eta = \frac{K_b T}{6\pi 10^8 r_{head}}
\]
where B is the 1 Hz intercept of the portion of the data with a slope of -2 and $r_{\text{bead}}$ is the radius of the bead. An example data set (simulated data) with these parameters labeled is shown in Figure 46. In practice this approach is more difficult to apply in instances where the spring constant is weak due to the fact that the cross over will be at a low frequency where system drift has a tendency to corrupt the data.

Figure 46: Example PSD showing components used to determine the viscosity and spring constant.

Using the same principals described above to determine the spring constant for a bead in laser trap, it is possible to determine the spring constant for a bead tethered to DNA or attached to a cell membrane. In Figure 47, experimentally obtained data showing the change in spring constant as DNA is extended is shown. In the case of DNA, the spring constant isn’t Hookean (linear) for all forces, as demonstrated by the worm-like chain (WLC) model (Figure 48) that has been shown to fit force vs. extension data for dsDNA very well. However, for small displacements around a given extension it
may be approximated as being linear. As a result, if we are able to determine a spring constant for DNA at a given extension and then inject small amounts of position noise we should be able to determine the corresponding force for an input current. This resulting calibration will be both bead specific and location specific.

Figure 47: DNA manipulation alters the spring constant.

(A) Repeated extensions of the same dsDNA strand as seen by the 3DFM-U1. The decreased amplitude of position fluctuations with extension (shown by yellow trace) indicates that the spring constant is changing with extension.

(B) Example data set used to determine the spring constant for a bead attached to DNA at different extensions of the DNA. In this data, the spring constant changes by a factor of two between the maximum extension from 20-50 sec. and the subsequent extension at approximately 110 sec.
Figure 48: Worm-like chain model describing force vs. extension of dsDNA.

(A) Log-linear force vs. extension plot for a 7.3 micron long dsDNA chain with a persistence length. Inset figure shows the linear-linear plot for the same parameters.

(B) WLC spring constant vs. extension.

As seen in Figure 48, the range over which the spring constant may be considered to be linear decreases with extension. Therefore the input position fluctuation about the point where the spring constant was determined should be smaller at higher extensions. Since the spring constant is much larger here at high extensions, thermal fluctuations of the probe’s location are significantly smaller, increasing the sensitivity for small position changes caused by additional current sent to the magnets.

With the force-extension behavior of DNA well known (Figure 48), it should be possible to compare results generated using the calibration technique described above to values expected from the WLC model to validate this technique. This is the remaining step in our development of this in situ calibration technique that is to be applied to tethered probes.

5.5 Conclusion
Calibration techniques discussed in this section provide the ability to determine a force vs. coil current relationship for magnetic beads manipulated using the 3DFM. For the case where a general calibration over an entire field of view is desired, a Stokes Law calibration procedure performed on a large collection of beads will allow the variation in forces with respect to pole tip location to be determined at various currents. This calibration will vary by approximately ± 10% due to variation in the magnetic contents of the bead alone, and will not apply to beads already tethered to a target molecule or membrane. For the case of a tethered bead, we have described an in situ calibration technique that should allow for the calibration of a specific bead using methods related to calibration techniques used in laser traps. Final verification of this method is still pending, but initial experiments performed on a bead attached to a single dsDNA molecule suggest that the approach is valid.

5.6 Acknowledgements

The Stokes Law calibration section in this chapter, specifically the collection of data and development of magnet drive and data analysis programs, received significant contributions from both Jeremy Cribb and David Bober.
5.7 References


Chapter 6: Beating Cilia Manipulations Using the 3DFM

This experiment was performed using our 1st generation 3DFM system[1]. Here, applied forces are much lower than what is obtainable from the second generation system, but the higher bandwidth of the feedback portion of the 1st generation stage used for particle tracking made laser tracking possible. This is part of an on going investigation into the forces exerted by cilia and the response of cilia to external force. Our interest in cilia, particularly human lung cilia, stems from our desire to aid in the development of a model that describes mucociliary clearance in the healthy and diseased lung.

6.1 Introduction

Cilia are ubiquitous actuating structures in biology, present in unicellular, simple multicellular and complex multicellular organisms including vertebrates. The rapid oscillation or “beating” of cilia is used for locomotion and translocation of food particles by unicellular ciliates such as paramecia. Cilia are also used for transporting food particles and fluids by stationary multi-cellular organisms such as tunicates[2]. Of the many functions served by cilia in humans, we are primarily interested in their role in the clearance of infectious agents and particulates from the lung, also known as mucociliary clearance.
The alveolar surface of the human lung is bathed in a thin aqueous layer covered by a thicker and more complex layer of mucus (see Figure 49). Mucus is a nonuniform viscoelastic fluid composed of mucin proteins, glycosaminoglycans and cellular remnants such as actin and DNA. The mucus layer sticks to and traps particulates, bacteria and other infectious agents for removal. The beating cilia propel this layer out through the airways toward the throat[3, 4], where the mucus and its entrained detritus are swallowed and disposed of safely in the gut. A basic understanding of this process and how it can fail is fundamental to several important research areas: environmental factors affecting lung function, developing new drug delivery methods, studying the underlying causes of such diseases as cystic fibrosis[5], and the development of developing new treatments for these diseases.

Figure 49: In the healthy human airway the coordinated beating of cilia propels mucus, trapped dust and pathogens out of the body. The cilia beat within the periciliary liquid (PCL) layer between the mucus and the cell surface.
The pattern of the ciliary beat, and the force applied by the forward, or “power” stroke of the cilia tips as they engage the mucus, are essential to understanding how the cilia propel mucus. Previous experiments studying the beat pattern have employed optical microscopy and high speed video imaging[6-9], both inherently two-dimensional instruments. The measurements of forces exerted by cilia have heretofore been limited to the compound cilium of single cell organisms[10] and to the flagella of bull sperm[11]. In this study, we have used the 3DFM to track the motion of cilia in living human lung cell cultures and to explore their response to applied forces.

6.2 Methods

For cilia preparations, human airway epithelial cell cultures were obtained and grown on T-COL membrane supports (Costar, Cambridge, MA), as described by Matsui[12]. Cultures were deemed ready for use when evidence of cilia motion or “hurricanes” were visible with a 10x objective on a phase contrast microscope. The ciliated cultures were washed with PBS, and then the apical surface was treated with 10 mM DTT in PBS for 5 min. Gentle washing of the cultures with 3 changes of PBS was used to remove the mucus layer. The apical surface was then treated with a 1/1000-1/10,000 dilution of biotinylated wheatgerm agglutinin in PBS (Vector labs, Burlingame, CA), for 10 min, and washed 3 times with PBS. Streptavidin-coated superparamagnetic polystyrene microspheres of either 0.9 (Spherotech, Inc. Libertyville, IL ) or 1.0 µm diameter (Dynal Biotech, Oslo, Norway) were added undiluted to the apical surface of the cultures and pipetted many times onto the centers of the cultures. Cultures were then
incubated at 37° for 10 min, and beads were pipetted again onto the centers, then washed gently once with PBS to remove unattached beads.

To allow for use in our system, the culture and membrane were then cut from their plastic support and gently cut into fourths with a sharp razor blade and scalpel. Each fourth was then placed onto an 18×18 mm glass coverslip (number 1, Fisher Premium (Fisher Scientific, Pittsburg, PA), with the apical side up. Approximately 10 uL Airway medium and Hepes-buffered airway medium (25 mm, pH 7.5) were added to each specimen. A second coverslip was placed over the first, using a border of 2 layers of double sided tape (3M Corporation, St Paul, MN) to create the specimen chamber deep enough to allow the cilia to beat unrestricted. The cilia continued to beat vigorously for at least an hour in these chambers, and often for several hours. On occasion, after extended experimentation (typically greater than 2 hours) and an observed decrease in ciliary bead frequency, specimen chambers were reopened and the media replaced. This resulted in an increase in the ciliary beat frequency back to its initial levels.
6.3 Results

Magnetic beads were attached to beating cilia and tracked using our laser tracking system. A real-time 3D trajectory of a bead attached to the cilia was measured, the plot of which is shown in Figure 51, with an approximate beat frequency of 15 Hz. Depending on the age of the culture a range of beat frequencies were observed, with healthy beating seen for the first hour or so (Figure 52). The bead size of 1 µm and the spacing between cilia being less than 300 nm precludes our being able to definitively rule out multiple attachments at this time. A stroke length of 3 µm was measured for the trajectory of the cilia beat. Our tracking system is capable of measuring the beat cycle in full 3D, significantly including the z motion of the cilia tip. This vertical component of motion is understood to be a critical factor in the operation of the cilia-mucus system as it allows the cilia to retract into the less viscous lower layer during the retraction stroke. During the power stroke, the cilia are at their greatest extension, allowing them to couple more effectively to the more viscous overlying mucus. This z motion is observed in our 3D traces.

Magnetic forces were applied to the cilia via the attached magnetic beads in an initial attempt to explore the response of cilia to forces. Figure 53 shows repeated force application of force to the same bead-cilia complex. In Figure 54, sequential excitation of individual poles results in the average position of the orbit of the cilia being shifted in the direction of the energized pole. Since the poles lie at the corners of a tetrahedron for
this system, an energized pole will in general pull a bead both laterally and vertically at the same time. This was observed. The applied magnetic force, estimated to be about 2 pN, appears to be enough to shift the beat trajectory significantly without stalling the cilia. This is consistent with the measurements performed on sperm flagella where a stall force of about 250 pN was measured[10]. Current experiments are being conducted to measure the force necessary to stall a beating cilium.

Figure 51: Three dimensional trace showing the motion of a bead attached to cilia over approximately 2 beat cycles.
Figure 52: Power spectrum of tracked bead showing a beat frequency of approximately 15 Hz.

Figure 53: Y axis displacement for a bead attached to beating cilia as a force of about 2 pN is applied in twenty second intervals.

Figure 54: 3D position of a magnetic bead attached to cilia with no force applied and force application towards four different pole pieces. Maximum applied force is estimated to be 2 pN.

6.4 Conclusions
The results of these experiments indicate that our magnet-based manipulation device with three dimensional laser tracking may be used in experiments involving biological specimen. The ability of this first generation system to alter the beat pattern of the cilia but not detectibly diminish the amplitude of the beat indicates that the forces required to stall beating cilia may be significantly larger than those generated here. As a result, similar investigations utilizing the high force capabilities of the magnet based manipulator discussed in Chapter 2 are currently ongoing.

6.5 Acknowledgements

Construction of the first generation 3DFM was aided by the efforts of Dr. Jeremy Cummings, Leandra Vicci, and Kurtis Keller. Tissue cultures used in this investigation were provided by Dr. Hiro Matsui, with specimen preparation and general consul provided by Dr. William Davis. Dr. Tim O’Brien developed the bead attachment protocol as well as the method used for removal of the cultures from their culture wells. Data collection and analysis assistance was provided by Jeremy Cribb and Ben Wilde. Dr. David Hill and Ashley Estes are currently performing investigations into the forces required to stall beating cilia.
6.7 References


Chapter 7: DNA manipulations

Whereas the cilia experiments described in previous chapter will require the generation of large forces (estimated in excess of 800 pN) for successful completion, the relevant forces for single molecule experiments are significantly lower. This chapter will begin with a discussion of polymer models relevant to DNA, including the worm-like chain (WLC) model, used to describe the behavior of these molecules under tension. In addition, chain entropy associated with stretching and relaxation will be introduced to prime further discussion in Chapter 8. After this theoretical section, results from DNA manipulation experiments utilizing the 3DFM will be presented, including an investigation involving the application of broad band force and an investigation into the forces associated with the disruption of nucleosome-DNA binding.

7.1 Introduction

Deoxyribonucleic Acid (DNA) is a right-handed double helix consisting of a sugar phosphate backbone and attached bases connected to a complimentary strand by hydrogen binding. The base pairs in this structure are separated by .34 nm, with 10 base pairs per turn of the helix. DNA encodes the sequence of amino acid residues (amino acid after –OH group removed) in proteins.

7.2 Polymer Models
Single stranded DNA (ssDNA) and double stranded DNA (dsDNA) can be modeled as part of a category of polymer chains known as ideal chains. Ideal chains are polymers in which interactions between monomers that are far apart on the chain do not exist, even if the conformation of the chain causes the monomers to approach each other in space. It is the conformation of this ideal chain, and restrictions placed upon its movements, that serve as a starting point for most molecular models in polymer physics.

The variety of conformations available to an ideal chain is mainly a result of variations in the torsion angles of the chain bonds. Changes in the torsion angle of a bond will result in energy variations, with the lowest energy conformation being the trans state of the torsion angle. Consecutive trans states of torsion angles result in a rod-like zig-zag conformation of the chain (Figure 55). When the entire chain takes on this conformation, it has its largest possible end to end distance ($R_{\text{max}}$). This distance is referred to as the contour length of the chain and is determined by the following equation[1]:

$$ R_{\text{max}} = nl \cos \left( \frac{\theta}{2} \right) $$

Where $n$ is the number of main-chain bonds and $l$ is the length of an individual segment.
Figure 55: Consecutive trans-conformations of a polymer chain with \( n=8 \) main chain bonds.

The average end-to-end vector of an isotropic collection of ideal chains with \( n \) backbone atoms is \( <R_n> = 0 \). This is an ensemble average, \( < > \), and can be determined by taking many conformations of the same chain or a single conformation of many chains. If we assume that our polymer has a constant bond length and no correlation between the different bond vector directions, we have one of the simplest models of an ideal polymer, the freely jointed chain model. For this case, the mean-square end-to-end distance is given by the equation:

\[
<R^2> = nl^2
\]

Flexible polymers can be described in a way that takes into account all of the universal properties that are independent of local chemical structure. This is done with the use of an equivalent freely jointed chain (FJC), a structure that has the same mean-square end to end distance, \( <R^2> \), and maximum end-to-end distance \( R_{max} \) as the polymer, but has \( N \) freely jointed effective bonds of length \( b \) (bond length \( b \) know as the Kuhn length).

Again from Rubinstein[1], this results in the following expressions for the equivalent freely jointed chain:

- **Contour length**: \( Nb = R_{max} \)
- **Mean-square end-to-end distance**: \( <R^2> = Nb^2 = bR_{max} \)
- **Kuhn monomer length**: \( b = <R^2>/ R_{max} \)

### 7.2.1 Freely Rotating Chain Model
If the differences between the probabilities of the different torsional angles in the polymer are ignored, and the probability of all torsional angles from $-\pi$ to $\pi$ are considered equal, the result is the freely rotating chain model. In addition to the aforementioned constraints, this model assumes that all bond lengths and angles are constant. This results in a mean-square end-to-end distance given below.

$$\langle R^2 \rangle = n l^2 \left( \frac{1 + \cos(\theta)}{1 - \cos(\theta)} \right)$$

In describing this model, the number of main-chain bonds in a persistence segment, $s_p$, is introduced. This is related to the length scale at which local correlations between bond vectors decay according to the following expression:

$$s_p = -\frac{1}{\ln(\cos(\theta))}$$

### 7.2.2 Worm-like Chain (WLC) Model

The worm-like chain model (a.k.a. Kratky-Porod model) is a special case of the freely rotating chain model restricted to very small bond angles. From Rubinstein[1], for small bond angles, the $\cos(\theta)$ can be expanded about its value of unity at $\theta = 0$.

$$\cos(\theta) \approx 1 - \frac{\theta^2}{2}$$

and for small $x$, $\ln(1-x) \approx -x$, resulting in the denominator $-\frac{\theta^2}{2}$. Therefore, the number of main-chain bonds in a persistent segment for the WLC model will be
\[ s_p = 2/ \theta^2 \]

The persistence length is the end-to-end length of the segment described above, and is equal to:

\[ l_p = s_p l = l(2/ \theta^2) \]

with the corresponding Kuhn length equal to twice the persistence length. According to Rubinstein[1], the mean-squared end-to-end distance of the worm-like chain is:

\[ \langle R^2 \rangle = 2l_p R_{\text{max}} - 2l_p^2 [1 - \exp(-R_{\text{max}}/l_p)] \]

The important difference between worm-like chains and freely jointed chains is that worm-like chains are not assumed to be completely rigid on length scales shorter than the Kuhn length, they can fluctuate and bend. It is this fluctuation of the contour of the chain rather than the changes in bond rotation that result in conformational changes and the model’s dependence of extensional force on elongation near complete extension.

**Radius of Gyration**

While determining the mean-square end-to-end distance for a linear polymer has been shown to be a trivial procedure, the lack of two well defined ends in branched or ring polymers necessitates the use of another size defining parameter. As a result, the ubiquitous radius of gyration may be used to define the size of all polymers. Again from Rubinstein, the square radius of gyration is defined as the mean square distance between
monomers in a given conformation and the center of mass of the polymer. For an ideal linear chain, the radius of gyration is:

\[ <R_g^2> = \frac{b^2 N}{6} = \frac{<R^2>}{6} \]

**Chain Motion as a Random Walk**

According to Boltzmann’s Law, a molecule always tends towards its lowest energy state. However, molecular collisions prevent a molecule from spending all of its time at this lowest energy state and instead force the molecule into states with higher energy. These collisions cause the molecule to move in random, frequently changing directions, also known as a random walk.

The collection of possible conformations for an ideal chain can be mapped onto a random walk where the length of each step is constant and the direction of each step is independent of all previous steps. The probability for such a function is highest at the origin and falls off quickly away from the origin. Because of the shape of this function it is usually approximated using a Gaussian. The mean-square displacement of a random walk of \(N\) steps from the origin is equal to the mean-square end-to-end vector of a chain of \(N\) monomers with the length of each monomer, \(b\), equal to the step size of the random walk, \(<R^2> = Nb^2\). This displacement is composed of displacements from each of the three independent dimensions, with the contribution from each dimension being one-third of the total and equal to \(Nb^2/3\). The corresponding 1 dimensional probability function, useful for determining the likelihood that the chain vector will lie between \(\vec{R}_x\) and \(\vec{R}_x + d\vec{R}_x\), will be,
\[ P_{1d}(N, \vec{R}) = \frac{1}{\sqrt{2\pi} <x^2>} \exp\left(-\frac{x^2}{2 <x^2>}\right) \]

For three dimensions this will be the product of three independent distribution functions, which, substituting for \( <R^2> \) gives the following:

\[ P_{3d}(N, \vec{R}) = \left( \frac{3}{2\pi Nb^2} \right)^3 \exp\left(\frac{3R^2}{2Nb^2}\right) \]

The resulting probability distribution for the end-to-end distance \( \vec{R} \) will be the probability for the vector to be in the spherical shell (because 3D) with a radius between \( \vec{R} \) and \( \vec{R} + d\vec{R} \). The Gaussian approximation used here will be valid only for end-to-end vectors that are significantly shorter than the maximum extension of the chain due to the fact that it predicts a finite probability for \(|R| > Nb\). For real chains this should be zero. This has been addressed by Marko and Sigia[2], resulting in the following expression that describes the response of the polymer to an extensional force, \( f \), as

\[ \frac{fb}{k_b T} \approx \frac{1}{2} \left( \frac{R_{\text{max}} - <R>}{R_{\text{max}} - <R>} \right)^2 - \frac{1}{2} \]

which is frequently written as:

\[ f(r) = \frac{k_b T}{p} \left( \frac{1}{4} \left( 1 - \frac{r}{L} \right)^2 \right) - .25 + \frac{r}{L} \]

where \( k_b \) is Boltzmann’s constant, \( T \) the temperature measured in Kelvin, \( r \) is the extension, \( p \) the persistence length, and \( L \) the contour length.
Figure 56: WLC model Force vs. Extension curve for a persistence length (half Kuhn length) of 50 nm and a contour length of 7.3 um.

**Chain Energy and Stretching**

The entropy, $S$, of an ideal chain is the product of the natural logarithm of the number of states $\Omega$ and the Boltzmann constant $k_b$.

$$S = k_b \ln(\Omega)$$

If $\Omega(N,R)$ is the number of conformations of a freely jointed chain with $N$ monomers and an end-to-end vector $\vec{R}$, the entropy is then a function of $N$ and $\vec{R}$ and thus:

$$S(N, \vec{R}) = k_b \ln\Omega(N, \vec{R})$$

When force is applied to the chain, placing it under tension and increasing the end-to-end length, the total number of possible conformations for each segment is reduced. As a result, the entropy of the chain is reduced and the free energy is increased. This free energy increases quadratically with the magnitude of the end-to-end vector of the chain.
satisfying Hooke’s law (Energy = \( \frac{1}{2} kx^2 \)) where \( k \) is the spring constant. The force necessary to keep the chain stretched to a conformation defined by a vector \( R \) is thus

\[
-\vec{f} = \frac{3k_bT}{Nb^2} \vec{R},
\]

with \( \frac{3k_bT}{Nb^2} \) being the linear entropic spring constant. To discuss the range of extensions over which this is valid we turn to Figure 57. Here the circles surrounding sections of the chain with diameter \( \xi \), called tension blobs, indicate the length scale above which the chain appears to be elongated. Below this length scale the conformation of the chain is randomized by thermal forces. This means that the force (with units of energy/length) required to stretch a chain with a tension blob diameter \( \xi \) is \( k_bT/\xi \).

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Figure 57: An elongated chain may be divided into tension blobs. Tension blobs define the length scale below which the conformation of the chain appears random due to thermal fluctuations. Above this scale the chain will appear to be stretched.

When the diameter of the tension blob is reduced to the Kuhn length (2x persistence length), the limit of the linear entropic spring region is reached. Below this blob diameter
any elongation of the chain is a result of the straightening of the Kuhn length. For
dsDNA with a persistence length of 50 nm, the force at the limit of this linear regime is 4
x 10^{-14} \text{N}.

When tension is removed from the extended chain, its desire to be in the lowest
possible energy (highest entropy) state will drive it to relax towards the unperturbed state.
This is known as an entropic driving force. Depending on the applied tension at the time
of relaxation, the chain will begin its relaxation process as either the non-linear or linear
entropic spring. If non-linear, the chain will relax in this manner until the size of the
tension blobs increases to have a diameter equal to the size of the Kuhn monomer.
After that point is reached, the molecule will relax with a linear spring constant.

7.3 DNA Specimen Preparation

The complete procedure used to modify dsDNA to allow for the attachment of
one end to the surface of a coverslip and the other to a magnetic bead is described in the
appendix. A description of the end product is shown in Figure 58. This prep serves as
the basis for the attachment method used in all of the experiments discussed in the
remaining sections of this chapter. Using this sample preparation, we are able to hold
one end of the DNA in place via an attachment to the coverslip surface and pull the other
end for the purpose of applying a tension force to the chain. It is the effect of this tension
force that we are interested in determining in many of the following experiments.
Figure 58: DNA/Chromatin sample preparation showing the attachment of a magnetic bead to one end of the DNA and the attachment of the other end of the DNA to the coverslip surface

7.4 DNA Investigations

Using our aforementioned magnet based manipulation system, we are able to collect position vs. time data as we manipulate both DNA and chromatin specimen. With proper calibration, we can then convert this into force vs. extension data that may be analyzed by comparing it to various models or by looking for specific events during force application. When manipulating DNA, previously published results[3] indicate that the force vs. extension profile should fit the worm-like chain model very closely under 10 pN of applied force (the entropic range where the structure of the DNA is not being altered by the applied force). Above this limit, at approximately 60 pN, the application of force will cause the structure of the DNA to shift from the usual B-form to an overstretched S-form (refs).

7.4.1 Low Force DNA Manipulations
As previously stated, at low applied forces DNA remains in the entropic regime.

In Figure 59, DNA labeled with Sytox Orange (Invitrogen, Carlsbad, CA) is manipulated and images captured using a high sensitivity camera (Cascade II, Photometrics, Tuscon, AZ). In addition to the single molecule of DNA, the autofluorescent 1 micron magnetic bead is also visible in the images.

![Figure 59: Fluorescently labeled DNA.](image)

(A) Intensity plot for fluorescently labeled DNA stretched by the application of approximately .25 pN of force.

(B) Montage of fluorescently labeled DNA images, each frame separated by .05 sec, where the applied force is held constant at approximately .25 pN.

As would be expected from the worm-like chain model, as the force applied to the DNA increases, so does the spring constant, resulting in smaller fluctuations of the attached bead’s position. This is seen in Figure 60 where a bead attached to DNA has
been extended numerous times. The path traveled by the tracked bead, indicated in this figure by the yellow trace, gets narrower with extension. The green wireframe shows the position of the bead at the last time in the data set.

Figure 60: Repeated extensions of the same dsDNA strand as seen by the 3DFM-UI. The decreased amplitude of position fluctuations with extension (shown by yellow trace) indicates that the spring constant is changing with extension.

7.4.2 High Force Manipulations: Transition from standard B-form to overstretched S-form

With the application of large forces (over 50 pN), DNA has been shown to take on an overextended conformation indicated by a sudden increase in extension with force. Such an event is shown in Figure 61. Here, at approximately 65 pN of applied force, the position of the magnetic probe attached to the DNA moves approximately 1.2 microns in
a relatively short period of time. When the force is decreased below this threshold, the DNA will change back from the overstretched S-form to the standard B-form.

Figure 61: Observed B-S form transition at approximately 65 pN.

7.4.3 High Force White Noise Manipulations for Probing the B to S Form Transition

To utilize the high bandwidth capabilities of the 3DFM, we have recently started investigations of this overstretched transition with the application of white-noise force. In this experiment, a broad-band drive signal is sent from the computer to the amplifier to drive the magnets with white current. “White” indicates that there is energy (in the form of noise at controlled rms amplitudes) over all frequencies. The production of white noise flux by the magnetic system will cause additional energy to be imparted on the bead. Since the bead’s power spectrum has a slope of -2 (due to Brownian motion or “brown” noise) the net result will be a vertical shift in the power spectrum that depends on the amplitude of the input signal. A simulated white noise experiment for a free bead in solution is shown in Figure 62. The additional energy (red trace) from the white noise
source results in the same power spectrum shift that would be expected if temperatures were elevated significantly. In Figure 8 b, actual data collected using a white noise drive signals with different amplitudes are shown. As the amplitude of the white noise is increased, so is the vertical shift of the power spectrum.

![Figure 62: Simulated and experimentally obtained white-noise data.](image)

(a) Simulated white-noise (elevated temperature) PSD for a 2.8 micron bead in high viscosity fluid.

(b) Experimental PSD for 2.8 micron bead driven with different amplitudes of white-noise.

Using drive signals similar to those responsible for generating the power spectrum traces shown in Figure 62 (b), 2.8 micron beads attached to DNA were manipulated for the purpose of determining if the force required to observe the B-S form transition is reduced. For the data shown in Figure 63, it is not obvious that the additional white noise results in a lowered force threshold due to the fact that the 2nd DC (lacking white-noise) drive signal (5th force routine applied to same bead-DNA complex) followed the same trend as we saw when increasing the noise amplitude. In the figure shown at the right in Figure 63 it is apparent that with the increase in the noise amplitude the DNA is more
likely to shift back and forth between conformations, as seen by the spikes (indicating rapid displacement changes) in the zoomed-in traces.

Figure 63: B-S transition observed with different amplitude white-noise drive signals. (Right) As the amplitude of the noise signal is increased (green and red traces mainly), the position fluctuations in for the attached bead increase rapidly prior to the DNA changing forms for an extended period of time.

7.5 Chromatin Manipulations

Previously published results for chromatin (the condensed form of DNA) manipulations have shown that at high forces (approximately 20 pN, depending on the loading rate) nucleosomes will begin to disassociate from the DNA, resulting in a sudden elongation of the polymer[4-10]. This disassociation and the resulting increased extension are known as a nucleosome disruption event. In the experiments described below, we demonstrate our system’s ability to cause and detect nucleosome disruption events using magnetic forces. At this time this data was first published[11], it represented the first investigation of nucleosome disruption events using magnetic forces.
The difference between disruption events observed with a magnet based manipulator compared to a laser trap are discussed at the end of this section.

### 7.5.1 Chromatin Prep

Using the method described in section three of the appendix, linearized lambda DNA (New England Biolabs, Beverly, MA; 48.5 kb) was labeled with digoxigenin-dUTP (Roche Molecular Biochemicals, Mannheim Germany) on one end and biotinylated dUTP (Roche Molecular Biochemicals), biotinylated dATP and dCTP (Invitrogen Corp., Carlsbad, CA) on the other. Next, chromatin was formed from the DNA by incorporation of yeast nucleosomes using high salt extraction of *S. cerevisiae* nuclear extracts, followed by a gradually decreased salt concentration to assemble nucleosomes onto the DNA. The chromatin was attached to the substrate using a digoxygenin/anti-digoxygenin coupling on one side, with the second end attached to the magnetic bead using a streptavidin/biotin linkage, as described in the literature[4].

### 7.5.2 Chromatin Results

Chromatin fibers were manipulated and extension (change in bead position) was monitored using the 3DFM and a ‘ramp and hold’ manipulation method (Figure 64 B). Fiber extension was monitored, with specific attention paid to sudden increases in overall extension (an indication of a possible nucleosome disruption event[4, 6, 7, 9, 12, 13]). Three consecutive extensions of the same fiber are shown in part A of Figure 64. For the initial application of force, where the maximum applied force was approximately 15 pN, the tension on the nucleosome (histone-DNA complex) was not large enough to cause a nucleosome disruption.
event. The overall extension of the chromatin fiber was significantly less (< 50%) than what would be expected for b-form DNA alone, indicating that the nucleosome organization of the fiber remained intact. In the second extension of the fiber, with a maximum force of approximately 24 pN, one nucleosome disruption event was observed during the ‘hold’ interval (Figure 64 C 1). The amplitude of the observed disruption events have been determined by taking the difference of the average of 1000 data points immediately before and 1000 data points immediately after the event. Using this method, the amplitude of this disruption event was determined to be 51 nm. For the third extension of the fiber (max force approximately 30 pN), three nucleosome disruption events were observed, with amplitudes of 144 nm, 68 nm, and 68 nm for the 1st, 2nd, and 3rd events (Figure 64 C, 2-4). Overall, the amplitude of the nucleosome disruption events shown in Figure 64 are in reasonable agreement with published results for “full” [8] nucleosome disruptions, with the second disruption event most likely being the result of the disruption of two nucleosomes.

In our second type of force experiment, our force ramp experiments, we see nucleosome disruption take place in groups (Figure 65). In this experiment we’ve applied force to the chromatin at a constant loading rate of 16.5 pN/second. For our first pull, or the first time we apply force to the chromatin, we see disruption events that correspond to forces of 7, 26, and 33 pN respectively. If we release our bead, or bring our force back down to zero and pull on the chromatin for a second time, we find that we reach 33 pN again, or the maximum force applied in the previous experiment, we see disruption events continue. If we zoom in on the seemingly larger disruption events at this force for both pull 1 and pull 2, we see that both pulls we have groups of disruption events that are approximately 65nm apiece.
It is important to note the amplitude of the disruption events as observed by the 3DFM may be slightly larger than those viewed using traditional laser tweezers due to the inherent differences in these force application methods. Traditional laser tweezers operate in a ‘position clamp’ mode where restorative forces act to maintain an object's position at the center of the laser trap. When the DNA that is wrapped around a nucleosome is released, it causes the force necessary to return the bead’s position to the center of the laser trap to decrease, thus reducing the overall tension on the chromatin fiber. Manipulation techniques based on magnetics, such as the 3DFM, inherently operate in a ‘force clamp’ mode where force remains relatively constant as position changes. For these chromatin experiments, the constant force delivered by the 3DFM will extend the DNA released during a nucleosome disruption event slightly more than the reduced force of the laser trap. In addition, the constant force will allow for investigations into the cooperativity exhibited by nucleosome-nucleosome interactions, a property that would be indicated by increases in the number of multiple nucleosome disruption events.
Figure 64: Chromatin manipulation

(a) Extension profile for three consecutive manipulations of the same chromatin fiber. For extensions where the maximum applied force was approximately 15 pN, no nucleosome disruption events were observed. In the subsequent traces (24 pN and 30 pN) the increased maximum force caused a total of 4 nucleosome disruption events (c 1-4), with possible multiple disruptions taking place in event 2.

(b) Force profile corresponding to each extension in (a).

(c) Individual disruption events
Figure 65: Chromatin ‘Force Ramp’ experiments showing nucleosome disruption events taking place in groups.

7.6 Conclusions

The results of this section demonstrate the ability of our manipulation system to be applied to investigations involving single molecules. These experiments require high force and position sensitivity, as well as compatibility with high NA optics and sensitivity cameras when imaging the molecule is required. Additionally, the unique high bandwidth force capabilities of this system should be useful for investigating the energy landscape associated with the transform of DNA from its normal conformation to its overstretched B form. Similar experiments involving DNA-protein interactions (such as the DNA chromatin experiment described here) and wide-bandwidth force application should also net interesting results.

7.7 Acknowledgements

Chromatin experiments discussed in this chapter were carried out in a collaboration with Dr. Kerry Bloom’s lab in the Biology department at UNC-CH.
Sample prep, including the formation of chromatin and the modification of DNA to allow attachment to a coverslip and magnetic bead surfaces was carried out by Julian Haase. E. T. O’Brien led the development of the specimen sample prep techniques used to modify the coverslip surface for DNA attachment, and Maxwell Ballenger aided in the day to day production of specimen as well as data collection.
7.8 References


Chapter 8: In Vitro and In Vivo Extended Polymer Relaxation Dynamics Studied Using the Taut and Stem and Flower Models

Existing polymer models describing the expected conformations of a stretching or relaxing molecule tethered at one end (see Chapter 7) may be used to extract polymer specific properties or properties of the media in which the polymer is relaxing. In this chapter, the observation of in vitro and in vivo polymer relaxation events will be discussed. This includes the first reported observation of the taut conformation for DNA relaxation as well as the first application of polymer relaxation models to extract in vivo persistence length and in vivo tension (produced by mitotic spindle forces) during cell division.

8.1 Introduction

Single molecule experiments involving fluorescently labeled DNA under tension have been used in a variety of investigations of polymer dynamics[1-11]. Traditionally these in vitro experiments have been performed using flow to extend the polymer, with the dynamics of the polymer being investigated at a specific flow velocity or after the flow is stopped, causing the chain to relax from its free end. Recent in vivo experiments
involving the stretching of chromatin, the compacted form of DNA, inside a cell's nucleus[12] and the subsequent relaxation of the stretched chromatin have motivated our investigation of a conformation not typically seen in these low viscosity in vitro studies involving the relaxation of DNA stretched by flow, but predicted by theory[13]. The low drag of the rod-like shape results in high relaxation velocities, necessitating the use of a high viscosity fluid in order to be able to observe this conformation during relaxation.

Figure 66: Summary of the expected conformations for the retracting polymer.

According to de Gennes[13], a stretched DNA chain subject to a tension force $f$ will be in a taut regime if $\phi$, where $\phi = f l_{po} / k_B T$ ($l_{po}$ the unperturbed persistence length, $k_B$ Boltzmann’s constant, and $T$ the temperature) is greater than one. If the force is suppressed at one end, the chain will retract with a fast time constant $\tau_1$, where $\tau_1$ is giving by the following expression:

$$\tau_1 = L_c^2 / D_0 \phi^{3/2}$$
Here $L_c$ is the contour length of the chain, and $D_o = 4 k_B T / k_v \eta I_{po}$, with $k_v = 1$ ($k_v$ is a constant related to the transverse diffusion coefficient)

After time $\tau_1$ the chain will relax from the rod-like or taut conformation to the stem and flower conformation as described by Brochard-Wyart[14]. Here, the flower describes a portion of the polymer where the tension is significantly lower than that of the remaining portion, the stem, of the chain. With time the flower consumes the stem, causing both the flower to grow and the stem to shrink according to the following expression:

$$L \approx (kTt/ \eta \rho)^{1/2}$$

Here $L$ is the length of the retracting polymer (the stem and the flower together) and $\rho$ the persistence length. Since this model is described by only these two regions, the above expression may also be used to describe the total change in length of the DNA chain.

For the stem and flower regime, a universal scaling method had been developed by Manneville[11] that allows the comparison of polymer chains of varying contour length and persistence lengths relaxing in fluids of different viscosities. For this approach, the length of the retracting polymer is divided by the half-length and the time by the half-time (length of time taken for polymer to retract to its half-length). Rescaled data fitting the stem and flower model will have a scaling exponent of 0.51[11].

The diagram shown in Figure 66 displays these anticipated retracting polymer conformations. This is a simplified view that ignores restrictions placed upon the polymer’s conformation by other polymers in solution as well as conformation that are combinations of those shown (ex: portion of stem in stem and flower taking on trumpet conformation).
Due to our desire to investigate the phenomena seen in our *in vivo* chromatin relaxation data, we will focus on the two conformations where the polymer is most extended, the rod-like (taut) and stem and flower conformations shown in Figure 66. To our knowledge, this is the first experimental investigation in any setting involving the rod-like conformation during the relaxation process. Additionally, this also represents the first application of these *in vitro* developed polymer models to data collected *in vivo*.

### 8.2 Materials/Methods

**DNA Prep**

*In vitro* samples were made by allowing concatamers of λ DNA to form via Biotin/Streptavidin and anti-Dig/Dig linkers. These long strands are attached to a coverslip surface with digoxigenin Fab fragments on the surface. FAB fragment attachment to the coverglass is accomplished by first plasma cleaning the glass, making it a high-energy negatively charged surface. The glass is then treated with APTES to modify the surface with amine groups which are coupled to the –COOH end of the FAB fragment during an EDAC reaction. The FAB treated coverglass is then soaked in BSA for 2 hours to reduce non-specific binding before the DNA is added. Dig/anti-Dig bonds between the molecule and the modified surface form DNA tethers at one end while the remaining free is stretched via thermal forces or flow during surface rinsing to binding sites on or near the surface.

**DNA Relaxation**
DNA stretched between two attachment points is cut using a photoscission process previously observed by Houseal[1]. Here, high intensity light is absorbed by the DNA, causing the helix to rupture. Rupture rates are controlled using an oxygen scavenging buffer and β-mercaptoethanol to prevent multiple breaks in one strand. DNA labeled with either Sytox Green or Sytox Orange is imaged using a 1.4 NA 100x oil immersion objective and a high sensitivity camera (Cascade II, Photometrics, Tucson, AZ) operating at 20 and 50 frames per second is used to collect images. For a discussion of the optimization of the imaging parameters please refer to Appendix 4. Polymer length is then measured from these images using the Image J (National Institutes of Health, USA) measurement tool.

**Chromatin Relaxation**

Mitotic spindles may be used as *in vivo* force applicators through the used of dicentric chromosomes[12] as shown in Figure 67. Here, packaged DNA (chromatin) on the same chromatid of a dicentric chromosome that has been stretched by spindle forces spontaneously ruptures and relaxes towards the spindle pole. These relaxation events are observed using a 1.4 NA 100x oil immersion objective, with images collected every 30 seconds using a Hamamatsu Orca II ER (Hamamatsu City, Japan) camera. The collection of images is then stabilized using the ImageTracker software package developed by Brian Eastwood (Dept. of Computer Science, UNC-CH) to assure that measurements are made from the same location with respect to the spindle pole.
Figure 67: Dicentric chromosome forces.
(a) Dicentric sister chromatids with microtubules attached to both centromeres on chromatid 2.
(b) Chromatid 2 with force application from both spindle poles.

8.3 Results

8.3.1 *In Vitro: Relaxation of DNA in Buffer*

Relaxing DNA in a low viscosity (1.0 cP) buffer spends the vast majority of its relaxation in the Stem and Flower conformation (Figure 68). Most likely the initial portion of the relaxation is done in the rod-like regime, but data acquisition limitations prevent us from seeing this initial conformation. Here, the intensity at the retracting end of DNA increases while that of the end closest to the coverslip remains constant, as would be expected for the Stem and Flower conformation. Fitting the Stem and Flower model to the position vs. time data and solving for the persistence length results in a value of 50 nm. This is within the expected values for dsDNA labeled with an intercalating dye (expected persistence length of 50 nm has been shown to change by up to 25%[15] when using similar dyes).
Figure 68: Relaxation of dsDNA labeled with Sytox Green in buffer solution over 1.5 seconds. The bright ball at the retracting end of the strand represents the flower, the portion below the flower being the stem.

This data has been plotted in Figure 74 (A) and (B) to allow comparison to the high viscosity sucrose relaxation data and the chromatin relaxation data. The relatively quick relaxation over a long distance results in the nearly vertical slope shown in Figure 74 (A). As seen in Figure 74 (B), the relaxation data has the expected .5 power law when rescaled using the universal scaling approach[11]. Since the microscale relaxation time is unknown due to the fact that there are no data points in the rod-like conformation, it is difficult to estimate the applied force pre-rupture using relaxation data alone. Figure 68 (A) is a montage of the DNA relaxation in low viscosity showing the formation of a flower at the relaxing end of the polymer. In Figure 69 (B), the peaks labeled 1, 2, and 3 show the buildup of DNA at the relaxing end, as would be expected for the Stem and Flower conformation. Here the integrated intensity across the chain does not remain constant, most likely due to photo-bleaching and noise.
Figure 69: DNA relaxation intensity plots (low viscosity).

(A) Montage of DNA relaxation with intensity mapped to color. The relaxation proceeds down the Y-axis towards zero. The buildup in intensity at the retracting end shows the presence of a growing ball or flower. The broad distribution of intensity seen in the retracting strands at the very bottom of the image may be representative of the trumpet conformation shown in Figure 1.

(B) Intensity profile for three individual frames, showing the increased intensity at the relaxing end indicative of the flower from the Stem and Flower model.

8.3.2 *In Vitro: Relaxation of DNA in High Viscosity Fluid.*

At high solvent viscosities, the drag on a polymer recoiling from a fully stretched state maintains the original rod-like shape long enough to be observed using traditional fluorescent microscopy techniques. As described above, here the drag is almost entirely monomeric and significantly lower than will be seen in the subsequent conformations. As seen in Figure 70, the rod-like conformation of the polymer holds up for multiple frames. This is shown in further detail in Figure 71 which shows the fluorescence intensity for the first few frames being distributed equally. As the polymer retracts in the rod-like conformation, the density of the fluorescent signal increases over the entire strand while the summed intensity remains relatively constant. Upon further retraction, a
small ball or flower appears at the relaxing end with a corresponding intensity that increases with time (Figure 70, 71). Using this data, we can first take the section where the polymer is in the Stem and Flower conformation (determined by the presence of a peak at the retracting end of the polymer) to determine the persistence length. Fitting the model and using the known viscosity of $598 \pm 2$ cP for the sucrose solution, the resulting persistence length is 40 nm.

Figure 70: DNA relaxation in high viscosity fluid over 4.2 seconds.

![Figure 70: DNA relaxation in high viscosity fluid over 4.2 seconds.](image)

Figure 71: DNA relaxation intensity plots (high viscosity).

(A) Montage showing relaxation of DNA in high viscosity sucrose solution with image intensity mapped to color and height.
(B) Intensity plot for three frames of the DNA relaxation. In trace 1 (black), the entire molecule has an approximately constant intensity value which is lower that the value for trace 2 (blue). In trace 3 (red) the stem and flower conformation is apparent by the increased intensity value at the retracting end (this is the broad peak between 26 and 30 on the x-axis).

Using the portion of the data where the relaxing DNA is in the Stem and Flower conformation and applying the universal scaling technique we obtain the traces shown in Figure 9 (A) and (B) for the sucrose data. Due to the relatively short retraction length and rate the trace in Figure 9 (A) remains near the origin. In Figure 9 (B), the predicted slope of .5 indicates that the portion of the data set we have selected is indeed relaxing while in the Stem and Flower conformation.

For the taught regime of the DNA relaxation, we determine a fast relaxation time constant (fit to the equation $A_0 e^{t/\tau}$) in order to determine the tension force on the chain. For the data shown in Figure 5, the resulting time constant of $\tau = 4.76$ sec together with the previously determined chain length allows us to determine a tension force of 1.1 pN.

The results of these experiments show that it is reasonable to expect that a polymer retracting in a highly viscous fluid will take on both the rod-like and stem and flower conformations, and that though the use of models that describe these conformations we may determine both the persistence length of the relaxing polymer and the tension force necessary to stretch the polymer pre-rupture.

8.3.4 In Vivo: Relaxation of Chromatin in Nucleus

As with the relaxing DNA in high viscosity sucrose, the in vivo chromatin relaxation similarly has a region of fast relaxation followed by a slower relaxation regime. The first few frames in the relaxation montage shown in Figure 72 display a
somewhat constant intensity near the retracting end of the fiber. Determining the conformation directly from the fluorescent images is increasingly difficult for this data due to fluorescent artifacts in the *in vivo* environment. When plotted with the hot colormap shown in Figure 73 (A) it becomes more apparent that without the interfering fluorescent artifact we may expect to see a distribution more like what was found in the high viscosity sucrose *in vitro* DNA relaxation. This suggests that we may again break up relaxation data into the rod-like and Stem and Flower regimes. Our assumption is validated by the expected .5 scaling exponent when using the universal scaling approach (two different chromatin data sets shown in Figure 74 (A) and (B)) with the Stem and Flower model. In Figure 9 (A) we see that both chromatin data sets have similarly slow relaxation times. However, since we do not know the viscosity of the nuclear extract, we must make an estimation of either the viscosity or the persistence length and then use the model to determine the other parameter. Published values for the intracellular viscosity as measured using the same probe size (100 nm) vary from approximately 10 P[16] to 520 P[17]. This wide range of possible values has led us to instead make an estimate of the persistence length of the retracting polymer based on the polymer’s initial length prior to rupture. For the dicentric chromosomes described here the region of fluorescently labeled DNA/chromatin is 10 kb, which would result in an extended length of approximately 3.4 microns. For this data set, the structure stretched between the two poles has an estimated pre-rupture length that corresponds to the completely extend length of this 10 kb, indicating that compaction is not present at the time of rupture. As a result, we will use the commonly accepted value of 50 nm for the persistence length of B-form DNA as the persistence length of this structure. With this assumption, the Stem and
Flower model may be applied to the appropriate portion of the data, yielding an intracellular viscosity of 288 P. This viscosity is relatively large and will be discussed in a later section. The fast relaxation time constant $\tau_1 = 167$ sec. (taken from the first 3 data points in the relaxation), combined with the estimated persistence length and the previously determined viscosity results in a tension force of .3 pN. Again, this value will be discussed in comparison with existing spindle force measurements.

Figure 72: Chromatin relaxation.

(A) Processed In vivo chromatin relaxation over 330 seconds post dicentric stretching.

(B) Example pre-processed images showing bright spot below relaxing chromatin
Figure 73: Chromatin relaxation intensity plots.

(A) Montage showing in vivo chromatin relaxation with image intensity mapped to color and height. The trough between X Pixels 28 and 32 is caused by the applied image processing routine.

(B) Intensity plot for three frames of the DNA relaxation.

Figure 74: Stem and Flower data analysis.

(A) Relaxation data fit to the Stem and Flower model.
(B) Universally scaled Stem and Flower data for *in vitro* DNA showing the expected scaling exponent of .5.

**8.5 Discussion**

**8.5.1 Analysis method**

As previously mentioned, the viscosity and force values obtained from the *in vivo* relaxation data deserve further comment. A comparison with the reported intracellular viscosity values as measured using a 100 nm bead (10-520 P) shows that reported viscosity for the ‘Chromatin 1’ data set (288 P) is within the range of published values. Similarly, the value obtained from the ‘Chromatin 2’ data set (133 P) is also within this range of expected values but significantly different from the ‘Chromatin 1’ measurement (Figure 75). The difference between these two measured values may be the result of the retracting polymers having different persistence lengths, or the polymers experiencing heterogeneous viscosity regions inside the cell.
Figure 75: Published intracellular viscosities (blue) with the range of viscosities currently obtained using our analysis technique show in green.

Existing measures of the force generated by mitotic spindles are shown in Figure 76 along with the value we’ve obtained from our ‘Chromatin 1’ data set. The lack of a taut regime in the relaxation of the ‘Chromatin 2’ data set prevents us from having more than one measurement for the tension force generated by the spindle.
Figure 76: Reported spindle forces for low loads including the value returned from this study (value 4).

At this point it seems reasonable to address the assumptions that were made in the analysis of the *in vivo* data, specifically that the persistence length was 50 nm and that it remained constant. For the only other investigation where the *in vivo* persistence length of a either DNA or chromatin was determined, a value of 170-220 nm[18] was obtained and determined to be a measurement of the persistence of DNA compacted into chromatin. Using this as an upper bound for our persistence length measurements, we may determine a corresponding viscosity that would be obtained from our data using the stem and flower model. The resulting viscosity (determined for a persistence length of 220 nm) is approximately 60 P, giving us a range of values to input to the into the taut force analysis of 50-220 nm for the persistence length and 60-290 P for the viscosity. Assuming that our time constant and contour lengths remain fixed (these are measured values from the raw data), the range of forces obtained from the taut conformation
analysis is approximately .09 to .3 pN. Again this range of forces may seem broad, but it is important to note that this adjustment of the parameter space does not change the outcome more significantly. Through the use of the in vitro arm of this experiment we should be able to better predict the in vivo persistence length of the molecule by collecting data in an environment that closely resembles what is seen inside the cell.

8.5.2 Implications

Recent unpublished results from the Bloom Lab at UNC have shown that a cohesion protein is responsible for holding sister chromatids together prior to their separation in early anaphase. Once this protein is cleaved from the chromosome, the chromatids may move towards the proper poles. One proposed idea is that a region of chromatin associated with the kinetochore is stretched all the way to a pole, as seen in our in vivo experiments, which is followed by the cleaving of the cohesion proteins (see Figure 77). When the cohesion proteins release the sister chromatids, they are then free to relax towards the poles. Results obtained in these initial experiments, in particular the ability of the spindle forces to decompact a region of chromatin and stretch it to a pole, suggest that this sequence of events is plausible.

Figure 77: Proposed model for the movement of sister chromatids towards poles due to the relaxation of a portion of a stretched region of chromatin.
(A) Cohesion proteins hold sister chromatids together as the centromere is pulled towards the pole, altering the compaction of DNA in that region.

(B) Cleavage of cohesion proteins allows the sister chromatids to move towards the poles.

(C) Relaxation of the portion of strongly stretched DNA causes the entire chromatid to move towards the pole.

8.6 Conclusion

The rod-like and Stem and Flower conformations in the relaxation of \textit{in vitro} dsDNA and \textit{in vivo} chromatin were analyzed by fitting length vs. time data to existing polymer models. In addition, these conformations were confirmed using fluorescence intensity to identify a shrinking rod in the rod-like or taut conformation and the formation of a flower in the Stem and Flower conformation. This investigation represents the first observation of the predicted taut conformation for DNA under tension as well as the first application of the model to data to determine the tension force. The application of the same models to \textit{in vivo} relaxation data obtained from chromatin stretched using dicentric chromosomes has been used to determine the intracellular viscosity as well as the tension force applied to the chromatin/DNA prior to rupture. We believe that this is the first application of these models to \textit{in vivo} data for the purpose of obtaining biologically relevant information.

8.7 Acknowledgements

The results presented in this chapter again stem from our collaboration with the Bloom Lab in the Biology department at the University of North Carolina at Chapel Hill. Specifically, the dicentric chromosome data discussed in the \textit{in vivo} arm of this
experiment was collected by their group. Tim O’Brien and Maxwell Ballenger both aided in the development of the *in vitro* system.
8.8 References


Chapter 9: Discussion

In this final chapter, the next steps to be taken in the evolution of our Three Dimensional Force Microscope will be discussed. This will include improvements to be made to the current system, possible topics for investigation using this device, and the development of a high throughput screening (HTS) device based upon techniques developed in the construction of this apparatus. This chapter will conclude with a brief overview of the accomplishments of the manipulation system as well as the polymer relaxation experiments.

9.1 System improvements

Referring back to table 1.1 (repeated below for simplicity) for the purpose of comparing manipulation techniques, it becomes obvious that the largest performance gaps separating magnetic manipulations devices from invasive techniques such as AFM are the maximum applied force and the trap stiffness. As a result, the primary goals for future improvements to this system should be to increase the maximum force it can generate as well as the trap stiffness.
Table 5 (same as 1.1): Properties of various manipulation modalities.

To increase the force while maintaining our existing flux generating coils either the saturation magnetization of the material used for the pole tips needs to be increased, or the sharpness of the tip, resulting in a higher gradient, needs to be increased.

Alternatively, if the main concern is the ability to apply large forces while maintaining...
full 3D control over probe location, decreasing the size of the space separating the pole tips in the hexapole geometry should be of main concern. In this case, extremely sharp tips will result in the field falling off too rapidly at the probe’s location in the center of the hexapole geometry. It is possible that alternative specimen chamber production techniques, such as glass etching, would aid in allowing the pole tips to be moved closer to the probe of interest while remaining separated from the actual specimen, eliminating any concerns about interactions between the specimen and the pole material.

The maximum stiffness of the magnetic trap, described as the position clamp technique in Chapter 2, Section 5, may be increased by improving the bandwidth of the system’s control loop. The most likely bottleneck in function of the currently implemented control loop is the network communication between the computer controlling the magnets and the computer giving position information. Having one computer that does both tasks instead of communicating over the network should drastically increase the stiffness of the trap.

9.2 Experimental Improvements

In the in vitro polymer relaxation experiments discussed in Chapter 8 forces of an unknown magnitude were applied to the DNA prior to the relaxation event and subsequently determined by applying polymer relaxation models to the resulting data. While the forces determined using this techniques appeared to be realistic, altering the experimental protocol to instead use a magnetic bead and the 3DFM to apply know forces to the DNA prior to rupture would add a level of confidence to this experiment. Additionally, since the properties investigated in the in vivo arm of the experiment arise
from a molecule that is at least temporally condensed as chromatin, carrying out these same experiments in the *in vitro* environment using chromatin would be beneficial in that it would aid in the determination of the effects of disrupted nucleosomes on polymer relaxation dynamics.

### 9.3 Future Directions

It is our belief that the manipulation device presented in this document will have a wide range of applications to biological investigations, even without making any of the improvements discussed earlier in this section. The range of forces, force sensitivity, force bandwidth, and imaging capabilities allowed with this device make it suitable for studies of cell membrane integrin binding forces, cytoskeleton remodeling under load, cellular calcium response to mechanical stimuli, driven bead micro-rheology, and the completion of our cilia stall force investigations discussed in Chapter 6. Additionally, the high bandwidth capabilities may be utilized to determine the ability of white-noise to produce experimental results typical of elevated temperatures without actually raising the specimen temperature.

While magnet based manipulation systems inherently provide means for manipulation of more than one probe at a particular point in time, the system discussed in this dissertation is only able to manipulate probes in one specimen chamber (one isolated environment) at a time. In future experiments, especially those where the investigating the rheological properties of multiple fluids is required, a system acting as multiple instances of a simplified 3DFM (perhaps only offering manipulation capabilities towards one or two poles) would be beneficial. Thin magnetic foil pole processing techniques
developed for this instrument that result in the creation of sheets of pole plates could be used to create sheets of poles to be driven by independent coils that would be associated with multiple specimen chambers. The resulting system would be capable of manipulating probes in separate specimen chambers independently, and as a result could perform multiple rheological investigations at a single point in time. This system, a type of high throughput screening (HTS) device is currently under construction in our.

9.4 Conclusions

The goal of the preceding chapters was to present a novel magnet based manipulation device capable of producing and measuring the response of forces exerted upon micro-scale and nano-scale biological structures. The end result, our thin-foil Three Dimensional Force Microscope, is capable of producing forces in excess of the largest previously demonstrated for a magnet-based manipulator, at a bandwidth exceeding 3 kHz, and full three dimensional forces while remaining compatible with high NA optics. This system and our previous generation low-bandwidth system have been utilized in conjunction with a laser tracking subsystem to manipulate biological structures ranging from cilia down to single molecules of DNA. In the final chapters of this document DNA was discussed in detail, with polymer models describing the behavior of DNA under tension and the relaxation of stretched DNA explained and then applied to data collected from \textit{in vitro} and \textit{in vivo} experimental systems. These DNA investigations resulted in the first experimental observation of the taut conformation in relaxation data as well as the first application of existing polymer relaxation models to \textit{in vivo} data. The original work presented in this document, specifically the thin foil based 3DFM and the polymer
relaxation data analysis, represent significant advancements in the field of Biomedical Engineering and should provide means for wide range of biological investigations in the future.
Appendix 1: 3DFM Laser Alignment Procedure

The procedure outlined in this document describes the initial alignment of our laser tracking subsystem. A complete list of parts used in this system is located in the parts list section of the appendix.

Alignment of the Nikon based 3dfm (Hercules) begins at the back of the microscope on the elevated breadboard. The purpose of the first part of the procedure is to get a level, collimated beam entering the back of the microscope that has the desired spot size (> 9.25 millimeters when using the 60x/1.2 WI Nikon objective)

1. Spatial Filter Stage’s Optics: In this first step of the alignment procedure, the laser will be connected to the adaptor plate, focused through a spatial filter, and collimated.
   a. Screw laser into adaptor plate. The FC connector at the end of the laser’s fiber optic connects directly to the adaptor plate at the back of the spatial filter stage’s optics assembly. Once the laser is connected, it should be secured to the strain relief clamp (post w/clamp behind stage).
   b. After the laser is connected to the adaptor plate and secured in the strain relief clamp, the laser must be directed through the spatial filter (15 micron pin hole). The beam is made small enough to pass through the 15 micron spatial filter using an 8 mm focal length lens. The position of the beam entering the lens is changed using adjustments A1 and A2. Beam convergence/divergence is adjusted using A3 to move the lens with respect to the spatial filter. The X-Y position of the spatial filter is
adjusted using A4 and A5. The alignment of these components is
determined by using a power meter placed on the far side of the spatial
filter. Adjustment knobs A1-A5 should be used to maximize the signal
reaching the power meter.

c. Once the spatial filter is aligned, Lens C1 is used to collimate the laser
after it leaves the spatial filter. When this lens is placed 1 focal length
from the spatial filter, the laser will be collimated. The optics selected for
this system will result in a collimated beam that is approx. 3 mm in
diameter.

Figure 78: Spatial filter stage with attached laser conditioning optics.

2. **Alignment of Breadboard Mirrors:** The goal in this section of the alignment
procedure is to maintain a constant beam height as the laser follows a path to the
back of the microscope. The beam follows an exact path through a series of optics designed to magnify the spot size and allow for the position and angle of the beam entering the microscope’s objective to be controlled. Later in the procedure, small adjustments will be made to M1 and M4 to make sure it follows a straight path through the back of the objective. This part of the procedure should be completed with lenses L1 and L2 removed from the optical path.

3. **Beam Alignment Using Iris Cage:** Once the beam has entered the back of the microscope, a dichroic mirror will redirect the beam so that it heads up towards the back aperture of the lower objective. It is important that the beam is centered on and perpendicular to the back aperture. To test this, place the iris cage in the turret location that the beam is passing through, making sure that the base is seated in the turret correctly (this will assure that the iris cage is straight). The iris cage should have the circular power meter attached to the top so that power measurements can be made while adjusting the beam. The procedure below should be followed to make sure the beam is straight and centered in the cage.

   a. Open the top and bottom iris
   
   b. Measure and record the power meter signal. This will be your starting point prior to making any adjustments.
   
   c. Using mirrors M1 and M4, maximize the signal on the power meter. Keep track of how far each adjustment knob was turned so that you can get back to the starting point if necessary.
   
   d. Close the bottom iris so that 10% of the signal is blocked
   
   e. Close the top iris so that 50% of the signal is blocked.
f. Repeat step c.
g. Open the top and bottom irises and record the signal on the power meter.
h. Repeat steps b-g until the signal does not improve
i. Record the intensity
j. Add lenses L1 and L2 to the system
k. Adjust the position of these lenses again to maximize the signal. The final intensity should be the same as recorded in step i

4. **Beam Alignment past Hot Mirror:** The goal in this stage of the alignment procedure is to make sure that the beam passes down the center of the tube that QPD camera and the QPD are connected to. This is done by attaching the round photodetector at the end of the tube (with lens and detectors removed) and maximizing the signal on the detector.
   a. Put top and bottom objectives in place
   b. Attach photodetector to top tube by screwing it into the threads on the outside of the tube
   c. Use condenser positioning thumbscrews to maximize the signal on the photodetector.

5. **Beam Alignment – Upper Optics:** In this section of the procedure the QPD and QPD camera to the left of the hot mirror will be aligned. In order determine the proper location for the QPD, a separate camera is used to select the proper location (based on clear focus of an image at the back aperture of the condenser)
of the camera with respect to the imaging optics. Position the FL = 38 mm lens approximately 60 mm from the Hot Mirror cube as shown in Figure 9. This lens demagnifies the back aperture so that it fits on the very small QPD. Once a clear image of the aperture is obtained (see Figure 8), the location of the camera with respect to a fixed reference point is noted so that the QPD may be placed in the same location. Since the QPD and the condenser aperture are conjugate planes, an image of the QPD and the bead’s diffraction may be obtained by imaging the back aperture. This is accomplished using a 25.4 mm focal length lens placed 40 mm from the cube holding the Hot Mirror and the QPD camera approximately 35 mm from the lens. Adjust the position of the QPD camera until a sharp image of the QPD is visible.

Figure 79: Imaging of the back aperture of the condenser with a camera placed in the QPDs location. A piece of tape with a black ink line is used to focus the image to make sure QPD is in the correct location.
Figure 80: Upper optics for tracking system.

6. **Alignment of Laser through Microscope**: This section of the procedure should be completed with lenses L1 and L2 removed from the optical path. The objective and condenser should be removed as well. You will need to position the Iris Cage (below) in the microscope’s turret.

Figure 81: Iris cage used to align laser as it exits microscope turret.
a. With the Iris Cage in place and both irises open, position the wand photo
detector above the Iris Cage. Measure and record the laser’s intensity. The
next steps will maximize this measurement.

b. Using the 3 adjustments on M4, maximize the signal on the photodetector.
These adjustments will control the position of the laser as it enters the cage.

c. Record the position of the adjustment screws on mirror M1. Keeping track of
how far you have turned these screws, adjust the mirror’s position to
maximize the signal measured at the top of the Iris Cage. This adjustment
will control the angle of the laser entering the cage (want laser to go straight
through the cage)
Final Alignment: With a bead in place at the specimen plane (and in the beam), oscillate the MCL stage in X and Y (approximately 500 nm for each axis) while monitoring the magnitude of the change in the X and Y QPD signals. Adjust the position of lens L3 to maximize the amplitude of the change in the X and Y signals. This is changing the location of the beam waist with respect to the specimen plane. When the bead is in the center of the laser in X and Y and at the waist in Z, the system will be the most sensitive to lateral translations. The same holds true for oscillations in Z although a slightly large oscillation amplitude may be needed due to the lower Z sensitivity of the system. This procedure may also be done using a razor blade to cut through the beam with the waist z position adjusted using the same lens.
Appendix 2: 3DFM Optics Daily Alignment Procedure

The procedure outlined in this document describes the sequence of events for daily operation of the Hercules model laser tracker subsystem. All of the as-built tracking systems work in a similar manner, but computer names mentioned in this document will be different for the other systems. This document assumes that all devices have been turned on (laser power supply, QPD power supply, MCL stage power supply, camera power supply, etc.), and that the laser has had adequate time to warm up (approximately 15 min.).

1. Insert Sample: Hercules uses a water immersion objective, so there must be water on top of the objective prior to inserting the sample.

2. Open Particle Tracker (Aurum): This is the software used to do the actual tracking. By opening this first the user can control the MCL stage using the knobs. Also, when the Particle Tracker program begins, the MCL stage is centered (this moves the sample). Because starting this software moves the sample, finding a bead before starting the Particle Tracker is of little use.

3. Click “Disconnect QPD”: Disconnecting the qpd allows the Virtual Bench software to gain access to the A/D board in the computer.

4. Open virtual bench (Aurum). This will allow the user to see the laser levels on each quadrant of the photodetector. User must make sure that the volt/division setting for each of the 4 channels representing the quadrants is the same.

5. Open amcap (Argentum) software that allows user to view camera that sees laser on qpd. To access argentumn, open an explorer window and type in the following address:
6. Open iris in front of qpd. User should be able to see iris on camera that views laser + QPD.

7. Using camera looking at brightfield image (light must be on), find the region of interest in the sample. This is done to make sure that the objective is at the location where it will be used during experiments.

8. Adjust the Z position of the condenser so that the image of the QPD on the QPD camera appears to be a square similar to below (light must be off). This is done using the large micrometer to the left of the halogen lamp. Make sure there are no poles blocking the QPD (image should look like below).

![Figure 83: Image of QPD](image)

a. When the condenser is at this position, the laser sum signal (add 4 quads together) should be 12-18 volts. The more opaque the sample, the lower
the sum signal will be. Also, using pole plates will cause a reduction in the sum signal.

Figure 84: Virtual bench software showing individual quadrant signal strength.

9. Using thumbscrews on the square mount in front of the QPD, move the QPD until all four signals (as seen on virtual bench) are approximately equal (must not differ by greater than 5%). Exit virtual bench, reconnect QPF on Particle Tracker software (click connect qpd button). This completes the alignment section. Now on to tracking!

10. Find bead and position it underneath laser. Find the bead by moving the adjustments on the K stage (this is the stage that sits atop the MCL stage).

11. Turn off the brightfield source: This is done so that the image of the QPD can be seen on the QPD camera. If the bead is centered in the laser you should see an image similar to the one below. If the image of the bead doesn’t look like below, move the MCL stage in x, y, or z until it does. You should see rings moving on the qpd
12. Track bead: Make sure all gains in the output-I gains section are positive. They should be $x = .01$, $y = .01$, $z = .005$. Click on agnostic tracking to initiate tracking algorithm. This will move the bead in $x$, $y$, and $z$ to get calibration signals needing for program, then initiate tracking automatically.

13. To view trace of bead moving in real-time, open 3DFM-UI on desktop of Argon. When this program is open, go to ‘Connection: Tracker Connection: ---make sure remote tracker is **StageTracker@aurum-cs:4500**, then click **DONE**

If bead is not centered, use the MCL control knobs to move the bead a round until it looks like the image above

Troubleshooting: If an image of the laser does not appear on the qpd camera, and the sum signal as seen by virtual bench is less than 2 volts:

1. Make sure the laser is plugged in.

2. Make sure nothing is blocking the laser’s path

3. Adjust the position of the upper optics assembly to center the condenser over the objective

   a. There are two ways to tell when this has been accomplished. First, the brightfield illumination source transmitted through the objectiv should be centered on the filter cube below the objective. Second, you should have an image of the QPD visible on the QPD camera.
Appendix 3: DNA Preparation Method

The method described in this section describes how commercially available DNA is modified to allow for the application of force. Using our manipulation system we apply a tension force to the molecule by stretching it between a glass coverslip and a magnetic bead.

\textbf{λ Dig-Biotin (multiple biotins) Prep}

Linearized \textit{λ} DNA (Ne England Biolabs, Beverly, MA: 48.5 kb) is labeled with digoxigenin-dUTP (Roche Molecular Biochemical, Mannheim Germany) using the Kenlow reaction (New England Biolabs). This linear strand of DNA with two labeled ends is then cut using XbaI to expose two more sites for labeling. The newly exposed site is triple labeled with biotinylated dUTP (Roche Molecular Biochemcals) and biotinylated dATP and dCTP (Invitrogen Corp., Carlsbad, CA) using the Kenlow reaction. The resulting pieces of DNA are labeled on both ends, one with Dig antibody and the other with 3x biotin. It is these labeled ends that are used to attach the DNA to the coverslip (Dig-AntiDig interaction) and a magnetic particle to the DNA (Biotin-Streptavidian interaction)
Appendix 4: DNA Imaging

In order to obtain the highest quality images of DNA for our specific application, investigations were carried out to determine the ideal microscope configuration and specimen prep. Here, the goal was to maintain a reasonable signal to noise ratio (SNR) while obtaining the highest resolution (most pixels per unit area) possible. Previous investigations using the Sytox family of dyes found that they were best for this application. As a result, all images presented here have either Sytox Green or Sytox Orange as the label.

To begin, the auto-fluorescence of the various beads used to manipulate DNA was tested. Since the intensity of the bead’s signal is so large compared to the DNA, the auto-fluorescence of the bead can bleed into surrounding pixels, making it difficult to image the faint DNA.

Figure 85: Auto-fluorescence of 1, 2.8 and 4.5 micron beads.
As seen in Figure 85, the intensity of the 1 micron beads is approximately half that of the 2.8 and 4.5 micron beads. As a result, they should be used for applications where high quality images of DNA under tension are required.

In the following tests, force was applied to DNA labeled with Sytox Orange using the 1 micron beads discussed above. This particular dye was chosen because its excitation wavelength is longer than that of Sytox Green, resulting in there being more time to image the molecule before photoscission takes place. For all images the dye was added at an approximately 1 to 10,000 dilution from the stock concentration. At this concentration it is reasonable to expect that the molecule is saturated.

Figure 86: Maximum magnification of DNA using 100x objective, 4x tube, 1.5x multiplier, and 2x2 binning.

(a) Intensity mapped to color and height

(b) Example fluorescence images
To begin, DNA was imaged at the highest magnification possible with the ‘Hercules’ model system (Figure 86). This was accomplished using the 100x oil immersion objective, the 4x tube in front of the camera, and the 1.5x multiplier on the microscope. Images had to be binned using 2x2 binning in order to obtain any noticeable signal. The resulting image has 33 nm/pixel but a signal to noise ratio (SNR) of only approximately 1.2.

Figure 87: DNA imaged using 100x objective, 4x tube, 1x multiplier and 2x2 binning.

By removing the 1.5x multiplier, an SNR of approximately 2 was obtained with a calibration of 44 nm/pixel (Figure 87). In Figure 88, the SNR was improved further (to approximately 4) by removing the 4x tube (100x objective, 1.5x multiplier). Here the calibration is 66 nm/pixel.
Using only the 100x objective, a SNR of approximately 5 is obtained along with a calibration coefficient of 88 nm/pixel (Figure 89). For most applications, the increase of the SNR from 4 to 5 is not worth the change in the calibration coefficient. As a result,
the imaging system used in Figure 88 (100x objective, 1.5x multiplier) is the method of choice