

A SOFTWARE-BASED CONTROLLER FOR SINGLE-MOLECULE
EXPERIMENTS USING AN ATOMIC FORCE MICROSCOPE INTEGRATED WITH
A MAGNETIC MANIPULATOR

by

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ABSTRACT

A SOFTWARE-BASED CONTROLLER FOR SINGLE-MOLECULE EXPERIMENTS USING AN ATOMIC FORCE MICROSCOPE INTEGRATED WITH A MAGNETIC MANIPULATOR

Single-molecule methods have become powerful tools to study biomolecules because they allow direct observation of the complex molecular kinetics. Among them atomic force microscope (AFM) is one of the most popular tools due to its capability of nanomanipulations. Various information of biomolecular bonds such as force level, life time, length and their relationship can be derived by pulling molecules in different ways with AFM. In this thesis, a software based PID controller for force clamped experiment that pulls the molecules in a constant force is developed with LabVIEW. Then the controller is verified and characterized in piezo actuation mode and magnetic actuation mode using a customized AFM. Lastly, force clamped experiments with heparin-FGF2 and biotin-streptavidin are performed with the controller and the probabilities of their lifetimes are analyzed statistically.

ÖZET

MANYETİK MANİPÜLATÖRE BAĞLI ATOMİK KUVVET MİKROSKOBUyla YAPILAN TEK MOLEKÜLLÜ DENEYLER İÇİN YAZILIM TEMELLİ KONTROL CÜ

Tek-molekül teknikleri, karmaşık molekül yapılarının hareketlerini doğrudan gözlemlenmesini imkan kıldığından dolayı, biyomolekülleri incelenmesi için güçlü bir metod haline gelmiştir. Atomik kuvvet mikroskobu, nano boyuttaki cisimlerin hareket ettirme kabiliyetinden dolayı en sık kullanılan tek-molekül kuvvet spektroskopisi tekniği olmuştur. Atomik kuvvet mikroskopunu kullanarak biomoleküllerin bağ yapıları hakkında daha geniş bilgilere ulaşılabilir, Örneğin moleküller arası etkileşim, bağlardaki kuvvet seviyeleri ve kopma süreleri gibi bir çok bilgilere, AKM ile molekülleri birbirinden çekerek yada iterek ulaşabiliriz. Bu çalışmamızda moleküller arasındaki kuvveti sabit tutarak, iki molekül arasındaki etkileşimi anlayabilmemizi sağlayan bir yazılım tabanlı PID kontrollü LabVIEW programı geliştirilmiştir. PID kontrolü daha sonra kendi tasarladığımız manyetik ve piezo hareket ettirme sistemi ile entegre edilmiş atomik kuvvet mikroskobu ile test edilmiştir. Bu testler için Heparin ile FGF2 ve Biotin ile Streptavidin molekülleri arasındaki bağlar incelenmiş ve raporlanmıştır.

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LIST OF SYMBOLS

A	Signal amplitude
B	Magnetic flux density
D	Piezo constant
e	Error
f	Frequency
F	Force
F_c	Clamping force
F_p	Force measured according to piezo
F_{photo}	Force measured according to photodetector
k	Spring constant
K_d	Differential gain
K_i	Integral gain
K_p	Proportional gain
M	Magnetization
S	Deflection sensitivity
t	Time
T	Sampling time
T_d	Derivative time
T_i	Integral time
u	Controller output
v_d	Speed of deflection signal
V	Voltage
V_c	Clamping force represents in voltage
V_p	Voltage applied on piezo
V_{p0}	Offset voltage applied on piezo

V_{ph}	Voltage readout from photodetector
Δu	Controller output increment
Δv	Relative velocity
ΔZ	Change of displacement
μ_0	Magnetic permeability in vacuum
χ	Magnetic susceptibility

LIST OF ACRONYMS/ABBREVIATIONS

AFM	Atomic Force Microscope
BSA	Bovine Serum Albumin
CAD	Computer-Aided Design
CLK	Clock signal
DAQ	Data Acquisition
DI	Deionized
DS	Deflection Sensitivity
FGF	Fibroblast Growth Factor
PBS	Phosphate Buffered Saline
PD	Photodetector
PID	Proportional Integral and Deferential

1. INTRODUCTION

Since its invention in 1986 [1], atomic force microscopy (AFM) has been developed into a versatile tool. With demonstrated resolution on the order of angstrom, which is more than 1000 times better than the optical diffraction limit, AFM has been widely used to image stiff material surfaces [2,3] and crystals [4]. The capability of functioning in liquid environments with variety choices of cantilevers makes it very suitable for imaging soft [5] organic materials such as bacterium [6] and proteins [7] as well. High speed AFM can even achieve video of proteins *in vivo* [8,9].

AFM is not only an imaging technique, but also has been frequently applied in studying mechanical properties of biological samples, such as elasticity and viscosity [10,11]. The capability of force measurement with piconewton resolution enables utilization of AFM for various applications in biomolecular science. For example, force-induced domain unfolding in single protein can also be measured with the force-measuring mode of the AFM [12,13].

In conventional AFM force spectroscopy experiments, as shown in Figure 1.1, the molecule that is anchored on a cantilever tip is pulled out from the surface with a constant speed through piezoelectric actuation. The pulling force causes bending of the cantilever and is measured by the laser reflection off the cantilever with a photodetector. In this way an increasing force over a wide range is applied to the molecular bonds and creates a complex time series of unfolding events and resulting in the saw-tooth pattern [14,15].

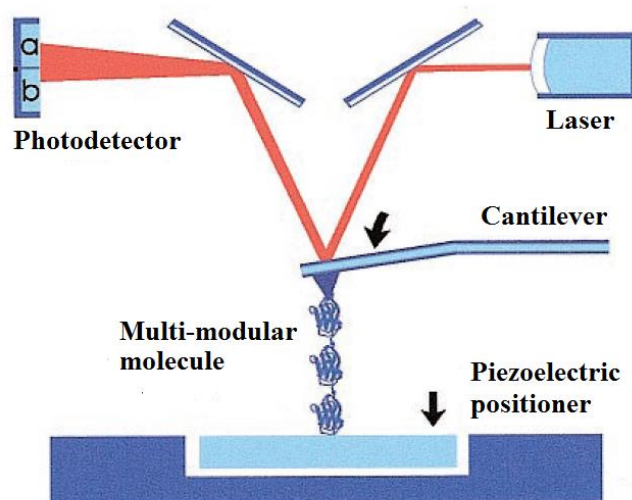


Figure 1.1. Schematic of Conventional AFM Force Spectroscopy Experiment.

AFM pulling experiments are also performed to characterize intermolecular forces between pairs of single molecules, such as protein-ligand [16] and antigen-antibody [17]. The experiments are cycling in nature and each time the cantilever tip, where one from the molecule pair is immobilized on, is brought into contact with the surface, where the other from the pair is immobilized on, and hold for a while, then pull back in a constant velocity. Force curves provide information about rupture forces and molecular distances. By varying the speed of the cantilever in each experiment, the relationship between force and probability of rupture events can be obtained.

Dynamic force spectroscopy [18], where the loading rate of the molecular bonds are varied by adjusting the cantilever pulling speed, allows the determination of potential barrier widths and the corresponding natural transition rates over those barriers. This method has been applied to different molecular pairs such as individual ligand–receptor pairs [19] or membrane [20] proteins.

The force-clamp method is another force-measuring mode of AFM, which was

introduced by Julio M Fernandez, *et al.* [21,22]. The force on the cantilever in force-clamp mode is kept constant using a feedback mechanism while the molecular bonds are loaded. Such a direct way to examine the mechanical stability of elastic proteins is more desirable because the mechanical unfolding is a force dependent process [16,23]. In this way the unfolding kinetics of titin and ubiquitin are successfully captured, and force dependency of biochemical reactions are also measured. But the difficulty to integrate it with the AFM system and the hard to tune nature of PID feedback controller are the short comings.

An all-digital force-feedback technique was implemented by Daniel J Muller, *et al.* [24]. This simple, fast software-based PID controller with a few milliseconds response time and around 200us for each feedback loop in combination with a commercial AFM yields a comparable performance to recent hardware based solutions. Both multi-domain titin Ig27 and membrane protein nhaA unfolding are successfully measured.

The aim of this research is to develop a software-based PID controller, which is implemented in PXI Platform using LabVIEW (National Instrument) to perform biomolecule force clamp experiments using a custom-made AFM setup. The AFM system is integrated with a magnetic manipulator that allows molecular pulling and molecular force-clamp experiments.

In this thesis, the controller software requirements are analyzed for piezo actuation and magnetic actuation respectively, modules in the software are programmed according to the operation needs and optimized by avoiding redundant functionalities, and velocity algorithm for digital PID control is then implemented in the software.

The system is capable of performing molecular pulling and molecular force-clamp experiments using piezo and magnetic actuation methods. Several experiments have been performed to verify the functionalities of the PID controllers for both actuators. Force

resolution limits and time behavior of the AFM with the controller are also discussed.

Force clamped pulling experiments for heparin-FGF2 and biotin-streptavidin were also performed using the software controller with piezo actuation and magnetic actuation. During the force clamped experiments, the biomolecules were pulled in a constant force, the lifetime of molecule bonds were measured. Statistical analysis of the lifetime of both biomolecular bond pairs were performed with data collected from these experiments.

2. THE EXPERIMENTAL SETUP AND THE CONTROLLERS

2.1. The AFM Setups

Figure 2.1 shows the experimental setup to realize a cantilever-actuated atomic force microscope with integrated electromagnets. The AFM head is manufactured using 3D printing as shown in Figure 2.2. The cantilever-actuated system, which can entirely fit inside a hemisphere, is chosen to reserve the bottom half of the space for additional functionalities.

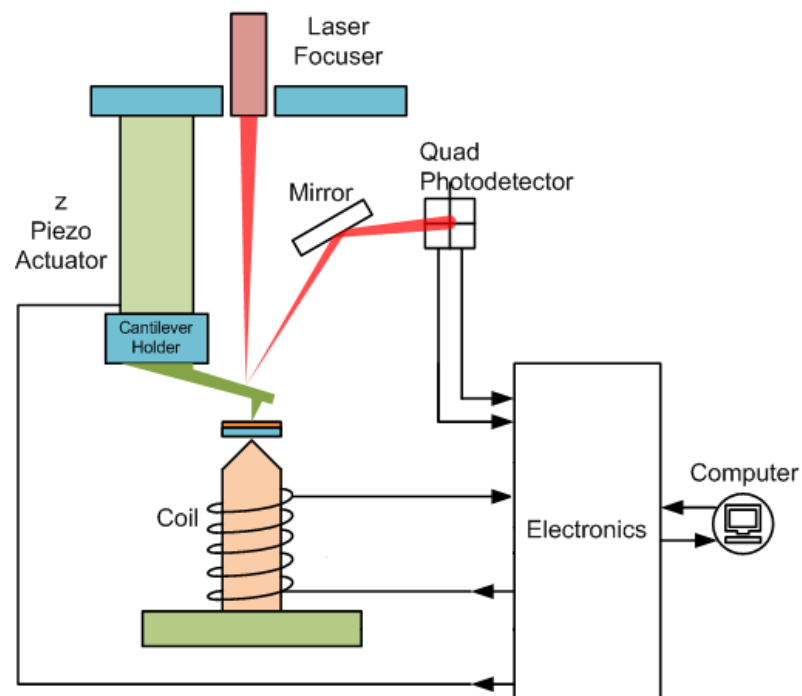


Figure 2.1. Cantilever Actuated AFM system.

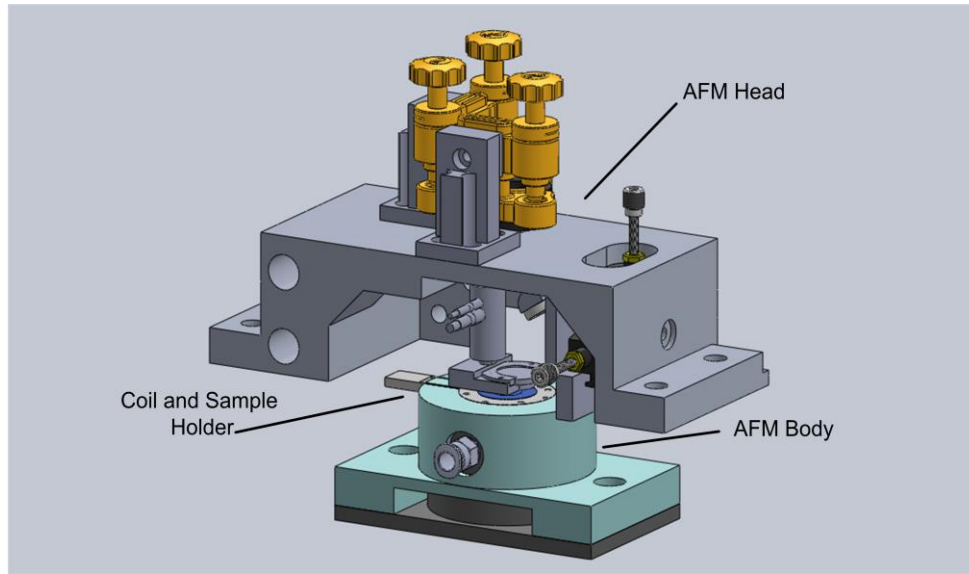


Figure 2.2. CAD Drawing for the AFM System.

In this AFM setup, the piezoelectric tube, where the cantilever is mounted on at an angle of 12° , provides a fine-tuning Z position actuation. A low noise laser beam is focused on the cantilever and the reflected beam off of the cantilever is steered on a quadrant photo detector using a mirror, which is placed at an angle of 33° to measure the position of the cantilever. A sample holder placed below the cantilever houses magnetic beads and biomolecules on top of the magnetic actuation coils. The maximum distance between the tip of the coils and the tip of the cantilever is determined to be $500\mu\text{m}$ to maintain adequate magnetic force on the nanowires. This distance is controlled precisely using a mirostage that positions the coils and the piezo actuator of the cantilever.

Figure 2.3 shows the relationship between the force applied on the cantilever and the laser spot location on the photodetector. When cantilever with spring constant k experience a force F in Z direction, according to Hooke's law, the cantilever will bend to the same direction as F by distance ΔZ .

$$F = k\Delta Z \quad (2.1)$$

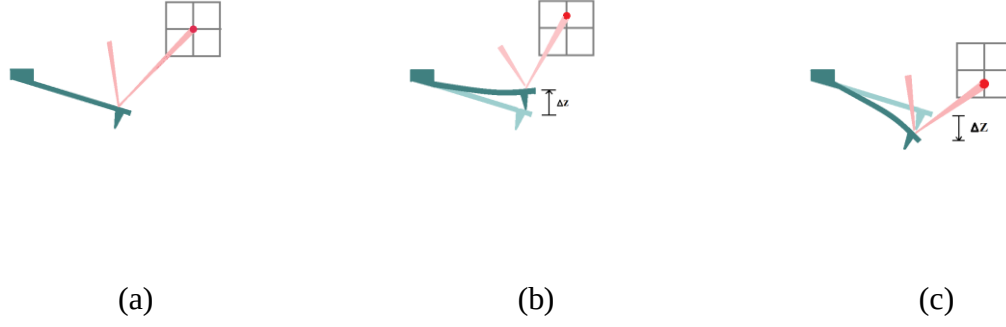


Figure 2.3. Relationship between Force on Cantilever and Photodetector Readout.

Figure 2.4 shows the schematic of the laser beam deflection measurement. The angular displacement change of the cantilever, which is proportional to ΔZ , is amplified by a long beam path, results in a considerable displacement change of the laser reflected on photodetector. This displacement change, which is proportional to the angular deflection of the cantilever as well [25], is detected by a position-sensitive photodetector whose output signal is collected by a differential amplifier. Therefore the voltage readout from the photodetector V_{ph} is proportional to the force applied on the cantilever.

$$V_{ph} \propto F \quad (2.2)$$

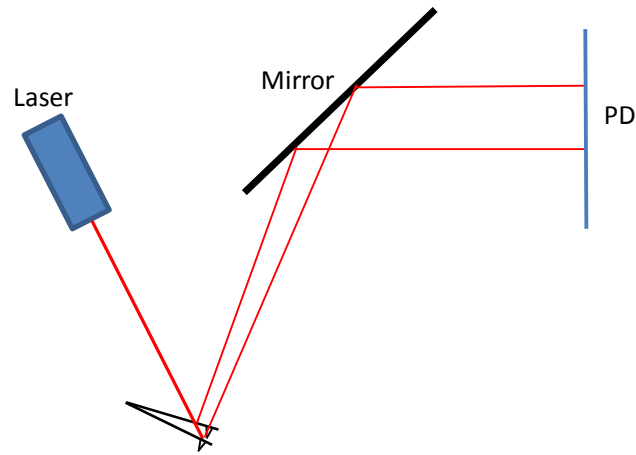


Figure 2.4. Laser Beam Deflection Measurement.

The specifications for molecular force clamp experiments are shown in table 2.1.

Table 2.1. Specifications for Molecular Force Clamp Experiments.

Specification	Definition and dependence (Force along z axis, closed loop operation)	Value
Maximum force	Force to break molecular bonds/rupture force	1000 pN
Maximum displacement	Nanowire displacement at bond breakage	5 μm
Force resolution	Incremental steps in force	5 pN
Response time	Rise time for step response	≤ 1 ms
Bandwidth	Maximum operation bandwidth of actuator	50 kHz

2.1.1. Piezo Actuation

The piezo actuator (P-840.1, Physik Instrumente, Karlsruhe, Germany) used in this setup is 32mm in length and 12 mm in diameter. It utilizes piezoelectric effect that is linear and reversible, converts electrical energy directly into mechanical energy and vice versa. Piezoelectric effect neither produces magnetic field nor is affected by magnetic fields.

Therefore, it is compatible with the magnetic actuation in the system. The piezo actuator, driven by a voltage amplifier covers a travel range of 15 μm with a transduction coefficient of 15 nm/V. The short response time which is only a few microseconds and the low power consumption which generates insignificant levels of heat make it very suitable for biomolecular experiments.

2.1.2. Magnetic Actuation

Magnetic actuation system is designed for pulling experiments for magnetic cantilevers or for non-magnetic cantilevers on which magnetic particles are attached. During magnetic actuation experiments, the cantilevers are not in contact with the surface, therefore the effects of noise from the piezo and thermal drift are reduced. The magnetic coil actuator is driven by a power amplifier. A magnetic field gradient is generated to provide magnetic force on a point-like magnet m , this force can be represented as below [26],

$$F_m = \frac{1}{\mu_0} \nabla(m \cdot B) \approx \frac{1}{\mu_0} (m \cdot \nabla) B \quad (\text{when } \nabla \cdot m = 0) \quad (2.3)$$

For superparamagnetic nanoparticles in a nonmagnetic medium [27],

$$m = V \mu_0 M = V \mu_0 \chi H \quad (2.4)$$

Where M is the magnetization of the particle and χ is the magnetic susceptibility. Therefore

$$F_m = \frac{V \chi}{\mu_0} (B \cdot \nabla) B \quad (2.5)$$

The magnetic particles should also overcome the hydrodynamic drag force, for spherical particle with radius r in liquid with viscosity η , here Δv is the relative velocity of the particle and the liquid.

$$F_d = 6\pi\eta r\Delta v \quad (2.6)$$

The single coil system can be also replaced by a four coil system to perform twisting experiments, as shown in Figure 2.5.

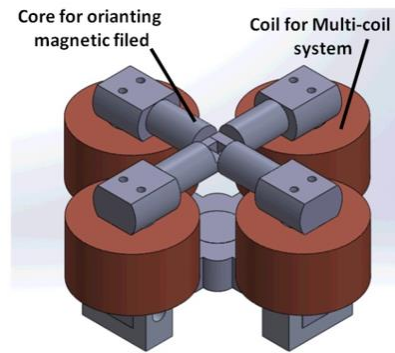


Figure 2.5. Four Coil System.

2.2. The Controller

The Block diagram of the electronics required for the single-coil system is shown in Figure 2.6. The AFM system is controlled by a PXI embedded controller (NI PXI-8102, National Instruments, TX, USA) via the DAQ card, which has 8 analog inputs, 2 analog outputs and 24 digital inputs/outputs. The sampling rate of the DAQ card is 1.25MS/s, which is adequate for bio-molecular experiments.

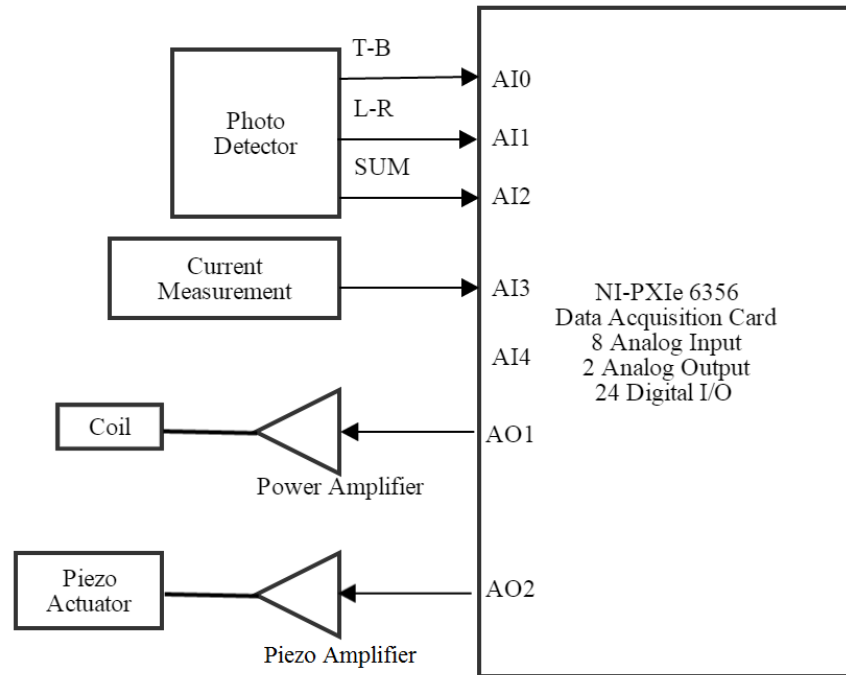


Figure 2.6. Electronics for the Single-Coil System.

DAQ card reads the voltages from the photodetector (PD) and current from the coil, sends voltage signal to the piezo amplifier and power amplifier. In order to implement force-clamp conveniently, all the forces and distances are converted in terms of voltages. Controller minimizes the error between the setpoint force that is expressed in units of Newtons and the photodetector readout that is expressed in units of Volts. The variable conversion requires a calibration routine implemented in the software.

LabVIEW 2012 Professional on Windows7 is used for developing the controller for the AFM. The loop speed of the software is a key factor to the performance of the controller, thus reducing the computational effort, which can be achieved by using low level VIs, reducing the number of VIs in the loop and avoiding redundant functions, is crucial. Therefore although the fundamental principles and main functions of piezo actuation mode and magnetic actuation mode controllers are quite similar, they are written in 2 separate

programs. Another VI, called MANAQA VI, which is shown in Figure 2.7, is used during the preparation of each experiment.

2.2.1. VI for Preparation

MANAQA VI is used in the preparation of experiments for laser alignment and deflection sensitivity measurement.

The first step is laser alignment. To align laser on the tip of the cantilever, the positioning screws on the top of the AFM head are adjusted to change the x and y position of the income laser beam. Laser spot location on the cantilever is verified by observing the interference patterns of the laser (Figure 2.8). To align the deflection signal to the photodetector, the angle of the mirror and the position of the photodetector are adjusted. Laser spot location on the photodetector can be verified from the deflection and left and right signal shown on MANAQA interface. Sum signal is then maximized by fine adjustment mentioned about.

The second step is to measure the deflection sensitivity. A triangular wave with a DC offset, generally 5V, which is half of the range of the DAQ voltage output and provides the freedom to move the cantilever both upwards and downwards, is applied to the piezo tube by setting the parameters of Piezo Drive on the interface. Then cantilever is brought in contact with surface by adjusting the microstages manually to move either the AFM head downwards or the AFM body upwards, meanwhile the deflection signal is observed on the waveform chart until a waveform similar to the Deflection Signal in Figure 2.7 appears.

Deflection sensitivity S is calculated from the recorded deflection signal as below:

$$v_d = \frac{V_1 - V_2}{t_1 - t_2} \quad (2.7)$$

$$S = 2DfA / v_d \quad (2.8)$$

Where (V_1, t_1) and (V_2, t_2) are two points on the same approach curve. $v_d(\text{V/s})$ is the speed of the deflection signal. A (V) and f (Hz) are the amplitude and frequency of the triangular signal that applied on the piezo respectively. Piezoelectric constant D ($\mu\text{m/V}$) is provided by the manufacturer, here it is $1.5\mu\text{m/V}$.

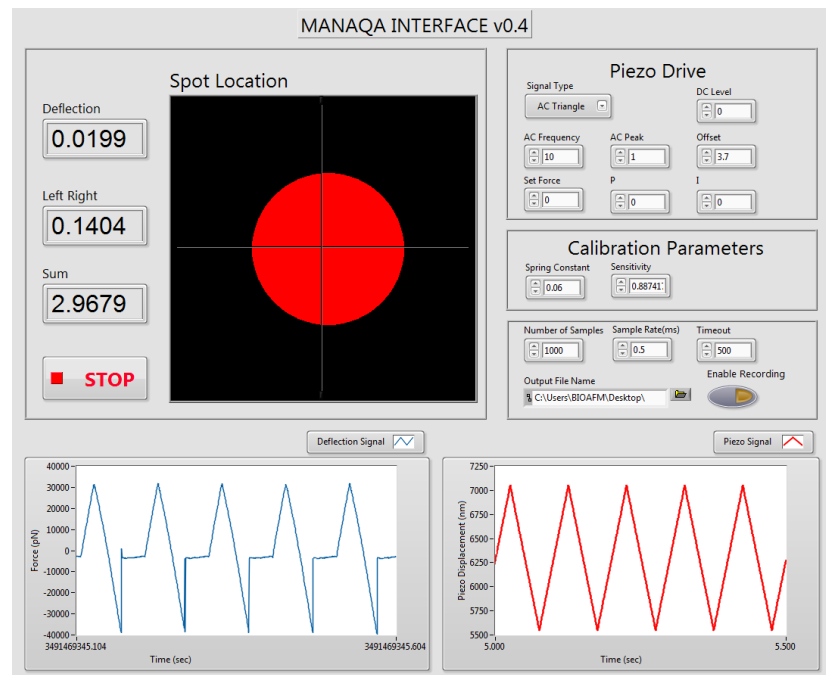


Figure 2.7. MANAQA Interface.

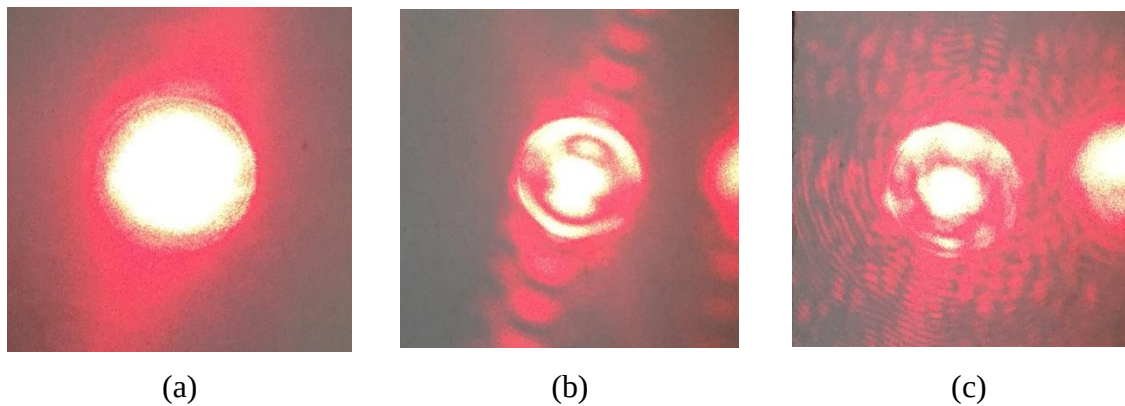


Figure 2.8. Interference Patterns When Laser is (a) not on the Cantilever, (b) on the Leg of a Triangular Cantilever and (c) on the Tip of the Cantilever.

2.2.2. VI for Force Clamped Experiments

The controller software can be divided into 4 major parts: measurement I/O, PID controller, interface and file I/O. The software follows a Producer/Consumer structure to further increase the efficiency and reliability of the controller. The block diagram of the software is shown in Figure 2.9. Data flow is controlled by connecting these blocks with logic functions.

All the modulus that require precision timing or real time response, including measurement I/O, controller and indicators are placed in the producer while loop which is a timed while loop controlled by the timer on the DAQ card. The data to be written will be stored in the buffer in a queue and written in files in large chunks in the consumer loop which is a normal while loop without timing. Figure 2.10 shows the implementation of producer-consumer structure in LabVIEW, in such fashion the controller speed won't be limited by the writing speed of the hard drive. When only basic measurement and file I/O functions are in the loop, a maximum loop speed of 7 kHz has been achieved. Controller speed for the system is limited by the CPU speed and the buffer size of the computer and the DAQ card.

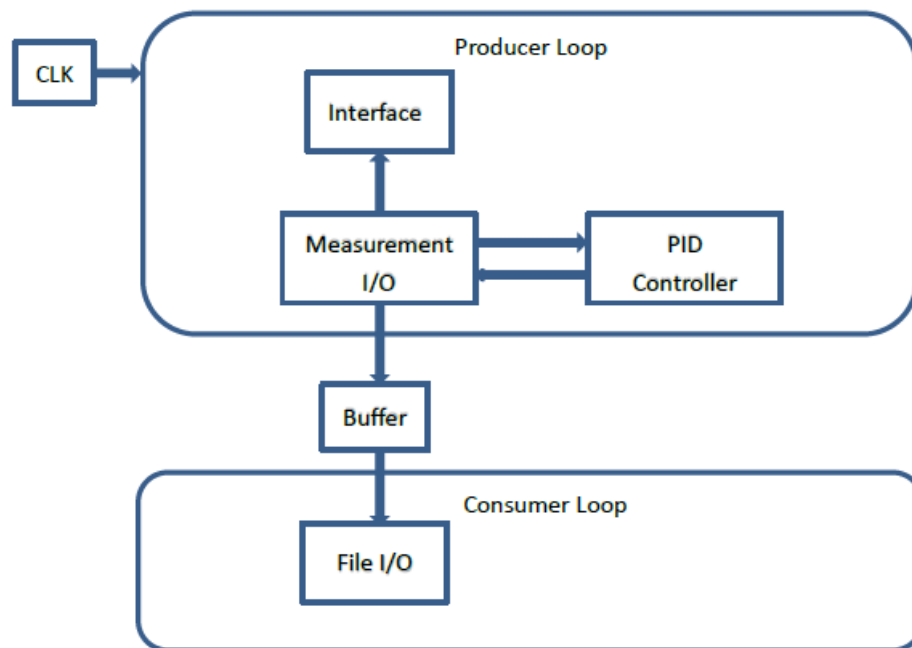


Figure 2.9. Block Diagram of the Controller Software.

CLK is an external timer embedded on the DAQ card. It provides sub-millisecond time resolution, which is much faster and more precise than the default timer resolution, 15.6ms, of Windows7 system. 4 kHz loop speed can be achieved with 100% CUP usage, but to avoid missing events of the samples due to black out, generally the producer loop speed, thus the sampling and actuating frequency, is limited to 2 kHz. That's adequate for bio-molecular experiments, which require 1 kHz detection bandwidth.

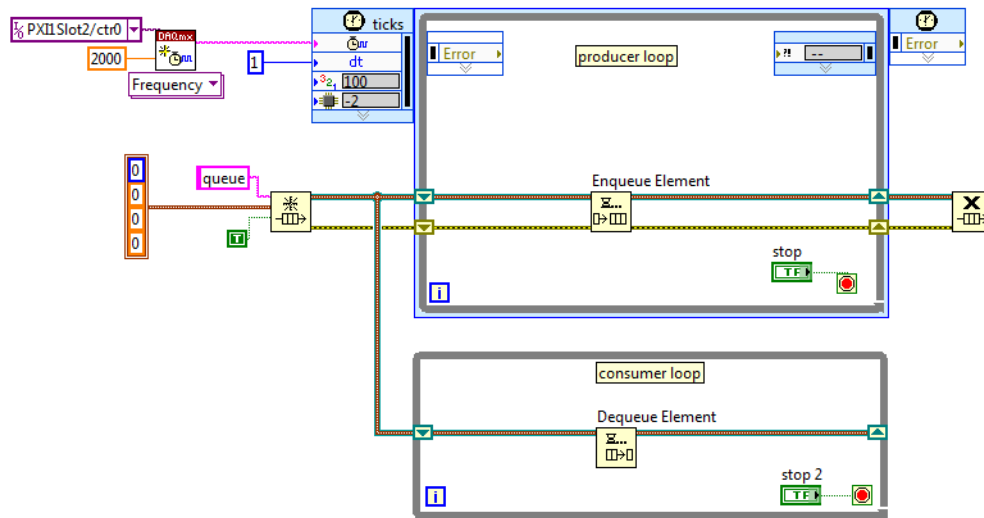


Figure 2.10. Producer-Consumer Structure in LabVIEW.

Measurement I/O, shown in Figure 2.11, includes sampling data and sending actuation signals. Low level DAQmx VIs are chosen as they are more flexible and consume less CPU effort compare to DAQ Assistant. DAQmx Read VI continuously takes N samples from feedback signal in each loop then mean value of the samples is calculated as the observation. In piezo actuation mode, the feedback is the deflection signal from photodetector, which indicates the actual condition of the cantilever and the force applied on the samples; in magnetic actuation mode, besides for the deflection signal the current applied on the coil is also measured. Meanwhile DAQmx Write VI sends back the required actuation signals to piezo or coil amplifier once in each loop. Create Chanel, Start Task, Stop Task and Clear VIs are placed outside the while loop so that the tasks only start once when program is launched, and keep reading or writing data until the program stops. Consequently the efficiency is further improved.

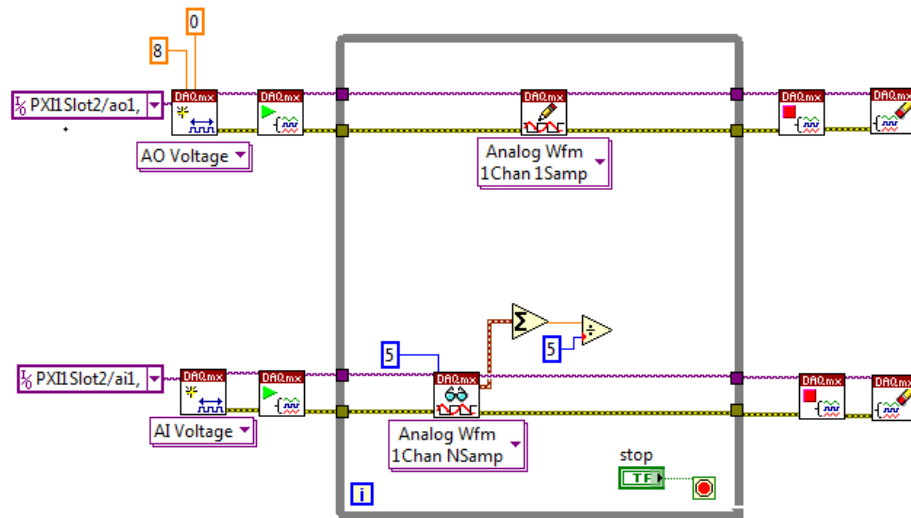


Figure 2.11. Measurement I/O Programmed in LabVIEW.

The closed-loop controller is designed especially for force-clamp experiments. The PID controller is programmed to drive both the piezo and the coil amplifier of the AFM system (Figure 2.12). Controller calculates the error between the setpoint force and the observed signals and determines the require actuation to reduce the error.

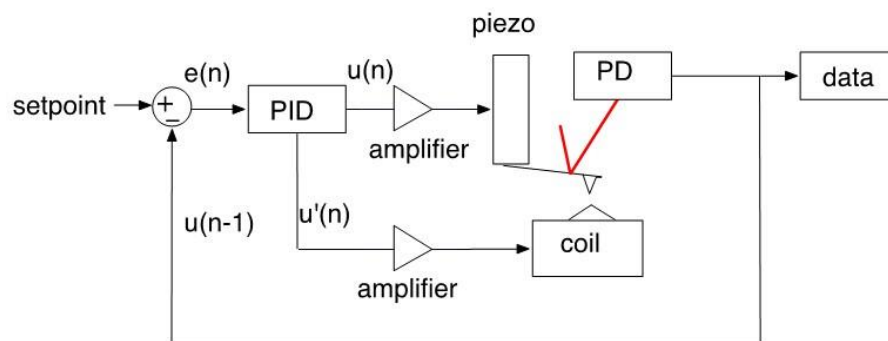


Figure 2.12. Block Diagram of the Controller and AFM System.

When detailed knowledge of the controlled process is absent, the PID controller has historically been considered to be the best controller [28,29], it's simple, straightforward and reliable. A velocity algorithm is chosen for the closed-loop controller because it can avoid large sudden changes of output and windup.

The outputs of the PID controller, u , is the voltage sent to the piezo amplifier in piezo actuation mode and the voltage sent to the power amplifier in magnetic actuation mode. In both case $u(t)$ can be modeled for a continuous time system as follows:

$$u(t) = K_p \left\{ e(t) + \frac{1}{T_i} \int_0^t e(t) dt + T_d \frac{de(t)}{dt} \right\} \quad (2.9)$$

For a discrete time system, the output $u(n)$ can be expressed as:

$$u(n) = K_p \left\{ e(n) + \frac{T}{T_i} \sum_{j=0}^n e(n) + \frac{T_d}{T} [e(n) - e(n-1)] \right\} \quad (2.10)$$

So, the incremental of output to piezo $\Delta u(n)$ can be expressed in terms of error $e(n)$ and constants as below:

$$\begin{aligned} \Delta u(n) &= u(n) - u(n-1) \\ &= K_p \left[\left(1 + \frac{T}{T_i} + \frac{T_d}{T}\right) e(n) - \left(1 + \frac{2T_d}{T}\right) e(n-1) + \frac{T_d}{T} e(n-2) \right] \end{aligned} \quad (2.11)$$

$$T_i = K_p / K_i \quad (2.12)$$

$$T_d = K_d / K_p \quad (2.13)$$

Therefore,

$$\Delta u(n) = (K_p + K_i T + \frac{K_d}{T})e(n) - (K_p + \frac{2K_d}{T})e(n-1) + \frac{K_d}{T}e(n-2) \quad (2.14)$$

Here $e(n)$ is the error between photodetector readout and clamping force at moment n , K_p , T_i , T_d and T are proportional gain, integral time, derivative time and sampling time interval, respectively. Although the derivative term can predict error and decrease overshoot, it's very sensitive to noise and can make the system unstable. Unless it is needed, T_d is usually set to be zero.

When T_d is 0, the controller algorithm can be simplified as PI control

$$\Delta u(n) = (K_p + K_i T)e(n) - K_p e(n-1) \quad (2.15)$$

The PID controller and PI controller can be programed as Figure 2.13. They are programed in a Sub-VI so that they can be reused when being called. It's obvious that PI controller consumes much less memory and calculation effort than PID controller, so normally PI Sub-VI is called instead of PID Sub-VI.

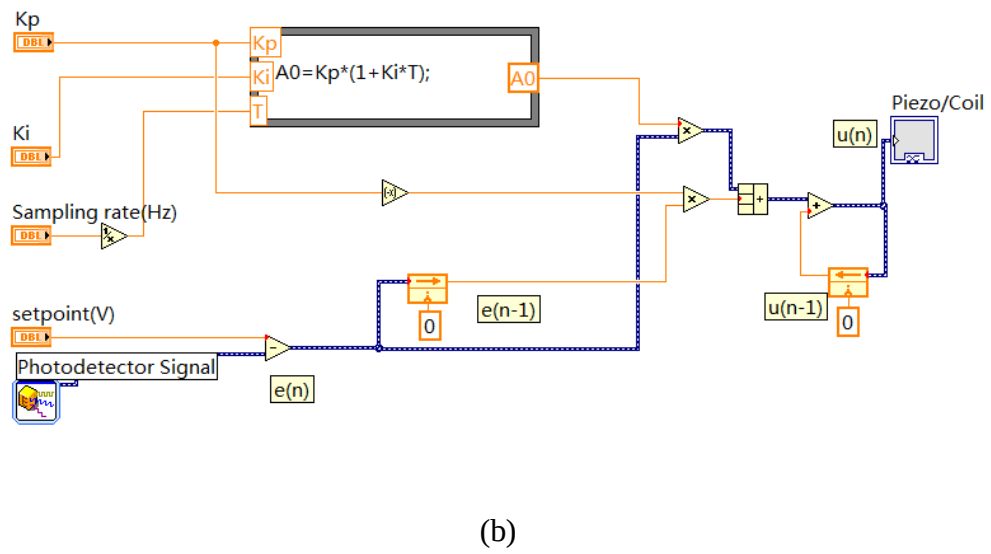
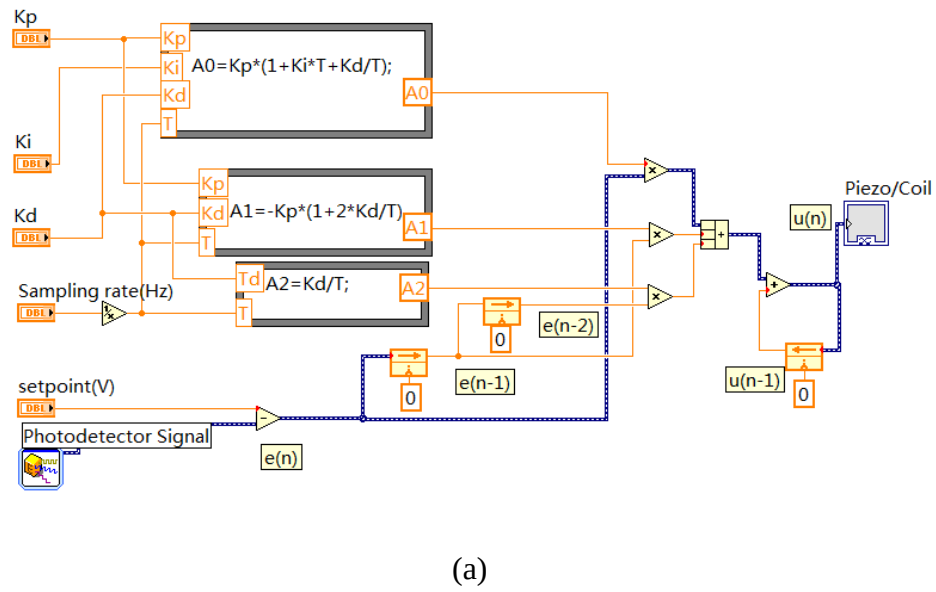


Figure 2.13. Controller Programed in LabVIEW (a) PID and (b) PI.

Since the photodetector readout is a voltage signal, to implement the algorithm the clamping force should also be converted into voltage V_c .

$$V_c = \frac{10^{-6} F_c}{kS} + V_0 \quad (2.16)$$

Here F_c (pN) is the expected clamping force, k (N/m) is the spring constant of the cantilever obtained by thermal fluctuation method, V_0 (V) is the deflection offset when cantilever is out of contact with the surface, S ($\mu\text{m/V}$) is the deflection sensitivity.

User interface comprises indicators such as waveform charts and Boolean lights and controls such as buttons and variable inputs. It allows users to monitor the conditions of the AFM and to take action during the experiments.

The interface for piezo actuation force clamped experiments is shown in Figure 2.14. In case of piezo actuation, the global values and controller parameters should be entered before running the program. Bio-molecular coated on the coverslip can be picked up in the “1 pick up” Run Mode by adjusting the “Bias Voltage” to the piezo and bring the cantilever in contact with the coverslip. Then pulling force clamp can be conducted in “Pulling Force Clamp Mode” by setting the “move cantilever” to a value between 1 and 3 then click the “Pull” button. Once the “Pull” button is clicked, controller will move piezo upwards and keep the cantilever at “Pulling Force” until rupture event appears if bio-molecular is picked up or will move piezo all the way upwards until the setting value of “move cantilever”.

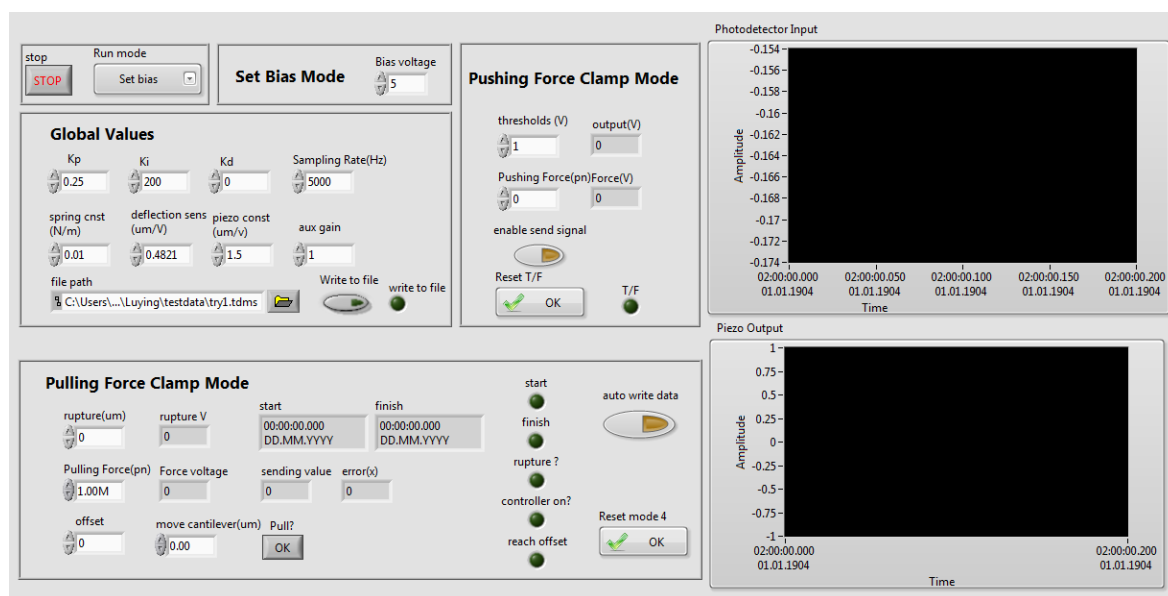


Figure 2.14. Piezo Actuated Force Clamp Interface.

The interface for magnetic actuation force clamp is shown in Figure 2.15. The experiment procedure is a little different from the piezo actuation. The global values and controller parameters should be entered before running the program. Bio-molecular coated magnetic samples can be pick up using the “1 pick up” Run Mode, sending signal to coil allow a magnetic field being generated near the tip of the coil and collect those samples, DC value to piezo controls the Z-position of cantilever and can bring the cantilever in contact with the samples. After the cantilever and samples in contact for a few seconds, the Run Mode is switched to “2 Force Clamp” mode, meanwhile signal to coil will automatically be set to 0. Then cantilever is brought out of contact from the surface by setting the “DC to piezo to 0”. Pushing the “Pull” button will turn on the controller and keep the pulling force to the “Set Force” until rupture if samples are picked up or keep increasing the current sent to the coil until reaching “Current Limit”. The controller will automatically switch off and current will set to be 0 if a rupture event is detected or the current reaches a predetermined threshold level.

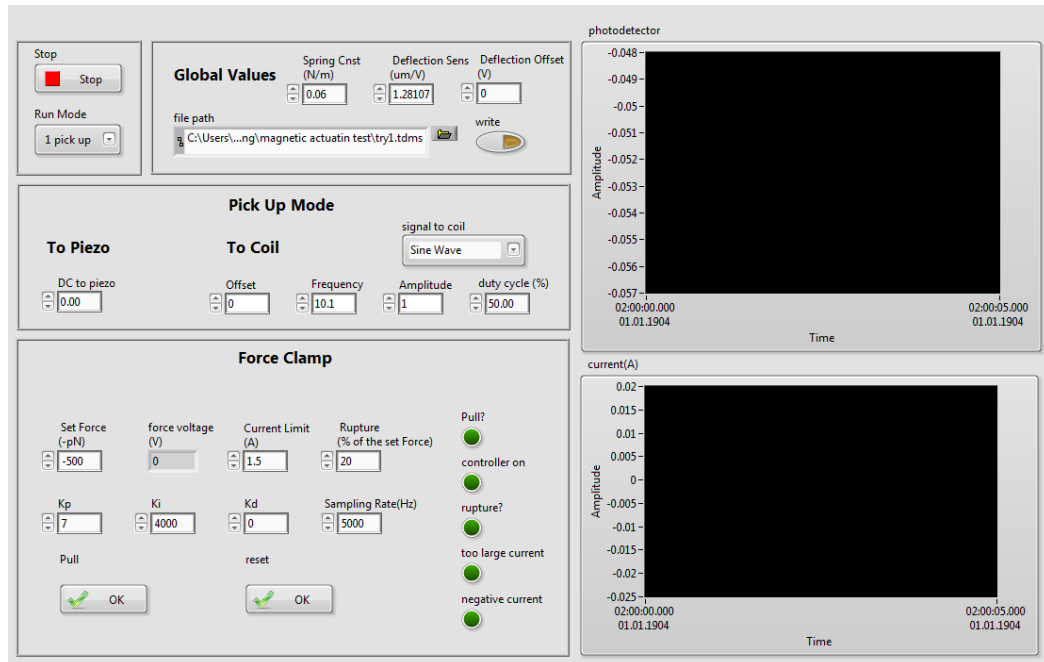


Figure 2.15. Magnetic Actuated Force Clamp Interface.

File I/O is for recording the useful data during the experiment. In piezo actuation mode, the data need to be record are time, voltage signal sent to piezo and deflection signal from photodetector. In magnetic actuation mode, the data need to be record are time, voltage signal sent to piezo, voltage signal sent to the current amplifier and current signal that send to the coil.

The open file and close file functions are placed outside of the while loop as shown in Figure 2.16, therefore the file won't be open and closed repeatedly when data are streamed to hard disk. All the data to be written by TDMS Write are taken from the queue in big chunks by setting the NI_MinimuBufferSize property to multiple of 512 bits.

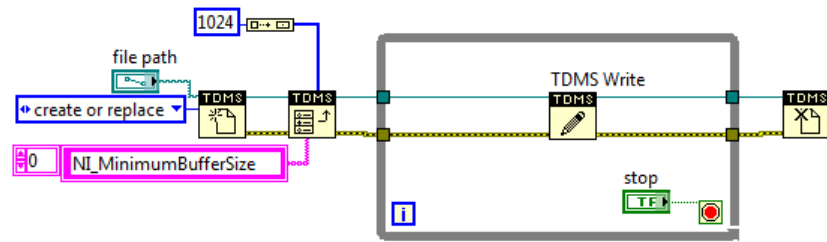


Figure 2.16. File I/O Programmed in LabVIEW.

3. CHARACTERIZATION OF THE CONTROLLER

The force level in biomolecular experiments usually ranges from several piconewtons to a few hundred piconewtons. The probability of picking up biomolecules by cantilever depends on the sample preparation procedures, but generally is set to a value between 10% and 40% to guarantee single-molecule interactions. Therefore to verify the capability of the controllers and further improve their performance conveniently and efficiently, some experiments where higher force levels on the order of nanonewton and high probability to form adhesion are specially designed.

To perform force clamp experiments, proper parameters for PID controller should be found by following an empirical method in Appendix A. The performance of PID controllers can be specified by step response which can be described by the following quantities [30].

Overshoot. The maximum peak value of the response curve that exceed final steady-state value. Overshoot is not desired in biomolecular experiments as a large force can break the bonds between molecules easily.

Rise time. The duration of time it takes for the response signal to reach a specified threshold value from an initial value, and it is defined as 80% of the steady-state value here. Shorter values of rise time are desired to ensure the controller respond fast.

Settling time. The duration of time it takes for the response signal to reach the final steady-state value and remain in an error band that is symmetrical about the final value, and the error band is defined as 10% here.

To compare data conveniently, the recorded photodetector signals and piezo signals

are converted from voltage to force F_{ph} and F_p in units of piconewtons respectively following the way below,

$$F_{ph} = 10^6 (V_{ph} - V_0) Sk \quad (3.1)$$

$$F_p = 10^6 (V_p - V_{p0}) Dk \quad (3.2)$$

Where $S(\mu\text{m}/\text{V})$ is deflection sensitivity, k is spring constant of the cantilver, D is piezo constant which is $1.5\mu\text{m}/\text{V}$ here. V_{ph} and V_0 are deflection signal read from the photodetector and deflection offset respectively, V_p and V_{p0} are the voltage applied on piezo and offset voltage on piezo respectively.

It's obvious that the minimum detectable force depends on the deflection sensitivity and spring constant of the cantilever. The cantilever used in biomolecular experiments usually has spring constant range from $0.01\text{N}/\text{m}$ to $0.06\text{N}/\text{m}$ and deflection sensitivity range from $0.35\mu\text{m}/\text{V}$ to $1\mu\text{m}/\text{V}$.

3.1. Piezo Actuation Force Clamp Controller

3.1.1. Verification of the Functionality

In the experiment performed to verify the functionality of the controller in piezo actuation mode, a silicon nitride cantilever was immersed in deionized water that models the environment of bio-molecular experiments and placed over silicon substrate; the unspecific adhesion force between the cantilever and substrate models the bonding between biomolecules; controller tries to keep the force on the cantilever at the setpoint.

Figure 3.1 shows a typical force clamp data from this experiment in which the spring constant of the cantilever was 0.06N/m and deflection sensitivity was 0.9 μ m/V. Controller brought the cantilever upward from contact position, which is not shown in here, by decreasing the voltage sent to the piezo until the deflection signal detected by photodetector reaches the setpoint, which was 1000pN here. The controller actuated the cantilever to a new setpoint when the setpoint was set to a new value. Once the adhesion between cantilever and coverslip ruptured, as shown in the end of c, controller turned off and brought cantilever to the top most position.

The experimentally determined noise levels are 42.2pN, 40.5pN and 73.4pN in a, b and c where controller was on respectively. The noise shown in c is dominated by the environmental factors such as mechanical vibration of the table and the acoustic noise. This demonstrates the controller is capable of suppressing low frequency disturbance such as mechanical vibration and thermal fluctuations.

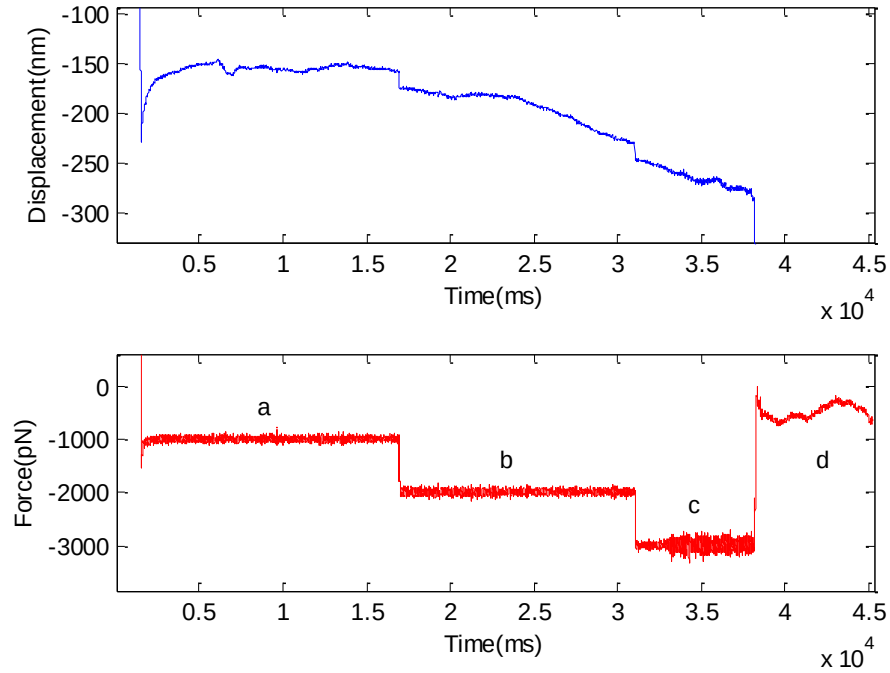


Figure 3.1. Force Clamped Data with Silicon Nitride Cantilever and Silicon Substrate in Water.

3.1.2. Step Response

The delay due to the actuator, piezo, can be omitted in case of piezo actuation because the response times of piezo actuators is only a few microseconds which is insignificant compared to the sampling time. Therefore the temporal response of the system is limited with the parameters of the PID controller. Experiments using silicon nitride cantilevers and silicon coverslips were performed to observe the step responses of the AFM in piezo actuation mode with different PID parameters.

Figure 3.2 shows a time trace for the step response of the AFM under underdamped PID control setting with piezo actuation when an instantaneous step force of 1000pN was applied. The percentage overshoot was measured 20%, rise time was measured 9ms and

settling time was measured 22ms.

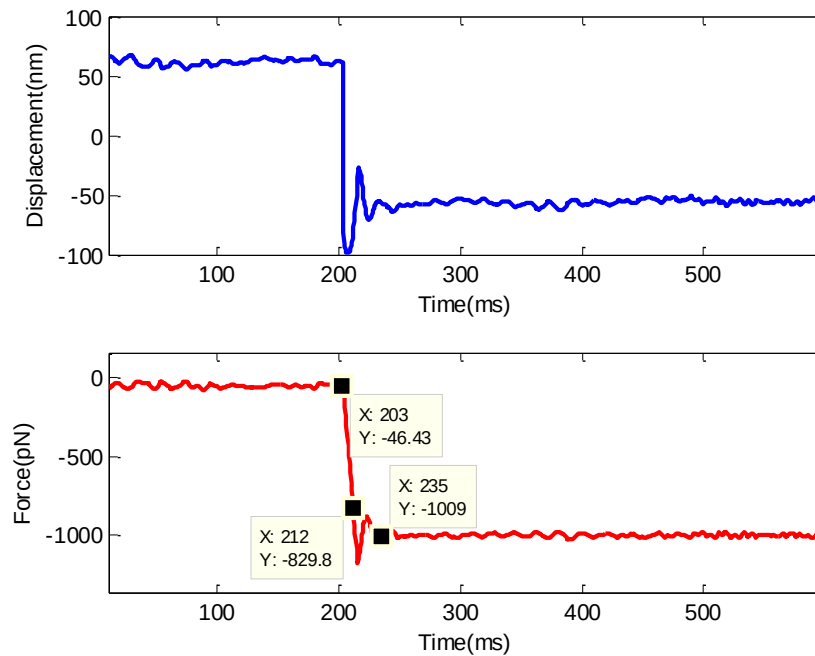


Figure 3.2. Step Response of the AFM under Underdamped PID Control Setting with Piezo Actuation.

Figure 3.3 shows a time trace for the step response of the AFM under overdamped PID control setting with piezo actuation when an instantaneous step force of 1000 pN was applied. The response did not exhibit overshoot, the rise time was measured to be 17.5ms and settling time was measured 40ms. Usually a slightly overdamped system is more desirable in biomolecular experiment.

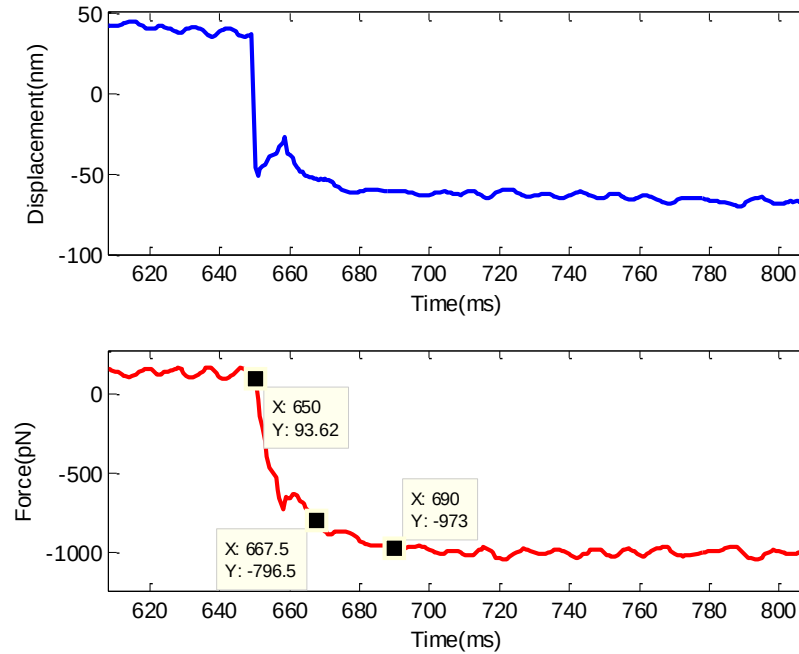


Figure 3.3. Step Response of the AFM under Overdamped PID Control Setting with Piezo Actuation.

3.1.3. Force Resolution

Figure 3.4 shows a time trace recorded during a biomolecular experiment, corresponding to a nonspecific adhesion event. The spring constant of the cantilever was 0.01N/m and deflection sensitivity was measured 0.5318 $\mu\text{m}/\text{V}$. While low frequency disturbance could be compensated by the controller, high frequency noise from the electronics or the laser remains as can be seen in Figure 3.4. The experimentally determined value of the noise on the photodetector signal in force clamped portion where the controller was on is measured 7.52pN.

An adhesion/rupture even on the order of few tens of piconewtons, which is more than twice the noise level, can be detected easily.

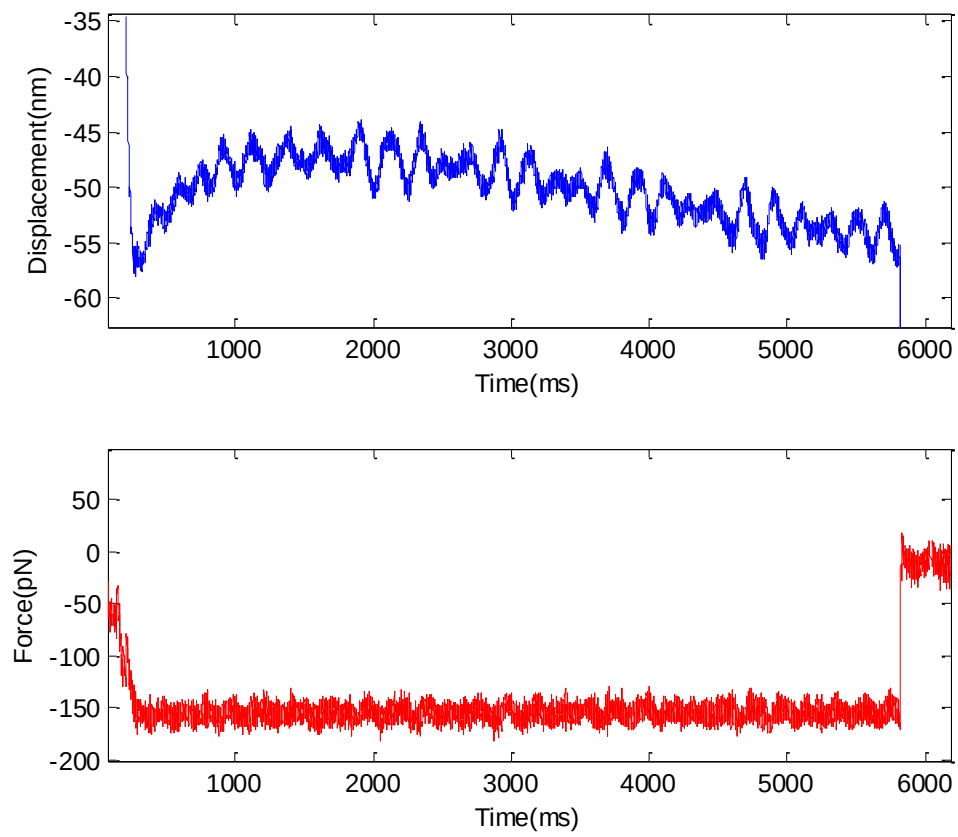


Figure 3.4. Nonspecific Adhesion during a Biomolecular Experiment.

3.2. Magnetic Actuation Force Clamp Controller

3.2.1. Verification of the Functionality

In the experiment performed to verify the functionality of the controller in magnetic actuation mode, as shown in Figure 3.5, cantilever was attached to magnetic beads through nonspecific adhesion to simulate molecular bonds, then was placed in deionized water. The cantilever was aligned well with the tip of the magnetic coil to guarantee the generation of adequate force levels.

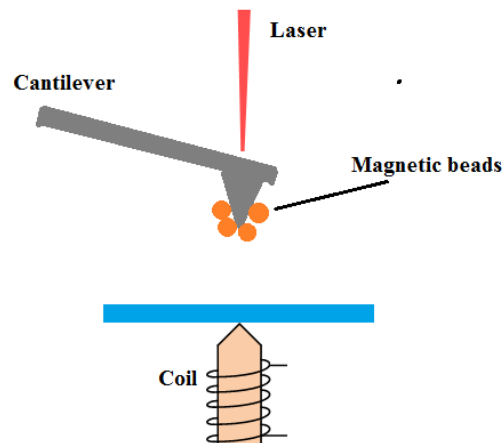


Figure 3.5. Schematic of the Verification Experiment.

Figure 3.6 shows a typical force clamp time trace from this experiment in which the spring constant of the cantilever was 0.06N/m and deflection sensitivity was measured $0.61\mu\text{m/V}$. Controller pulled the cantilever downward from 0 force position by increasing the current on the magnetic coil until the deflection signal detected by photodetector reached the setpoint, which is about 550pN . The setpoint was changed with steps of 100pN . Consequently, the controller brought the cantilever to the new setpoint.

The experimentally determined noise levels are 17.7pN, 17.3pN and 14.7pN in b, c and d where controller was on respectively. This demonstrates the controller is capable of suppressing low frequency disturbance such as mechanical vibration and thermal fluctuations.

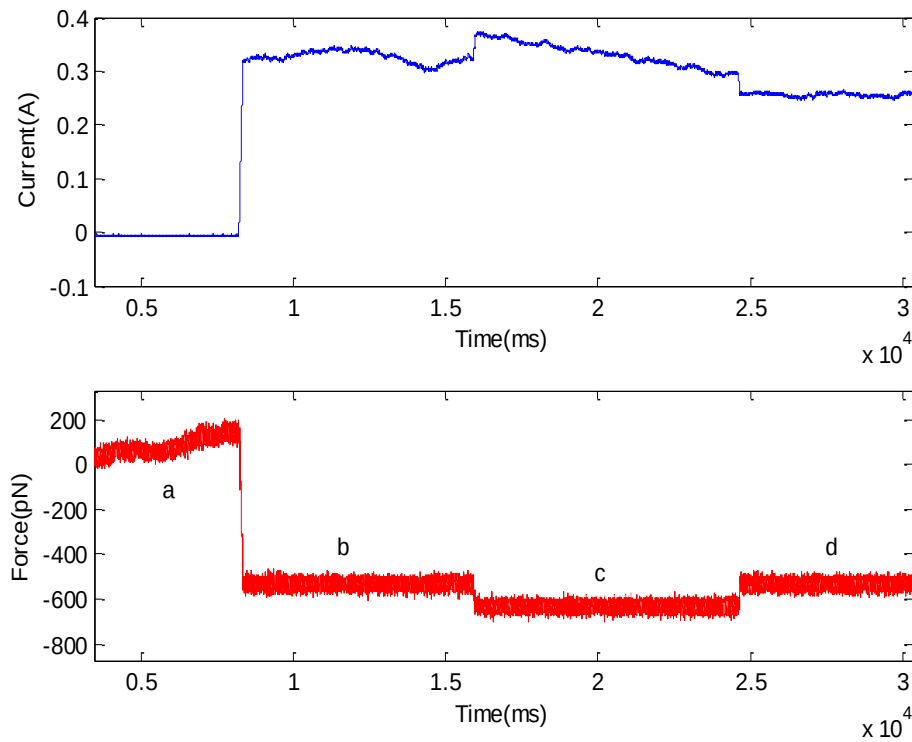


Figure 3.6. Force Clamped Data from Verification Experiments for Magnetic Actuated Mode.

3.2.2. Step Response

The delay due to the actuator and the magnetic coil, can be ignored as compared to the controller because the bandwidth of the coil and the amplifier is larger than 50 kHz. Therefore choosing proper PID parameters is very important. Experiments with magnetic beads attached cantilever were performed to obtain the step response of the AFM in magnetic actuation mode under different PID parameters.

Figure 3.7 shows a time trace for the step response of the AFM under underdamped PID control setting with magnetic actuation when an instantaneous step force of 500pN was applied. The percentage overshoot was measured 20%, rise time was measured 10ms and settling time was measured 40ms. The deflection sensitivity was measured $1.28\mu\text{m/V}$, nominal spring constant of the cantilever was 0.06N/m .

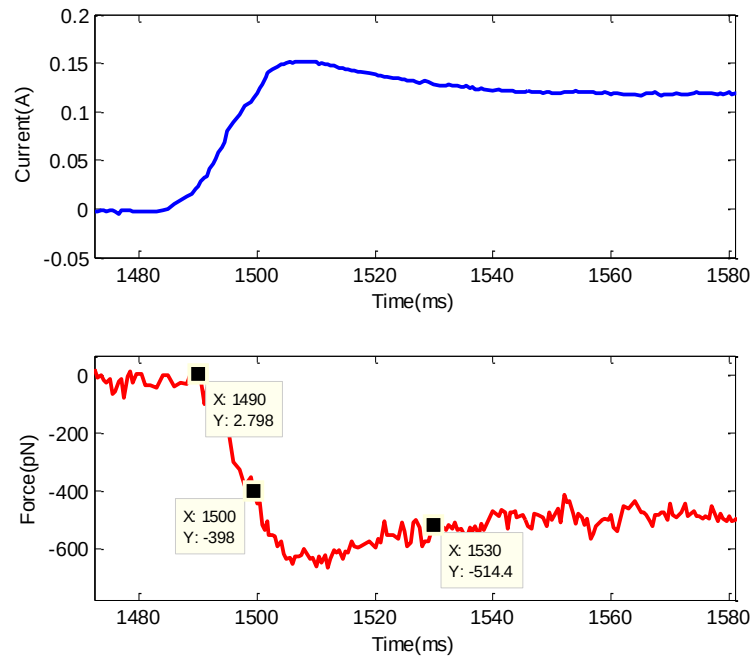


Figure 3.7. Step Response of the AFM under Underdamped PID Control Setting with Magnetic Actuation.

Figure 3.8 shows an example of 500pN step responses of the AFM under overdamped PID control with magnetic actuation. The step response doesn't exhibit overshoot, the rise time is 20ms and settling time is 44ms. Usually a slightly overdamped system is more desirable in biomolecular experiment.

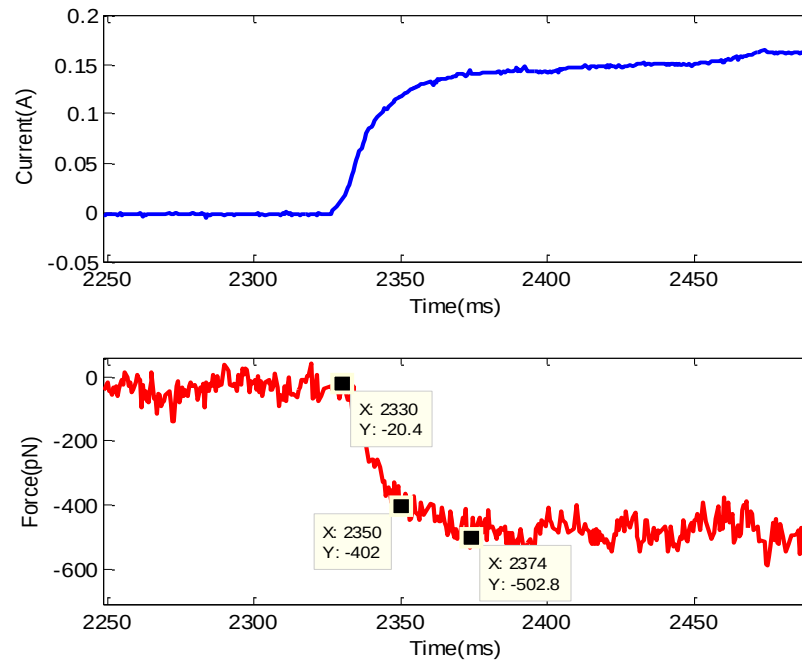


Figure 3.8. Step Response of Magnetic Actuated AFM with Overdamped PID Controller Parameters.

3.2.3. Force Resolution

Figure 3.9 shows a time trace of force measured by the cantilever and the current applied to the cantilever when a magnetic bead detached from the cantilever. The total force applied on the cantilever suddenly decreased when the magnetic beads detached. Consequently, the controller detected the change and increased the current applied to the coil, therefore increased the magnetic force applied to the remaining beads on the cantilever to bring the cantilever back to the setpoint. The spring constant of the cantilever was 0.06N/m and deflection sensitivity was measured 0.56 μ m/V. The noise measured during force-

clamped portion where the controller was on was measured 10.56pN.

This experiment models for sudden biomolecular rupture events with a rupture force of 75 pN.

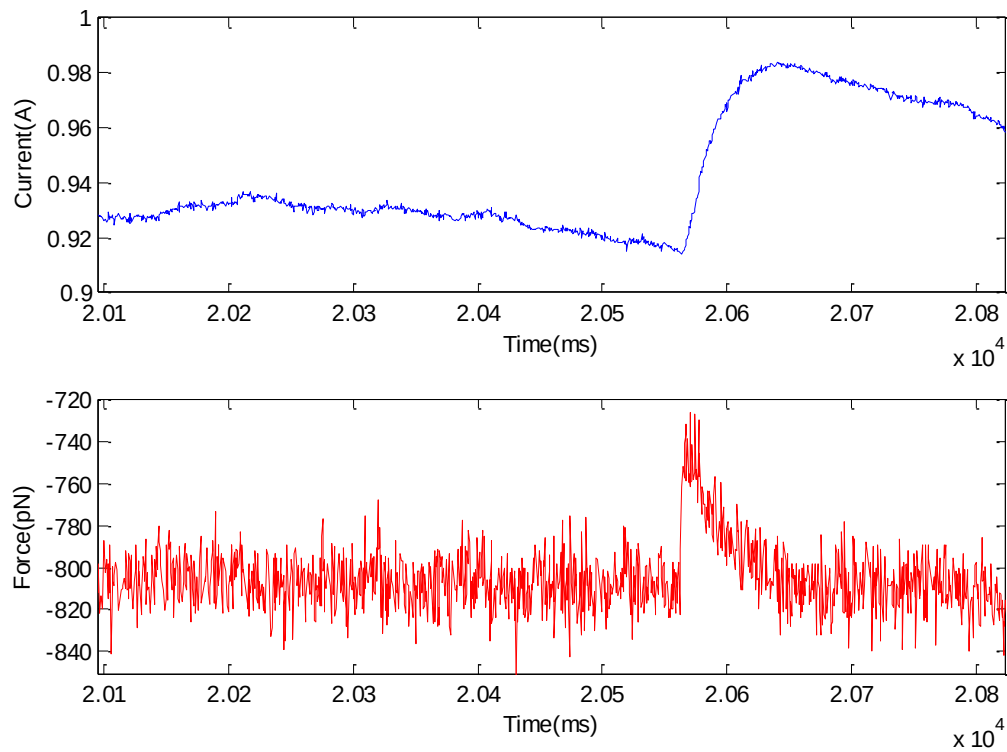


Figure 3.9. Rupture Event from Magnetic Actuated Force Clamped Experiment.

4. BIOMOLECULAR EXPERIMENTS

Heparin-FGF2 and biotin- streptavidin force clamp experiments were performed using piezo actuation and magnetic actuation to study the lifetime of the biomolecular bonds.

4.1. Heparin- FGF2 Experiments

Heparin is a highly sulfated polysaccharide, which plays important roles in fundamental biological process by binding to proteins. Fibroblast growth factor (FGFs) is a kind of protein presents in basement membranes and in the subendothelial extracellular matrix of blood vessels which has severe effects on growth, differentiation, migration and survival of wide variety of cells [31,32].

It has been hypothesized that, during both wound healing of normal tissues and tumor development, heparin sulfate differentially regulates the activity of FGFs, especially FGF1 and FGF2, thus mediating the formation of new blood vessels [33,34]. Although heprin is widely used as an injectable anticoagulant [35], its true physiological role remains unclear.

Force clamp pulling experiments were performed to investigate the interactions between heparin and FGF2 using both piezo actuation and magnetic actuation.

4.1.1. Sample Preparation

An incubation procedure at room temperature is required to prepare the sample for piezo actuated force clamp experiment for heparin-FGF2 pairs. The cantilever (Veeco probes, MLCT-C, nominal spring constant 0.01 N/m) was incubated in a droplet of FGF2 with a concentration of 112.5 $\mu\text{g/mL}$ for 30 minutes. The coverslip was incubated in heparin solution with a concentration of 20 $\mu\text{g/mL}$ for 30 minutes, then incubated in bovine serum albumin (BSA) solution with a concentration of 1% w/v for another 20 minutes to avoid non-specific adhesion between the tip and the substrate. After incubation steps, the functionalized cantilever was immersed in 100 μL phosphate buffered saline (PBS) that is placed on the heparin coated coverslip.

Figure 4.1 shows the schematic of heparin-FGF2 experiment with piezo actuation. If the tip of the cantilever happens to be right on a heparin molecule when being pressed on the surface, FGF2 on the cantilever may bond with heparin that is attached on the coverslip. Therefore the interaction between heparin and FGF2 can be investigated by pulling the cantilever upwards through piezo actuation.

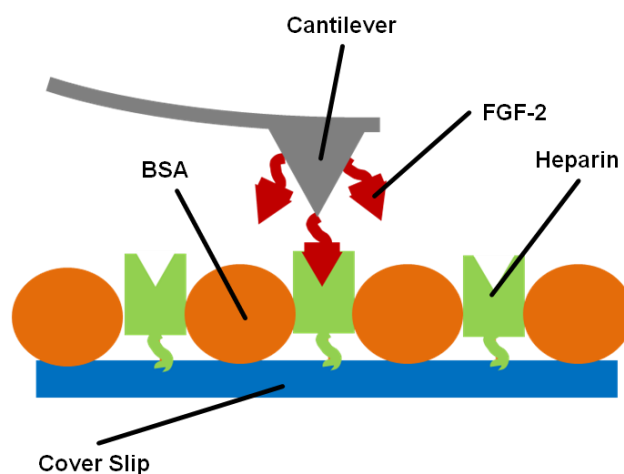


Figure 4.1. Schematic of Heparin-FGF2 Experiment with Piezo Actuation.

The sample preparation procedure for heparin-FGF2 experiments with magnetic actuation is slightly different. The cantilever (Veeco probes, MLCT-C, nominal spring constant 0.01 N/m) was incubated in a droplet of FGF2 with a concentration of 112.5 $\mu\text{g/mL}$ for 30 minutes at room temperature. The coverslip was then incubated in BSA solution with a concentration of 1% w/v for 30 minutes to avoid non-specific adhesion between the tip and the substrate. Then a 100 μL PBS droplet was used to create a meniscus around the cantilever on the coverslip and 2 μL solution of heparin-coated magnetic beads was injected into the PBS droplet. Functionalized cantilever was immersed in PBS after being well aligned with the tip of magnetic core along the long axis of the magnetic core.

Figure 4.2 shows the schematic of the setup for heparin-FGF2 experiments with magnetic actuation. The FGF2-functionalized cantilevers are brought in contact with heparin-functionalized beads. Then, the cantilever is pulled back and the magnetic actuator is turned on. If FGF2 and heparin form a biomolecular bond, then applying magnetic force on the beads can break the bond.

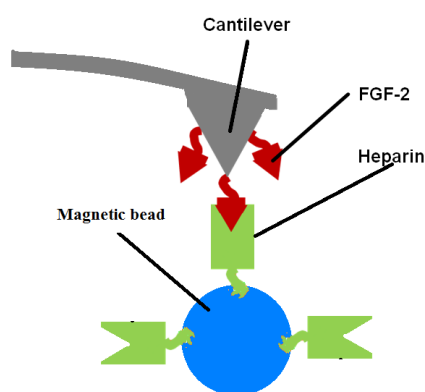


Figure 4.2. Schematic of Heparin-FGF2 Experiment with Magnetic Actuation.

4.1.2. Experiment Results

Figure 4.3 shows a typical time trace for heparin-FGF2 force clamp experiment with piezo actuation. During these experiments, life-time of the molecular bonds were measured. The life-time is defined as the duration of time between the instance when the molecular bond is loaded and the time instance of bond breakage. Figure 4.3 shows a life-time of 469ms for a clamping force of 150pN.

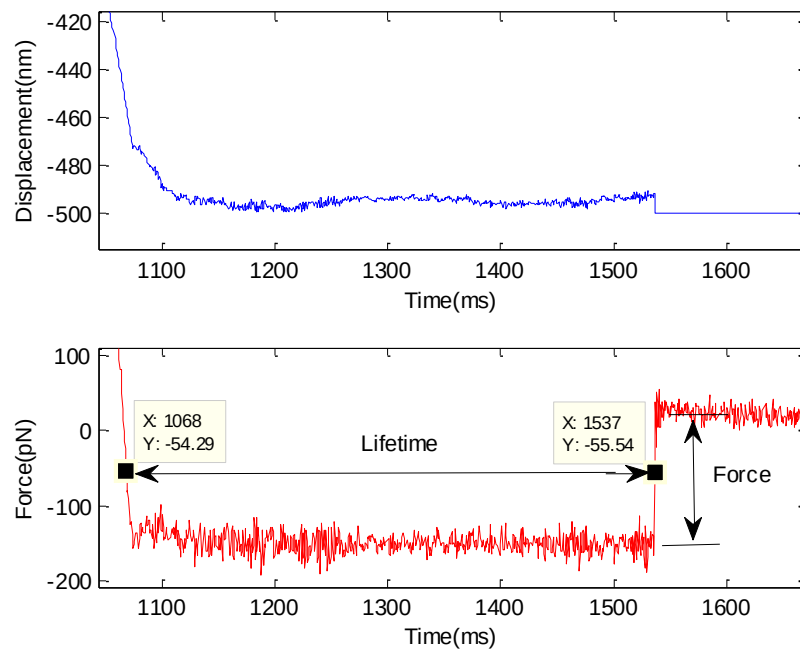


Figure 4.3. Heparin-FGF2 Force Clamped with Piezo Actuation.

Statistical analysis to estimate the lifetime for heparin-FGF2 bonds was performed after adequate number of data was generated. For each clamping force at least 30 rupture events were analyzed. The data were grouped by different force range, and then histograms of life-time in each force group were generated as shown in Figure 4.4. According to the histograms, the majority of the rupture events happen within 1000ms, therefore the rupture happening after that can be considered as non-specific adhesion. The probability of rupture decreases exponentially as life-time increase. Lifetime tends to decrease with increasing values of clamping force as expected for slip bonds.

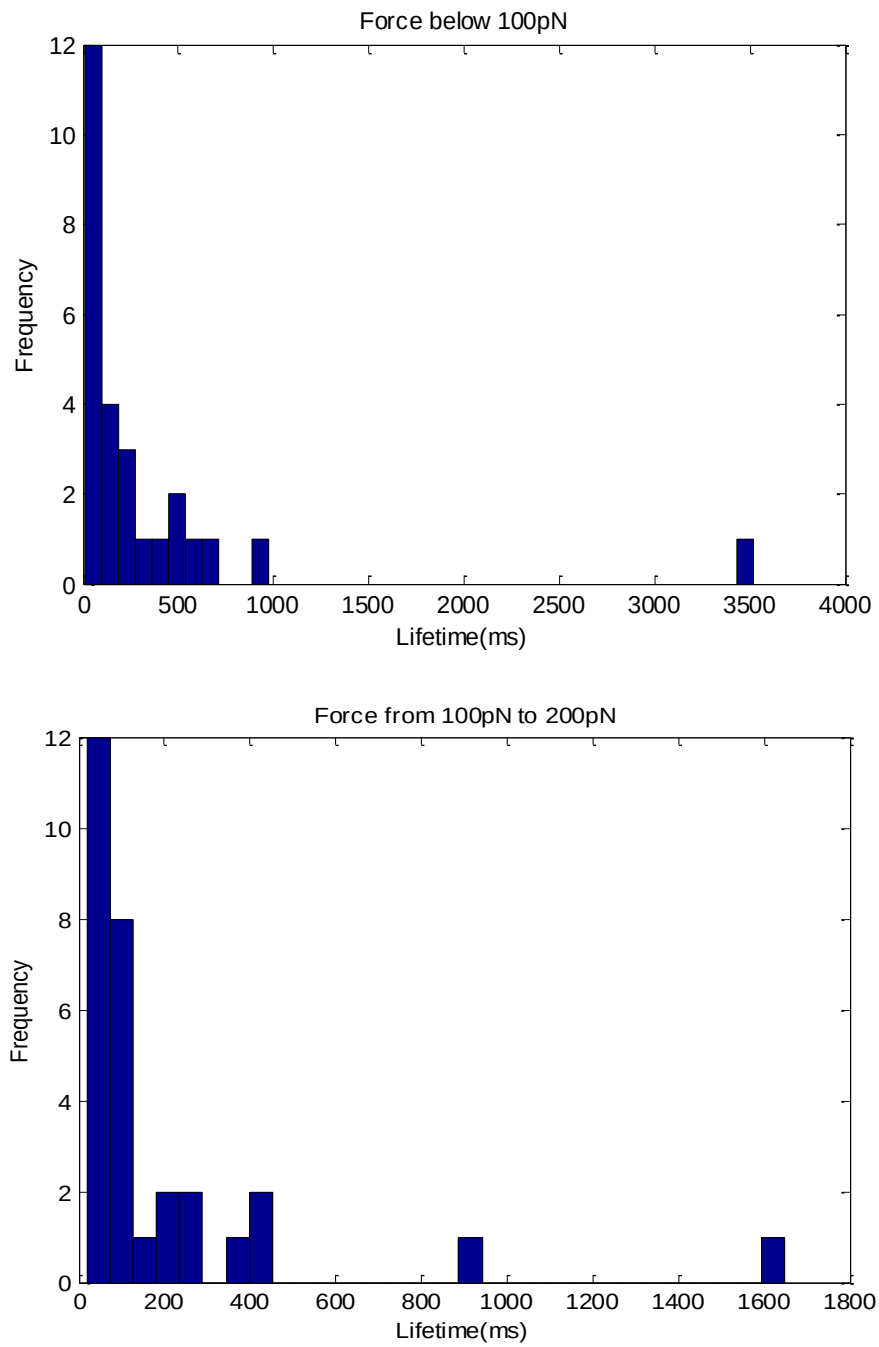


Figure 4.4. Histogram of Lifetime of Heparin-FGF2 in Different Force Levels.

The experiments to measure the life-time between heparin-FGF2 pairs were also performed using magnetic actuator. Figure 4.5 shows a time trace for a clamping for of 100

pN. The measured value for the life-time is 350ms.

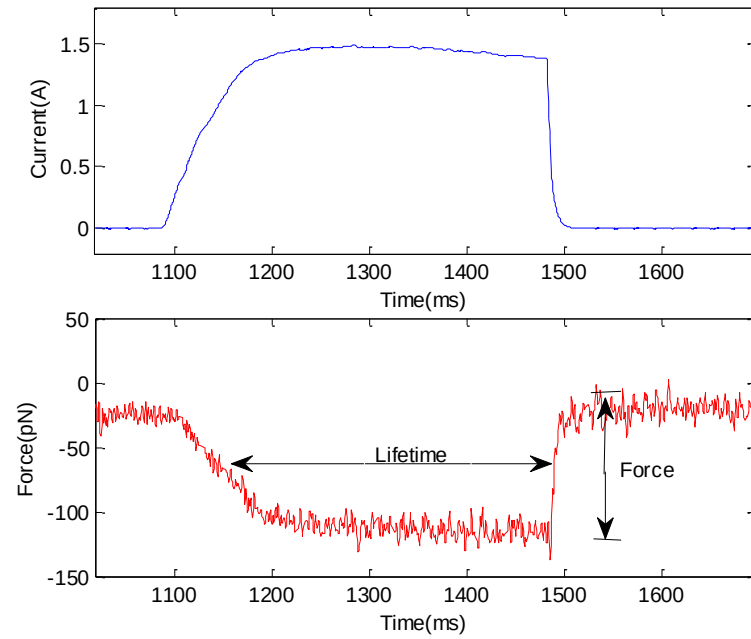


Figure 4.5. Heparin-FGF2 Force Clamped with Magnetic Actuation.

4.2. Biotin- Streptavidin Experiments

In addition to heparin/FGF2 experiments, biomolecular force-clamp experiments were performed using piezo and magnetic actuation methods to investigate the pairs of biotin and streptavidin. Biotin, also known as Vitamin H, is necessary for cell growth and the metabolism of fats and amino acids [36]. Streptavidin is a protein purified from fermentation filtrates of streptomycetes [37] which has very high affinity to biotin [38,39].

4.2.1. Sample Preparation

In the piezo actuated force clamp experiments, commercial cantilevers functionalized with biotin are used. The nominal spring constant of the cantilever is 0.06N/m. The cantilevers are actuated against a stationary cover slip that is uniformly covered with immobilized Streptavidin (VXP0010, Xenoprobe). The experiments are performed in 100 μ L PBS buffer at room temperature.

Figure 4.6 shows the schematics for the setup of biotin-streptavidin experiments with piezo actuation. The cantilever is actuated against the cover slip. Biotin and streptavidin form a weak bond with a certain probability when the cantilever is pressed against the cover slip. During retraction part of the experiment, the molecular bond is loaded and finally a rupture event is observed.

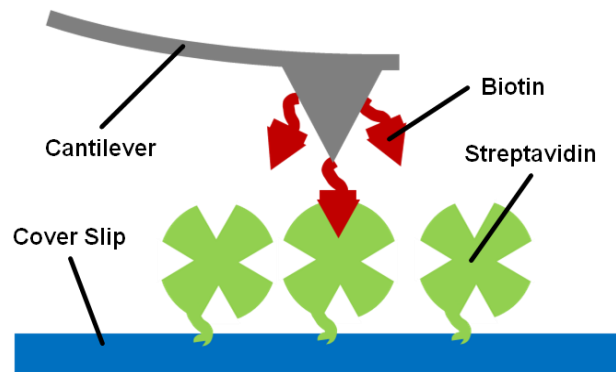


Figure 4.6. Schematic of Biotin-Streptavidin Experiment with Piezo Actuation.

In magnetic actuated force clamp experiment, the same commercial biotin cantilever as in the piezo experiment is used. The cover slip is first incubated with BSA in 30 minutes to prevent non-specific adhesion between the cantilever and cover slip. Then the cantilever is aligned with the tip of the magnetic coil and immersed in 100 μL PBS where 0.5 μL streptavidin coated magnetic beads (Dynabeads, M-270 Streptavidin) solution is injected in.

Figure 4.7 shows the schematic of biotin-streptavidin experiment with magnetic actuation. The biotin-functionalized cantilevers are brought in contact with streptavidin-functionalized beads. Then, the cantilever is pulled back and the magnetic actuator is turned on. If biotin and streptavidin form a biomolecular bond, then applying magnetic force on the beads can break the bond.

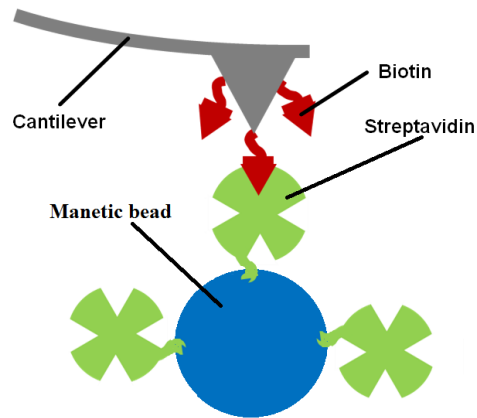


Figure 4.7. Schematic of Biotin-Streptavidin Experiment with Magnetic Actuation.

4.2.2. Experiment Results

Figure 4.8 shows a typical biotin-streptavidin force clamped data in piezo experiments. The lifetime and clamping force, which were calculated in the same way as in the heparin-FGF2 piezo experiments, are about 375ms and 180pN respectively.

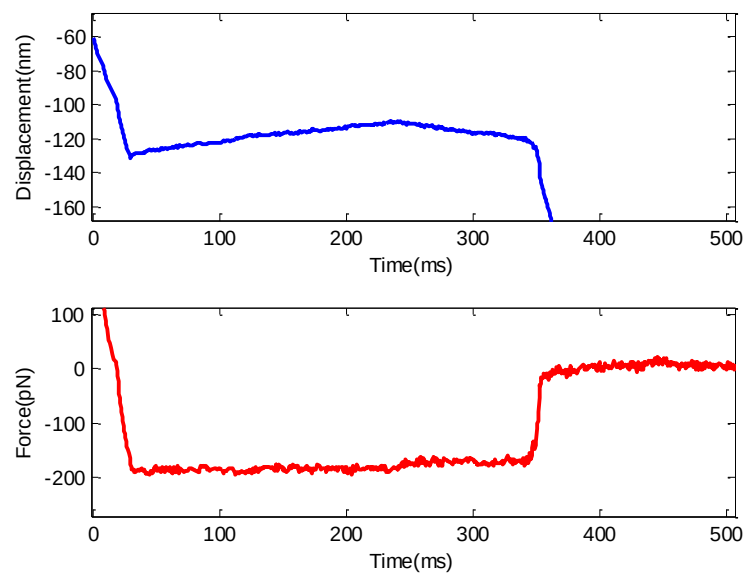


Figure 4.8. Biotin-Streptavidin Force Clamped with Piezo Actuation.

Since only 21 rupture event data are collected, the histogram of lifetime is directly generated without being grouped in different force levels as shown in Figure 4.9. The force here ranges from 50pN to 450 pN. The data with life-times that are too long or with rupture force levels that are too high are considered to be non-specific events, thus are not included in the statistic.

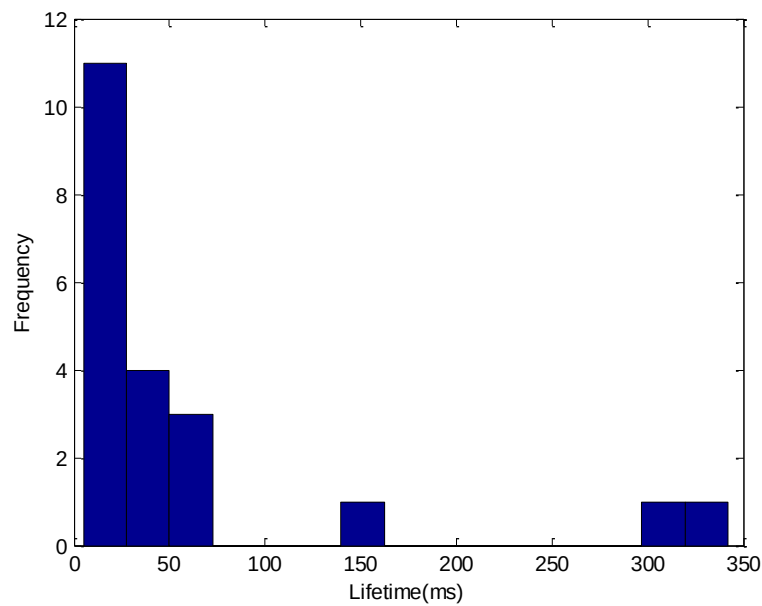


Figure 4.9. Histogram of Lifetime for Heparin-FGF2 with Piezo Actuation.

Figure 4.10 shows a typical biotin-streptavidin force clamped data in magnetic actuation experiments. A life-time value of 412 ms was measured from the total force applied on the cantilever reaches half of the setpoint to the rupture events. A clamping force of 135pN was measured at the instance of rupture. After the rupture event, where one of the bead was detached from the cantilever and total force on the cantilever decreased. Consequently, the controller increased the current on the coil to bring the cantilever back to the setpoint and stopped at 1520ms since the applied current to the magnetic core exceeded the maximum threshold value of 2A

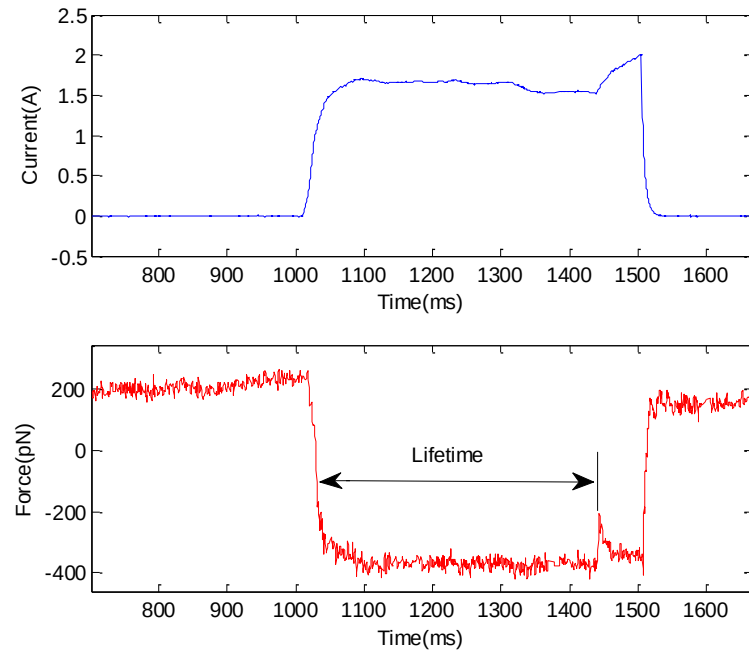


Figure 4.10. Biotin-Streptavidin Force clamped with Magnetic Actuation.

Multiple events in an individual clamping force period may be observed since biotin molecules on the cantilever can bond with multiple beads. If the rupture events due to multiple bonds are closed to each other with respect to time and if the individual events do not introduce significant increase in the current applied to the magnetic core, they still can be detected sequentially. The calculations of lifetime and rupture force values are the same as compared with single rupture events. Figure 4.11 shows an example of multiple events in a single clamped force period. The event that appears right after the second rupture is considered as a rebinding event.

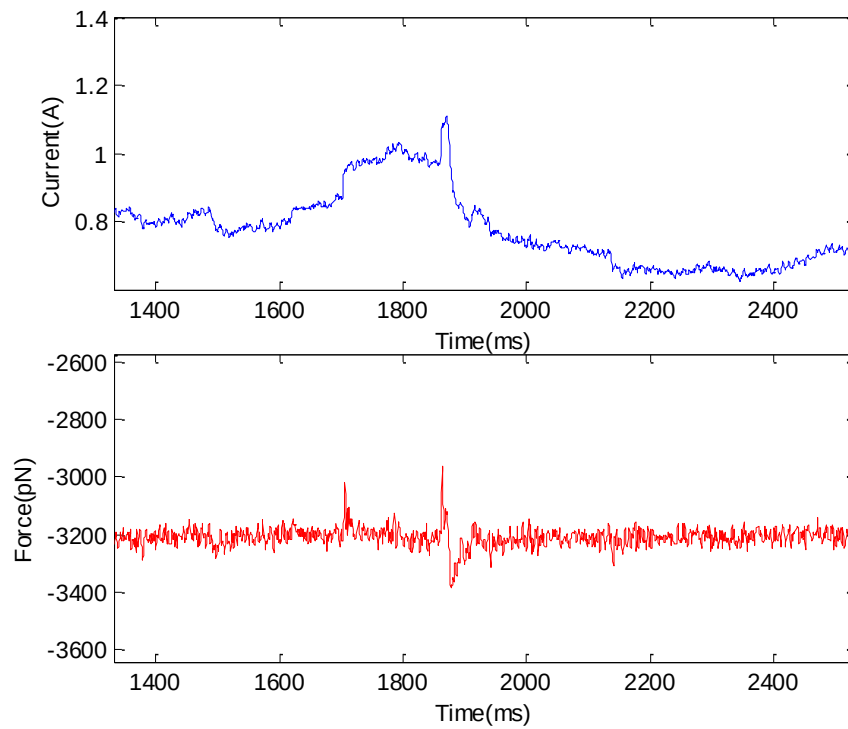


Figure 4.11. Multiple Events in Biotin-Streptavidin Force Clamped Experiment with Magnetic Actuation.

Statistical analysis to estimate the lifetime for biotin-streptavidin bonds was performed after adequate number of data was generated. For each clamping force at least 30 rupture events were analyzed. The data were grouped by different force range, and then histograms of life-time in each force group were generated as shown in Figure 4.12. According to the histograms, the majority of the rupture events happen within 2000ms, therefore the rupture happening after that can be considered as non-specific adhesion. The probability of rupture decreases exponentially as life-time increase. Lifetime tends to decrease with increasing values of clamping force as expected for slip bonds.

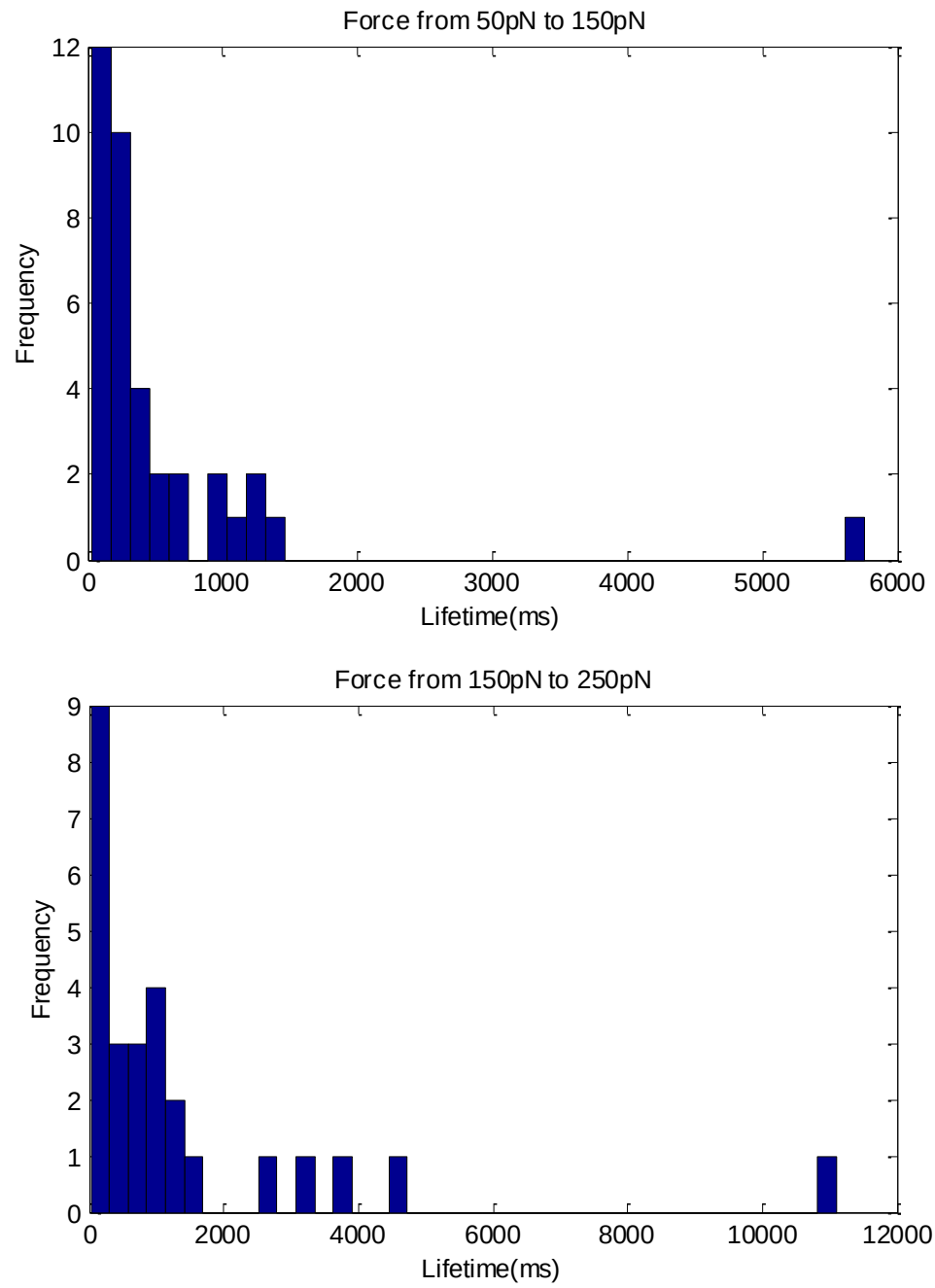


Figure 4.12. Histogram of Lifetime of Biotin-Streptavidin in Different Force Levels.

5. CONCLUSIONS

In this thesis, a PID controller is implemented in LabVIEW for an AFM system with dual actuation capability. The AFM system employs both piezo actuation and magnetic actuation for single-molecule experiments. The development of controller consists of requirement analysis, function realization and optimization. Biomolecular force clamp experiments were performed using this controller to study single-molecular events.

First, the specifications for the electronics and the requirements for the AFM setup were finalized. The procedure force clamp experiments using piezo actuation and magnetic actuation were determined separately.

Modules and logical data flow were designed based on the procedures of force clamp experiments. A velocity PID algorithm was implemented on the controller software. Software efficiency was optimized by reducing CPU effort.

The developed system was first characterized through dry experiments where the conditions for biomolecular force-clamp experiments were mimicked using silicon nitride cantilevers and silicon substrates as hard surfaces for piezo actuation. The biomolecular force clamp experiments using the magnetic actuator were mimicked using magnetic beads attached cantilevers. The force resolution of the AFM system was determined as <10 pN. The time resolution of the system was determined as 20ms during the experiments.

The developed system was employed to study single molecular bonds of heparin/FGF2 and biotin/streptavidin. Histograms of lifetime for heparin/FGF2 were obtained using piezo actuation. Histograms of lifetime for biotin/streptavidin were obtained using both piezo actuation and magnetic actuation. Statistical results indicate the probability of lifetime

decreases exponentially as lifetime increases as expected for slip bonds.

The force resolution of the system can be improved by decreasing the electronic noise and disturbance from the environment as a future work. Implementing a lowpass filter for the photodetector, better shielding of the cables and better connections between the devices are some common methods to decrease electronic noise and interference. Mechanical disturbance can be reduced by better isolation of the AFM system from the sources of mechanical noise, vibration and acoustic noise. Time resolution of the system can be improved by upgrading the hardware of the controller and implementing some advanced control algorithms rather than a classical PID controller.

APPENDIX A: AFM PID CONTROLLER TUNING GUIDE

The performance of a PID controller depends highly on three separate constant parameters, the proportional gain K_p , the integral gain K_i and the derivative gain K_d . It's crucial to choose these three gains properly before using the controller in an actual experiment.

There are several proven methods for tuning a PID controller when the mathematical model of the system is not available. However severe oscillation and large overshoot are highly undesirable for AFM systems, therefore those aggressive methods are not employed.

Usually manual tuning method and Ziegler-Nichols method [40] are used for tuning the PID controller for the AFM system here. The initial experiments for tuning the PID controllers were performed in air and use silicon nitride cantilever and silicon coverslip for piezo actuation and magnetic cantilever for magnetic actuation. The PID gains achieved from these experiments can be used as the starting point of other experiments. Further fine tuning is required for different kinds of samples.

A.1. Manual Tuning Method

Manual tuning method is basically a try and error process and requires some intuition and experience, but it's used most commonly when tuning the AFM system controller because severe oscillation can be avoided. An acceptable result can be achieved following the simple steps.

First K_i and K_d are set to zero. Increase K_p until a step response with small overshoot and some amount of steady state error is observed. Increase K_i until there's no steady state error in the step response. Then decrease K_p a little to reduce the overshoot and further increase K_i to decrease settling time. A very small, start from 10^{-7} here, K_d can be added when the step response is acceptable to further increase the performance.

There are some hints about how to change the gains by investigating the step response. If the step response oscillate frequently, then increase K_p . If overshoot and undershoot on the curve is large, then decrease K_p . If the time for eliminating steady state error is very long, then decrease integral time by increasing K_i . If the oscillation period is long, then increase integral time by decreasing K_i . Whenever oscillation appears K_d should be set to zero.

A.2. Ziegler-Nichols Method

Ziegler-Nichols method is also used in case of piezo actuation when cantilever is in contact with the surface, the upper and lower limits of actuation signal should be set carefully as it's a very aggressive tuning method. Setpoint force is set to be pressing force.

As in the method above, K_i and K_d are first set to zero. Proportional gain is increased slowly until the output starts to oscillate. This gain is defined as K_u , the oscillation period is defined as T_u . K_u and T_u are used to set the gains for different control types as shown in Table A.1.

Table A.1. Gains in Different Control Types with Ziegler-Nichols Method.

Control Type	K_p	K_i	K_d
P	$0.50 K_u$	-	
PI	$0.45 K_u$	$1.2 K_p / T_u$	
PID	$0.60 K_u$	$2 K_p / T_u$	$K_p T_u / 8$

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