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Design and demonstration of dual-beam optical tweezers

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DESIGN AND DEMONSTRATION
OF DUAL-BEAM OPTICAL TWEEZERS

A Thesis

Presented to

The Faculty of the Department of Physics

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

David R. Balsley

May 2001

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ABSTRACT

DESIGN AND DEMONSTRATION OF DUAL-BEAM OPTICAL TWEEZERS

by David R. Balsley

This thesis describes the design, construction, operation and preliminary calibration of a dual-beam optical tweezers assembly to be used for biophysics experiments. Three-dimensional trapping and steering was demonstrated both with a single trapping beam and with two independent beams. Polystyrene and silica spheres with diameters from one to five micrometers and yeast cells approximately four to six microns across were trapped and manipulated by focusing the 514.5 nm light beam from an Argon ion laser through a 100x microscope objective with 1.3 numerical aperture. Variations with time in the backscattered light collected with a photodiode detector were used to calibrate the axial strength of the trap so that it can be used for force measurements. Preliminary data show the axial spring constant to vary linearly with laser power from 0.74×10^{-6} to 2.87×10^{-6} N/m.

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Mike Andler contributed by building electronics for the quadrant detectors, but the greater contribution was his companionship and investigative spirit in the laboratory, which made the research process much more enjoyable.

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CHAPTER 1

1.0 Introduction

It was demonstrated in the early 1970s that dielectric particles with neutral charge interact with light beams in such a way that the particles can be manipulated and trapped by means of radiation pressure.^{1, 2} Early work manipulating dielectric particles with diameters on the order of a few microns led to optical trapping of single particles and even individual atoms using multiple laser beams.³ Arthur Ashkin was a principal figure in the development of various trapping techniques in both arenas. In the mid 1980s Ashkin and collaborators introduced another advancement when they proposed using the intensity gradient produced by focusing a light beam through a short focal length lens to allow trapping of dielectric particles in three dimensions with a single laser beam.⁴ This technique, known as "optical tweezers" or "laser tweezers," has since become a tool used in widespread applications, including cell biology, molecular biology, and biophysics.

A biophysics program is being developed in the department of Physics at San Jose State University. Several tools that use optics to investigate physical properties of biological systems will be used, including a Raman spectrometer, a Mach-Zender interferometer to be used to measure indices of refraction and an optical tweezers assembly. This thesis describes the theory, design, construction, and preliminary calibration of optical tweezers.

While the focus of this thesis is the development of laser tweezers for use in biophysics experiments, it is worth noting that considerable interest and many

developments in this tool have come from those involved in cell biology research. Soon after establishing the viability of trapping micron-sized dielectric spheres, Ashkin and his colleagues experimented with trapping colloidal tobacco mosaic virus.^{1, 5} After accidentally catching bacteria which had contaminated their sample, they began trapping and investigating the behavior of bacteria and yeast cells.

One issue of concern for these and any biological-based experiments is the sensitivity of the samples to heat or other radiation damage. Trapping has been demonstrated with wavelengths throughout the infrared and visible spectrum, and with powers as high as a few watts and as low as tens of milliwatts.^{6, 7} To avoid overheating and damaging biological cells, molecules or tissue it is important to trap with a beam of low power and of a wavelength which is not strongly absorbed by either the materials of interest or by the medium in which they are suspended, usually water or saline.

As further described in Sec. 3.1.1, there is a minimum in the absorption spectrum for this situation in the near infrared wavelengths, between about 750 nm and 1250 nm. The initial studies described here, however, were done using primarily non-biological samples (silica and polystyrene spheres) and employed the 514.5 nm beam from an Argon ion laser, taking advantage of the relative ease of using a beam in the visible range. Trapping of yeast cells was also demonstrated, as described in Sec. 4.1. The yeast did show signs of degradation when subjected to high-intensity trapping beams, becoming more opaque and difficult to hold in the trap. The quantitative measurements were done with silica spheres, which showed no such degradation.

Many cell biologists take advantage of the susceptibility of biological materials to localized heating and radiation damage by a focused laser beam to perform various types of "cellular surgery."⁸ Using a low-powered infrared beam to trap a cell of interest, a second, high-energy pulsed beam (sometimes known as "laser scissors"⁹) can be used to deactivate specific genes within the cell, or to puncture cell walls to allow the addition of genetic material.

As will be described below (Sec. 2.4), another feature of a calibrated optical tweezers system is its potential for use as a force transducer. A calibrated trap can be used to interact with cell membranes and individual molecules through the use of spherical "handles" of glass or polystyrene. From these interactions and the calibrated trapping force, measurements of interaction forces, tensile strength, and torque can be made. Cell biologists have used force measurements to do motility assays, measuring the swimming forces of motile cells such as human sperm.^{8, 10}

As the scale of biological investigation continues to move toward smaller structures, chemical and biological systems and processes can be understood in terms of the physical mechanics of interaction between structures such as molecules, membranes, and cells. Many of these biophysical studies can benefit from the use of optical tweezers, particularly as a means of measuring forces on these small scales.

Optical tweezers have been used in measurements of adhesion forces,¹¹ colloidal interactions and dynamics,¹² and a number of studies of the tensile strength and flexibility of large molecules such as actin filaments and DNA.^{13, 14} Another area for which optical tweezers have been a key tool is the study of molecular motors. Using single- and dual-

trap steerable optical tweezers systems, measurements of the piconewton magnitude forces and nanometer step displacements of such molecular motors as kinesin,^{15, 16} myosin,^{17, 18} lambda-exonuclease, and RNAP¹⁹ have been observed. These molecular motors are extremely efficient and present an opportunity to understand fundamental elements of biological functions such as muscle contraction, while also providing models for potential human-designed motors in nanotechnology applications. Recent studies involving the manipulation of single molecules with optical tweezers include investigations of the folding and unfolding of titin molecules,²⁰ the effect of tension on DNA polymerase activity,²¹ characterization of the torque generation of the flagellar motor of *E. coli*,²² and using fluorescence to observe a single enzyme unwinding a DNA molecule.²³

With biophysical studies such as those described above as a guide, the desired features of the optical tweezers constructed at San Jose State University can be characterized. The two primary modes of operation of optical tweezers are manipulation and force measurement. Each has different requirements.

In order to manipulate trapped objects the trapping beam must be steerable in at least the two radial dimensions, and preferably also in the axial direction. In order to manipulate two objects, for instance to hold two ends of a DNA or other strand-like molecule, or to hold one object still and move another close to it to determine interaction characteristics, it is necessary to have two separately steerable beams.

For force measurements, the trap is approximated as a harmonic well. Force can then be determined by means of Hooke's law. This requires a method of measuring the

displacement of the trapped object relative to the center of the trap during interactions of interest, and a calibrated system for which trap stiffness is known for a variety of trapping conditions.

In most experimental situations measurements are made either in one radial dimension or in the axial dimension. Radial trapping forces are several times greater than axial forces and are more convenient for many applications. There are, however, interesting applications for axial motion within the trap, including scanning membrane surfaces to measure topography of materials too soft to be measured with standard Atomic Force Microscopy.²⁴ It was a design goal to create a system that could be used for either application with relatively simple modifications.

CHAPTER 2

2.0 Trapping with Optical Tweezers: Theory

The operation of optical tweezers can be understood by determining the forces acting on the trapped object. The physical mechanism responsible for the dominant force is radiation pressure from the trapping beam. The radiation pressure is generally considered to comprise two components, a scattering force and a gradient force.

In typical optical tweezers the wavelength of light is between 500 nm and approximately 1000 nm (1 μm). Objects trapped with optical tweezers typically fall between 500 nm and 30 μm in diameter. When the wavelength of light is small compared to the diameter of objects it interacts with, a ray optics model can often be used to describe the interaction. In the regime where the wavelength is comparable to the dimensions of the trapped object, which is the case in the work described in this thesis, an electromagnetic model is more accurate. The ray optics model is nevertheless helpful as an intuitive guide to the trapping dynamics.

2.1 Ray optics Model

In the ray optics model, one imagines a parallel beam of light incident on the surface of a spherical object of refractive material such as silica or polystyrene suspended in a surrounding medium of lower refractive index (*e.g.*, water or saline, $n = 1.33$). If a ray is incident normal to the surface of the sphere, it will be partially transmitted and partially reflected. The transmitted ray will pass through the material and exit on the

other side of the sphere, again at normal incidence. In this situation, the transmitted portion of the ray experiences no change in direction from the incident beam, hence no change in momentum. The reflected portion, however, does experience a momentum change, and thereby imparts a reaction force, or scattering force, against the sphere in the direction of beam propagation.

If an incident ray is not normal to the surface, it will be refracted as it enters the sphere, and again as it exits, as indicated in Fig. 1(A). The final exiting ray thus experiences a change in momentum and imparts a reaction force to the sphere. The radial component of this force is in the direction to push the center of the sphere toward the original ray.

Now consider the case of a second ray, parallel to the first, incident at the same distance from the sphere axis but on the opposite side of the sphere [Fig. 1(B)]. The second ray will be refracted and reflected symmetrically to the first. The axial components of the forces imparted to the sphere by these rays contribute to the scattering force. The radial forces due to those two rays will be in opposing directions. If the intensities of the rays are equal the radial forces will cancel and there will be no net radial force. If the forces are not equal there is a net force on the sphere, known as the gradient force, directed toward the ray of greater intensity [Fig. 1(B)].

The net effect is that a refractive sphere illuminated by a beam of varying intensity cross-section will be drawn toward the highest intensity region of the beam while being pushed in the direction of propagation.

Figure 1: Ray Optics Model of Trapping Forces

This effect can be used for three-dimensional trapping by introducing a second light beam, of similar intensity and cross-section, aimed along the same axis but in the opposite direction to the first. This method has been used by Ashkin and others,¹ but is technically difficult and cumbersome. It is thus desirable to find a way to trap in three dimensions with a single beam.

The solution to this problem, developed by Ashkin in collaboration with Steve Chu and others,⁴ is found by focusing the trapping beam, thus introducing components of the gradient force which oppose the scattering force [Fig. 1(C)]. Under suitable conditions this axial gradient force can exactly balance the scattering force to produce a stable trap [Fig. 1(D)].

2.2 Electromagnetic Model

The ray optics model, while providing an intuitive understanding of the trapping process in optical tweezers, breaks down in the regime where the wavelength of the trapping beam is comparable to the size of the trapped object. In this regime one must instead consider the electrodynamics of the interaction.

Materials used in optical traps can be considered to be linear, isotropic, and homogeneous (l.i.h.) dielectrics. For a l.i.h. dielectric material in an applied electric field $\vec{E}$, a dipole moment is induced in individual molecules that is proportional to the applied field,²⁵

$$\vec{p} = \alpha \vec{E}, \quad (1)$$

where alpha is the polarizability of the dielectric, constant for a l.i.h. material and a tensor in general.

The potential energy of a dipole in an electric field is equal to the dot product of the dipole moment and the electric field: $U = -\vec{p} \cdot \vec{E}$.²⁶ The force on the dipole is then

$$\vec{F} = -\vec{\nabla} U = -\vec{\nabla}(-\vec{p} \cdot \vec{E}) = \alpha \vec{\nabla}(E^2). \quad (2)$$

The electric field inducing the dipole moment is the local field seen by the molecule. The field, and therefore the force, is oscillating at optical frequencies. Taking the time average of Eq. (2), it is seen that the net average force is proportional to the gradient of the *intensity* of the light, since intensity I is proportional to $\langle E^2 \rangle$:

$$\langle \vec{F} \rangle = \alpha \vec{\nabla} \langle E^2 \rangle \propto \alpha \vec{\nabla} I. \quad (3)$$

This result produces the same qualitative behavior described above for the ray optics model, *i.e.*, the refractive sphere will experience a force in an intensity gradient toward the highest intensity region of the beam. For a TEM₀₀ laser beam (Gaussian intensity cross-section), this will be toward the axis of the beam, and for a focused beam this will be toward the waist (or region of the smallest beam diameter). For a sufficiently strongly focused beam, there will be an equilibrium point on the beam axis just below the waist where this gradient force exactly balances the scattering force.

With this analysis it is also clear that the magnitude of the gradient force will be greatest where the intensity gradient is greatest. This leads to modeling the trap as a harmonic potential, as described in the next section. More detailed calculations of optical

tweezers forces using both ray- optics and electromagnetic models have been described by various investigators.^{6, 27, 28}

2.3 Harmonic Oscillator Model

For purposes of mathematical modeling of optical tweezers trap dynamics it is convenient to approximate the trap as a potential well with parabolic cross-section in each dimension. The magnitude of the radial force has been reported to be in the range of 25 to 30 times the axial force,⁶ making the three dimensional potential well oblate with its shortest axis along the beam axis. The forces in the two radial dimensions are approximately equal in stiffness, with some variation expected due to polarization and asymmetry of the beam cross-section.⁶ It has been shown that the harmonic approximation is valid in the radial direction for sphere displacements up to approximately one radius from the equilibrium position.⁷

Because the trapped object is suspended in a viscous medium (water or saline with a buffering agent to prevent sticking) the oscillations are damped. If the trapping beam and sample cell are held stationary, the only driving force is due to the Brownian forces from the thermal motion of the molecules of the suspending medium. These random forces can be approximated as delta functions with temperature-dependent frequency and magnitude.^{29, 30}

In order to calibrate optical tweezers for use in biophysics experiments, it is necessary to know the force applied to trapped objects under various conditions. Using the harmonic approximation this force can be represented by Hooke's law,

$$|\vec{F}| = kx \text{ or } \vec{F} = -k\vec{x}, \quad (4)$$

where x represents the displacement from the equilibrium position in a given dimension, and the spring constant k is a function of such factors as laser power, wavelength and intensity cross-section, particle diameter and refractive index, and viscosity of the suspending medium. The negative sign assures that the force is restorative, which will be the case within the potential well.

As so far described, optical tweezers can be modeled as a damped, randomly driven harmonic oscillator. The equation of motion for this system is then

$$m\ddot{x} + D\dot{x} + kx = F_B(t, T) \quad (5)$$

where m is the mass of the trapped bead, D is the Stokes damping coefficient due to the viscosity of the suspending medium, k is the trap stiffness, and $F_B(t, T)$ is the Brownian driving force. It is convenient to divide by the mass and rewrite this equation in the form

$$\ddot{x} + \beta\dot{x} + \alpha x = \mathcal{F}, \quad (6)$$

where $\mathcal{F} = \frac{1}{m}F_B(t, T)$, $\alpha = \frac{k}{m}$ and $\beta = \frac{D}{m}$.

The damping coefficient can be determined from Stokes' law for a spherical object with velocity $\dot{x}$ in a viscous medium,

$$F_S = -6\pi r\eta\lambda\dot{x} = -D\dot{x}, \quad (7)$$

where the damping coefficient $D = 6\pi r\eta\lambda$, r is the radius of the trapped sphere, η is the viscosity of the suspension medium, and λ is a correction term which depends on the proximity of the sphere to a planar boundary surface such as the cover slip or microscope slide. As with any friction force, the direction of the Stokes drag force is opposite to the

direction of motion, as indicated by the minus sign. For motion parallel to a planar boundary, the correction term can be written following Faxen's law^{30,31} as

$$\lambda = \frac{1}{1 - \frac{9}{16}\left(\frac{r}{h}\right) + \frac{1}{8}\left(\frac{r}{h}\right)^3 - \frac{4}{256}\left(\frac{r}{h}\right)^4 - \frac{1}{16}\left(\frac{r}{h}\right)^5} \quad (8a)$$

where r is the radius of the sphere and h is the distance from the center of the sphere to the boundary. For motion toward a planar boundary, the correction term is

$$\lambda = \frac{1}{1 - \frac{9}{8}\left(\frac{r}{h}\right) + \frac{1}{2}\left(\frac{r}{h}\right)^3}. \quad (8b)$$

In order to minimize spherical aberration and maximize trapping efficiency, a trapped object is typically held within a few diameters of the cover slip, so this term represents a five to ten percent correction to the standard Stokes coefficient and hence to the damping force.

The primary task of any calibration scheme is to determine the spring constant, k , or trap stiffness $\alpha = \frac{k}{m}$, as a function of the other parameters mentioned above, *i.e.*, characteristics of the trapping beam, the trapped object and the suspension medium. A number of techniques that have been used to determine the stiffness constant for optical tweezers are detailed below.

2.4 Calibration Methods

Conceptually, the simplest of the common calibration techniques is the direct use of Hooke's law to determine the spring constant. This can be done by translating the

sample cell while holding a trapped particle in place. As the suspension medium is swept past it, a trapped bead experiences a drag force which can be calculated through Stokes' law (with appropriate modification for nearby surfaces). If the relative velocity is constant, the bead is dragged away from the center of the trap until an equilibrium state is reached where $kx = 6\pi\eta\lambda\dot{x}$, or $\alpha x = \beta\dot{x}$.

In the above equation it is assumed that the measurement is taken over a long enough period that the motion due to the Brownian driving force averages to zero. Calibration is done by translating the microscope stage in a regular fashion at known velocities while measuring the displacement of the bead from the center of the trap. This can be done by focusing the image of the trapped bead onto a quadrant photodetector. As the bead moves within the trap its image covers varying portions of the four detector quadrants. The sums and differences of the four quadrant signals can be used to determine the bead displacement. For absolute measurements the detector must be aligned to the center of the trap and calibrated. The stage translations can be driven with a piezoelectric system for small, fast displacements, or with stepper motors for larger, slower motion.^{6, 7, 15-17} The stiffness coefficient can be determined from a plot of the displacement as a function of velocity, which will have a linear slope of magnitude $\frac{\alpha}{\beta}$.

The trap stiffness can also be determined from the converse process, leaving the sample cell in place while moving the sphere through the suspending medium by steering the trap. This works best for quick trap displacements, such as those produced with an AO modulator.⁷ The bead response to a sudden translation of the trap is of the form

$$x_B = x_T(1 - e^{-Ct}), \quad (9)$$

where the rate constant $C = \frac{k}{D} = \frac{\alpha}{\beta}$ can be determined from the displacement vs time curve.

The calibration methods described so far depend on large-scale motion of the bead relative to the trap center, averaging out Brownian motion. Another technique uses the small-magnitude, high frequency motion of the trapped sphere due to Brownian impacts to determine the trap stiffness directly. This technique involves measuring the bead position at a high sampling rate over a period of seconds or tens of seconds, then transforming the data to frequency space. While the motion of the bead is random in time, the frequency distribution of the amplitude of motion can be predicted theoretically. The trap stiffness can be determined by fitting the transformed data to a theoretical curve.

One of the appealing features of this calibration method is that it depends on relative displacement rather than absolute position, making alignment and calibration simpler. This method can also be used for either radial or axial motion, while those described previously are appropriate only for radial motion.

Starting with the equation of motion [Eq. (6)],

$$\ddot{x} + \beta\dot{x} + \alpha x = \mathcal{F}(t) \quad (6)$$

and noting that x , $\dot{x}$ and $\ddot{x}$ are all functions of time, the Fourier transform and its inverse are defined as

$$X(f) = \int_{-\infty}^{\infty} x(t)e^{2\pi i f t} dt \quad (10)$$

and
$$x(t) = \int_{-\infty}^{\infty} X(f) e^{-2\pi i f t} df . \quad (11)$$

Taking the time derivative of Eq. (11), it is seen that $\dot{x}(t)$ and $\ddot{x}(t)$ are related to $x(t)$ by

$$\dot{x} = -i2\pi f x \quad \text{and} \quad \ddot{x} = -4\pi^2 f^2 x \quad (12)$$

so that Eq. (6) can be rewritten as

$$[-4\pi^2 f^2 - i2\pi f \beta + \alpha] x(t) = \mathcal{F} \quad (13)$$

Taking the Fourier transform of this equation and solving for $X(f)$ gives

$$X(f) = \frac{F(f)}{-(4\pi^2 f^2 - \alpha) - i2\pi f \beta} , \quad (14)$$

where $F(f)$ is the Fourier transform of $\mathcal{F}$.

Parseval's Theorem states that the total power is the same in the time and frequency domains:

$$\text{Total Power} \equiv \int_{-\infty}^{\infty} |x(t)|^2 dt = \int_{-\infty}^{\infty} |X(f)|^2 df . \quad (15)$$

This leads to the definition of the power spectral density (PSD), the density of power in the frequency band f to $f + df$, as

$$P(f) = |X(f)|^2 . \quad (16)$$

In this case,

$$P(f) = \frac{|F(f)|^2}{(4\pi^2 f^2 - \alpha)^2 + 4\pi^2 f^2 \beta^2} \quad (17)$$

or, in terms of $\omega = 2\pi f$,

$$P(\omega) = \frac{|F(\omega)|^2}{(\omega^2 - \alpha)^2 + \omega^2 \beta^2}. \quad (18)$$

The Brownian driving force can be approximated using stochastic models³² as

$$|F(\omega)|^2 = 4k_B T \beta \quad (19)$$

where k_B is Boltzmann's constant, T is the absolute temperature and β is the damping constant (which will also be a weak function of temperature, through the viscosity).

The theoretical PSD for a typical optical tweezers trap is shown in Fig. 2. At low frequencies the denominator is dominated by the α^2 term, so that $P(f)$ is a constant value independent of frequency. At very high frequencies, the denominator is dominated by the ω^4 term, so that the $P(f) \propto \frac{1}{\omega^4}$. When plotted on a log-log scale, the function is constant at low frequencies and drops off approximately linearly with a negative slope at higher frequencies. The critical (or corner) frequency at which the PSD magnitude is half that of the low-frequency plateau value is $f_c = \frac{\alpha}{2\pi\beta}$,^{29, 30} which is proportional to the trap stiffness. The intuitive interpretation is that a stiffer trap will produce more high frequency motion, raising f_c and extending the plateau to higher frequencies.

In practice this calibration technique is implemented by collecting position data as a function of time in one dimension (axial or radial) at a relatively high sampling rate (a few kHz). These data are transformed to position as a function of frequency with a Fast Fourier Transform (FFT) program. The PSD is calculated from the FFT result. The resulting curve is fitted to Eq. (17) with α as the fitting parameter and β determined from the modified Stokes equation. In addition to simplifying the alignment and

Figure 2: Theoretical Power Spectral Density (PSD)
 Normalized PSD for damped, randomly driven harmonic oscillator. The corner frequency $f_c = 15 \text{ Hz}$ corresponds to a $3 \text{ }\mu\text{m}$ diameter sphere in a trap with spring constant $k = 17.9 \times 10^{-6} \text{ N/m}$.

calibration of the detector, an advantage of this method is the ability to monitor trap stiffness in real time during experiments.¹⁵

There is a related calibration technique that also uses position vs time data, but requires that the position information be calibrated to the actual motion of the bead. The average potential energy of a harmonic oscillator in one dimension is dependent on the RMS displacement of the trapped object from the equilibrium position as $U = \frac{1}{2}k\langle x^2 \rangle$.

If the source of energy is the thermal motion of the surrounding molecules, the equipartition theorem requires that this energy be equal to $\frac{1}{2}k_B T$. Equating these energy expressions gives the result that the trap spring constant $k = \frac{k_B T}{\langle x^2 \rangle}$. This method is

somewhat difficult to use in practice⁷ but can serve as corroboration for results from other calibration methods.

CHAPTER 3

3.0 Experimental Methods and Discussion

In this chapter the primary design considerations for an optical tweezers assembly are discussed, and the specific choices which were incorporated into the San Jose State University Optical Tweezers are described.

3.1 Design Considerations

The design of an optical tweezers assembly will involve four major elements: (1) the laser, (2) the trapping region, consisting of a microscope objective, sample cell and sample stage, (3) the delivery of the beam from the laser to the trap, which may include steering and other optical elements, and (4) optics for the observation and measurement of trapped objects and their motion.

3.1.1 The Laser

The primary considerations in choosing a laser for optical tweezers operation are wavelength and power. Trapping has been successfully conducted with wavelengths ranging from visible to near infrared and powers from a few milliwatts to a few hundred milliwatts.^{6, 7, 29, 33} Since the trap stiffness increases with power, the beam intensity should be chosen with the specific application in mind. Measurement of the piconewton-scale forces of a molecular motor system require a relatively weak trap, while trapping

and holding a motile object such as a sperm cell may require a much more powerful beam.

With any biological application, a primary concern is optical damage to the specimen. This can result from direct heating or from ionization and other mechanisms.^{8, 30} When practical, low-power trapping is generally preferable in order to minimize the danger of damage. One way to produce a trap with greater trapping force per milliwatt of power is to use a doughnut mode beam. The relatively low intensity along the beam axis and higher intensity of the outer rays which provide most of the axial gradient can improve trap efficiency by 50 percent or more.^{24, 29, 34, 35} Doughnut cross-section beams can also be used to trap particles with refractive indices lower than their suspension media, including air bubbles.^{36, 37, 38}

Appropriate choice of wavelength can also minimize damage. Most biological samples are suspended in an aqueous solution. As seen in Fig. 3, the absorption coefficient for water is low in the ultraviolet and visible range, but rises dramatically with wavelength toward the infrared. Conversely, the absorption coefficient for two common components of biological material, deoxyhemoglobin (HbO₂) and oxyhemoglobin (Hb) are high in the visible region and decrease with wavelength, dropping out in the infrared. The region around $\lambda = 1000$ nm is a minimum for the combined absorption, and is therefore ideal for trapping biological specimens. A common choice of laser in this range is the 1064 nm beam from a Nd:YAG laser. There are also tunable diode lasers available in this wavelength range.

Figure 3: Absorption Curve for Biological Material and Water.

The absorption curves for deoxyhemoglobin (Hb), oxyhemoglobin (HbO₂), and water (H₂O) are shown. Absorption is high in the visible wavelengths for Hb and HbO₂, and high in the infrared wavelengths for water, so that there is an overall minimum in absorption for near-infrared wavelengths (approximately 750 nm to 1250 nm).

Graph from Svoboda and Block, "Biological applications of optical forces." ³⁰

3.1.2 The Trapping Region

For the trapping region, the choices are simple. Many studies^{4, 6, 30} have shown that in order to produce a gradient force in the axial direction strong enough to overcome the forward scattering force, a high numerical aperture (N.A.) lens is required. This is generally a high quality microscope objective with a N.A. greater than 1.0 and magnification greater than 50x. Comparisons of various objectives have been included in studies by other investigators.^{6, 30, 33}

Objectives are typically designed for use with visible light, so that lens coatings are not optimized for the infrared wavelengths used most often for trapping. Transmission at these wavelengths is often relatively low, in the 40–60% range.^{6, 30} Such transmission measurements are difficult for high N.A. objectives because of total internal reflection and because the highly divergent beam emerging from the objective is difficult to collect with a photodetector. These problems can be overcome by using two identical objectives arranged confocally with a drop of immersion oil between them. The overall transmission of this system is the product of the individual transmission coefficients. If the two objectives are identical, each can be considered to have a transmission coefficient equal to the square root of the measured transmission through the pair.

Most traditional microscopes include a sample stage with micrometer translation in the x, y, and z directions (with the z-axis along the optic axis of the objective). Such a stage can be adapted for use in a trapping assembly by motorizing the motion and/or adding piezoelectric translators for fast, precise, sub-micrometer motion.

The primary modification of the standard microscope geometry of interest for tweezers is an inverted geometry with the specimen plane above the objective and the trapping beam incident from below. Trapping is most efficient within a few microns of the cell cover slip.^{6, 7, 30} The inverted geometry takes advantage of the fact that most objects of interest are more dense than their suspension medium, so that they tend to sink toward the bottom of the sample cell. In order to trap near the cover slip with the traditional geometry the cell must first be raised to bring the beam waist within range of the objects to be trapped, which have drifted down near the cell floor. Once trapped, an object can be raised toward the cover slip for optimum trapping during an experiment. The inverted geometry eliminates this issue.

3.1.3 The Beam Delivery Optics

The third main design element, delivery of the beam from the laser to the trapping region, involves more choices. The primary requirement of the delivery optics is to provide a beam at the rear pupil of the objective with the proper diameter and divergence to optimize trapping.

For efficient trapping, the transmission through the objective aperture should be fairly high, while assuring that the aperture is completely filled or slightly overfilled. Filling the aperture is desirable because it produces a diffraction-limited waist at the trap focus, and because rays from the outside edges of the lens contribute more to the axial gradient force while those nearer the beam axis contribute more to the scattering force.

A typical compromise between transmission efficiency and trapping efficiency for a Gaussian beam is to expand the beam so that 85% of the beam power is transmitted through the objective aperture. This approximately corresponds to the $1/e^2$ diameter of the beam.^{6, 7, 30}

The appropriate divergence for this beam will depend on the design of the objective. In a simple compound microscope the object is placed just outside the focal plane of the objective lens. The image produced by the objective lies in or near the focal plane of the eyepiece, which leads to a virtual image with large angular magnification. The distance between the focal planes of the objective and eyepiece lenses is known as the "tube length" of the microscope and is often between 160 mm and 180 mm for commercial microscopes. "Infinity corrected" objectives produce a collimated beam with no intermediate image.

Tweezers optics are designed to match the objective either by producing a waist in the trapping beam at the appropriate conjugate plane to the trap focus (approximately the tube length from the objective aperture), or by producing a collimated beam of the proper diameter for an infinity-corrected objective. Another way to describe this divergence matching is that the radius of curvature of the beam wave fronts must match that expected by the objective.⁶

It is desirable for most trapping applications to manipulate objects by moving the trap within the sample cell. Steering optics are therefore an important element of delivery systems. The goal of any steering system is to move the trapping beam waist within the focal plane of the objective by changing the angle of the beam at the entrance

pupil of the objective. The design challenge is to keep this pupil filled and the beam divergence constant while the angle is changed. In order to move the trap in the z-direction it may also be advantageous to be able to adjust the beam divergence over a narrow range.

Many of the steering methods in common use for tweezers approximate the requirement described above to first order. A single lens which focuses the trapping beam to a plane conjugate to the trap focus can be translated slightly in the x-y plane to provide x-y steering of the beam.⁶ Translation along the beam axis will also move the trap location in the z-direction by changing the beam divergence. Similarly, if an optic fiber is coupled to the laser the output end can be translated relative to a collimating lens to steer the beam in x, y, and z.^{6, 30} Either of these systems can be motorized for remote and/or automated steering.

More complicated systems provide better approximations of the desired steering. Some designs use a pair of mirrors, one for x-deflections and one for y-deflections, located between a beam waist and the lens that makes the waist conjugate to the objective. Changing the angle of these mirrors has the effect of changing the apparent location of the preceding beam waist within the focal plane, which produces corresponding motion in the trapping plane.^{7, 18} This design has also been used in conjunction with acousto-optic modulators⁷ which can produce very fast translations useful for specific applications. An AO modulator introduces a significant loss of beam power and quality, so its use is warranted only when speed is essential. Still other designs replace both steering mirrors with AO modulators.

One design that provides better than first-order approximation of the desired beam motion was introduced by Fällman and Axner.³⁹ Their system uses two pairs of lenses and a gimbal-mount mirror to provide steering in the x, y, and z-directions with excellent adherence to the desired goal of keeping the beam position and diameter constant at the objective pupil while adjusting the angle and divergence for steering.

A gimbal-mount is a mount that allows rotation about perpendicular axes that lie in the plane of the mirror. Typical optical mirrors rotate about axes that may not lie in the mirror plane. One result is that the beam strikes different spots on the mirror surface as the mirror is tipped. With a gimbal mount the beam spot is always at the same point, the center of the mirror face.

This is a key factor because in this design the surface of the gimbal-mount mirror is imaged at the objective pupil itself. The distance between the beam spot on the mirror and the objective pupil is constant, and since they are in conjugate planes, a change in beam angle at the mirror results only in a corresponding angle change at the objective, with no translation (to the degree that the thin-lens approximation is valid for the various optical elements), the ideal motion for x-y steering.

These two planes are made conjugate to each other by placing two lenses between them as shown in Fig. 4. The lenses are separated by the sum of their focal lengths, as in a standard beam expander or telescope. A major advantage of this design is that in addition to the steering properties, proper choice of the focal lengths of these lenses can fulfill the requirement of overfilling the objective aperture.

Figure 4: Radial Steering Optics

Afocal lens system images gimbal-mount mirror onto entrance aperture of microscope objective. Rotation of the mirror about its center changes the angle of the beam entering the objective without changing its size or position.

As in the figure, the distances between the four optical elements in the figure can be denoted by d_m , the distance between the mirror and the first lens, $f_1 + f_2$, the distance between the two lenses, and d_o , the distance between the second lens and the entrance pupil of the objective. The $\begin{bmatrix} A & B \\ C & D \end{bmatrix}$ matrix for the system can be calculated using standard matrices for the two thin lenses and three drift spaces as

$$\begin{bmatrix} -\frac{f_2}{f_1}; & f_1 + f_2 - d_m \frac{f_2}{f_1} - d_o \frac{f_1}{f_2} \\ 0; & -\frac{f_1}{f_2} \end{bmatrix}.$$

The fact that matrix element C is equal to zero identifies this as an afocal, or telescopic system (a collimated beam striking the mirror would result in a collimated beam at the objective). Matrix element D gives the angular magnification for the system of $-\frac{f_1}{f_2}$. This means that a change in angle $\Delta\theta_1$ at the mirror will result in a corresponding angular change $\Delta\theta_2$ at the objective related by this ratio. In turn, a change in angle $\Delta\theta_2$ at the objective produces translation of the trap position of approximately $\Delta r = f_{obj} \Delta\theta_2$, or

$$|\Delta r| = \frac{f_1}{f_2} f_{obj} \Delta\theta_1. \quad (20)$$

Identifying the input and output planes as conjugates is equivalent to requiring that matrix element B equal zero. Setting the expression for this matrix element equal to

zero produces a relationship between the focal lengths of the lenses and the distances between the four elements:

$$f_1 + f_2 = d_m \frac{f_2}{f_1} + d_n \frac{f_1}{f_2}. \quad (21)$$

To add steering in the z-direction, the divergence of the beam must be adjusted without changing the diameter of the beam at the objective aperture. This is equivalent to requiring that the diameter of the beam at the gimbal mirror be independent of changes in divergence. Fällman and Axner achieve this goal by inserting another pair of lenses before the gimbal mirror as indicated in Fig. 5(A). Matrix methods can be used to compare the size of the beam at distances d_1 and d_3 , before and after this lens pair, with a separation of d_2 between the lenses. The relationship between the output radius r_{out} and the input radius r_{in} is then

$$r_{out} = r_{in} \left[1 - \frac{d_2}{f_1} + \frac{d_2 d_3}{f_1 f_2} - d_3 \left(\frac{1}{f_1} + \frac{1}{f_2} \right) \right], \quad (22)$$

where the term in square brackets is element A of the matrix for the system. Rewriting this expression in the form

$$r_{out} = -r_{in} \left[\frac{d_3}{f_1} - \frac{(d_2 - f_1)(d_3 - f_2)}{f_1 f_2} \right], \quad (23)$$

makes it clear that setting the output distance d_3 equal to the focal length f_2 of the second lens has the effect of eliminating the distance between the two lenses, d_2 , from the equation.

The divergence of the beam can thus be varied by changing the spacing between the lenses without affecting the size of the beam spot at distance f_2 from the second lens.

Figure 5: Axial Steering / Beam Divergence Optics

- A) When d_2 is equal to the sum of the lens focal lengths ($f_1 + f_2$) a collimated beam input produces a collimated beam at the gimbal mirror.
- B) When d_2 is less than $f_1 + f_2$ the output beam diverges, but the size and location of the beam spot on the mirror are unchanged.
- C) A more compact afocal system using a diverging lens in combination with a converging lens.

By fixing the second lens a distance f_2 in front of the gimbal-mount mirror the divergence of the beam can be changed by adjusting the axial position of the first lens without changing the diameter of the beam at the objective aperture [Fig. 5(B)]. Setting the nominal distance between the two lenses equal to the sum of their focal lengths makes this another afocal system so that the overall effect on beam divergence is small.

Once again, the relative focal lengths can be chosen to increase or decrease the beam diameter if desired. In that case using one converging lens and one diverging lens in an afocal arrangement [Fig. 5(C)] allows this lens pair to be very compact (the distance between the lenses is then the difference in the magnitudes of their focal lengths rather than the sum).

For clarity, the complete optical system will be labeled as in Fig. 6, with lenses L1 and L2 forming the first afocal pair, responsible for varying the divergence of the beam and therefore the z-position of the trap, and lenses L3 and L4 imaging the gimbal-mounted mirror onto the entrance pupil of the objective, providing steering in the x-y plane.

This system is easily adapted to two-beam operation with the addition of two polarizing beam splitters (PBS's) as shown in Fig. 7. For a beam with both horizontal and vertical polarization components, the first PBS splits the beam according to polarization. Each beam is directed to its own set of lenses L1 and L2 and its own gimbal-mount mirror. The beams are recombined with the second PBS before entering L3. Lenses L3 and L4 make each mirror a conjugate plane to the objective aperture, so

Figure 6: Complete Optics for Radial and Axial Steering of a Single Trapping Beam.

that independent rotations of the two mirrors result in independent steering of the two beams.

The relative intensity of the two traps can be adjusted by changing the polarization of the incoming beam. For a plane-polarized beam, the introduction of a quarter-wave plate before the first PBS with its axis at an angle of 45° from the polarization axis will generate circular polarization, making the two traps approximately equal in intensity. Rotating the quarter-wave plate will change the ellipticity of the polarization and thereby the relative intensities of the traps.

Figure 7: Layout of Optical Tweezers Apparatus.

The arrangement allows independent use of Ar^+ or Nd:YAG lasers for trapping, or simultaneous operation (e.g., inducing fluorescence with Ar^+ while trapping with Nd:YAG).

3.1.4 Observation and Measurement

The final element of design is the optics for observation and measurement of the trapping region and trapped objects. Here the microscope objective plays a second role, imaging the region in the plane of the trap. Illuminating light shining through the sample cell into the objective will produce an image just as in normal microscopic applications. The object plane of the objective will coincide with the trap position if the divergence of the trapping beam has been tailored to match the objective. This is desirable for the practical reason that it simplifies imaging the trapped object as well as taking advantage of the fact that objectives are generally designed to minimize aberrations in this plane and its conjugate, thus producing a more uniform trap.³⁰

Figure 8 shows an arrangement for viewing objects in the trapping region and measuring their motion. A beam splitter separates the image from the incoming trapping beam. A dichroic mirror with different reflection and transmission properties for the trapping and illuminating beams aids this separation. A video camera focused on the image produced by the objective allows viewing and recording of the sample contents in the region of the trap. A CCD camera can be used to digitize and measure the sizes and motion of objects. The position and motion of trapped objects can also be measured with a quadrant photodetector as shown in the figure. A small-diameter quadrant photodiode minimizes dark noise and allows magnification of the image of a trapped object to fill approximately half the detector area.⁷ Sums and differences of the four quadrant signals can be used to measure the position and/or displacement of trapped objects.

Figure 8: Viewing, Recording and Measurement of Trapping Region

A video camera is used to view and record objects in the trapping region, while the quadrant detector output is used to measure radial position and displacement of trapped objects.

Friese *et al.*,^{24, 29} have shown that displacements of a trapped particle along the z-axis can be measured by means of backscattered light, allowing calibration of the axial trapping force following the Brownian motion analysis of Sec. 2.4.

In this method, light reflected back from a trapped object such as a polystyrene or silica bead is collected by the objective lens, creating an image of the trapped object much like that created by illumination from behind the sample. It is assumed that the total power of the reflected light is independent of the axial position for small displacements of a sphere that is larger than the waist of the trapping beam. This backscattered light is focused by the objective lens to an image at approximately the tube-length of the objective. This reflected image can be projected onto a photodiode with the aid of a beam splitter and additional lenses.

If, as indicated in Fig. 9, the detector is placed behind the image plane of this projection system, it will subtend a fraction of the backscattered light cone. As the image moves along the beam axis, reflecting the axial motion of the bead in the trap, the size of the light cone cross-section in the plane of the detector will vary. Since the detector area is constant, the fraction of the light collected by the detector will also vary. If the variation in average intensity over the illuminated region (the bead image) is small, the power collected by the detector will be proportional to the total power in the illuminated area by the ratio of the detector area to the illuminated area. This can be expressed as

$$P_{det} = P_{tot} \left(\frac{R_{det}}{R_{illum}} \right)^2 \quad (24)$$

where P_{det} and P_{tot} are the detector power and total power, and R_{det} and R_{illum} are the radii

Figure 9: Axial Displacement Detector Optics.

A trapped sphere is at average distance z from the microscope objective. Its image due to light backscattered from the surface of the sphere is at average distance Z from the projection optics. A detector is placed beyond the image, at distance D from the optics. The power measured at the detector due to backscattered light varies as the sphere moves in the axial direction. The variation in power, ΔP is approximately proportional to the axial motion, Δz [Eq. (29)].

of the detector and of the illuminated area, respectively. From the figure it can be seen

that $\tan\theta = \frac{R_{illum}}{D-Z}$ where D is the distance from the last optical element to the detector,

and Z is the distance from that optical element to the beam waist. The detector power can then be written

$$P_{det} = P_{tot} \left(\frac{R_{det}}{\tan\theta} \right)^2 \left(\frac{1}{D-Z} \right)^2. \quad (25)$$

If the trapped bead moves along the trap axis a distance Δz , the image before the detector will move a distance

$$\Delta Z = M_l \Delta z = (M_t)^2 \Delta z, \quad (26)$$

where M_l and M_t are the longitudinal and transverse magnifications of the lens system, respectively.

The variation in power at the detector ΔP due to axial motion Δz of the trapped bead can be approximated by

$$\Delta P = \frac{dP_{det}}{dz} \Delta z. \quad (27)$$

Assuming that $\tan\theta$ is approximately constant for small ΔZ , the derivative is

$$\frac{dP_{det}}{dz} = \frac{d}{dz} \left[P_{tot} \left(\frac{R_{det}}{\tan\theta} \right)^2 \left(\frac{1}{D-Z} \right)^2 \right] \approx 2P_{tot} \left(\frac{R_{det}}{\tan\theta} \right)^2 \left(\frac{1}{D-Z} \right)^3 \frac{dZ}{dz}. \quad (28)$$

Taking $\frac{dZ}{dz} \approx (M_t)^2$ from Eq. (26), and substituting Eq. (28) into Eq. (27) we can

then write

$$\Delta P \approx 2 \frac{P_{det} (M_t)^2}{D - Z} \Delta z. \quad (29)$$

The variation in power collected by the photodetector is therefore approximately proportional to the variation in the axial position of the trapped bead.

Since this technique measures only relative motion, the natural choice for calibrating the axial force is to use the Brownian motion frequency analysis described in Sec. 2.4.

3.2 Design Choices and Assembly

In choosing the specific design elements of the San Jose State Optical Tweezers assembly the design considerations described in the last section were considered in conjunction with the available resources. Manufacturers and model numbers are listed in Table 1.

3.2.1 The Laser

As noted, the primary considerations in choosing a laser for an optical tweezers assembly are wavelength and power. For biological samples, a wavelength close to 1 μm is ideal to match the minimum in the absorption spectrum. A Nd:YAG (Quantronix) laser that can be operated in CW mode with average output of several watts will be used for future experiments where biological materials are involved. For this early work, however, the relative ease of working with visible light led to the choice of a 2W CW Argon Ion laser (Spectra Physics). The 514.5 nm Ar^+ beam was used for trapping.

Table 1: Equipment and Supplies

Description	Specifications	Manufacturer	Model/Part number
Ar ion laser	2 W, CW	Spectra Physics	Head: 164 Exciter: 265
Nd:YAG laser		Quantronix	416
He-Ne laser	1.8 mW	Uniphase	Head: 1103P Supply: 1201
Quarter wave plate	QWPM-514.5-10.4	CVI Laser Corp.	
Dichroic reflector	LWP-45-R495-T550-PW-0604-0	CVI Laser Corp.	
Polarizing cube beam splitter	1 inch; coated for 400-700 nm	Newport (NRC)	10FC16PB.3
Microscope Objective	100x, 1.30 N.A.	Leitz Wetzler	A341506
Microscope Objective	100x, 1.25 N.A.	Leitz Wetzler	519804
Lenses (L1/L2)	double convex, plano-convex	Newport (NRC)	
Lenses (L3/L4)	coated doublets	Melles Griillot	
Dual shearing attenuator		Newport (NRC)	925B
Motor driven gimbal mount		New Focus	8812
Motor driven corner mount		New Focus	8809
Picomotor multi-axis driver		New Focus	8801
Picomotor control pad		New Focus	8620
Programmable motion controller		Compumotor	C4000
Stepper motors		Compumotor	S57-83-MO
Microscope slides	75 x 125 mm	Fisher Scientific Co.	
Cover slips	#0; 23 x 30 x 0.16 mm	various	
Double-sided transparent tape		Scotch (3M)	
Immersion oil	$n_c = 1.518$, $n_D = 1.515$	Zeiss	518C
Triton X	Octyl Phenoxy Polyethoxyethanol	Sigma Chemical Co.	T-6878
8 mm video camera	32x zoom	Samsung	SCA 20
Monochrome video monitor	29 cm diagonal screen	Zenith Data Systems	
Silicon photodiode	integrated Op Amp	Hamamatsu	S1406-03-04
Optical multimeter		ILX Lightwave	OMM-68108
Power/WaveHead with integrating sphere input	10 μ W to 1 W 460 to 1000 nm	ILX Lightwave	OMH-6720B
Power Meter	300mW-3W full scale	Coherent	
PC for data collection	100 MHz 486	HiQ	
ADC data acquisition board	DAS-8, 12-bit, 24 kHz	Keithley	Metabyte
Polystyrene latex spheres	1.069 μ m and 2.90 μ m	Duke Scientific	
Silica glass spheres	Hypersil, 5 μ m nom.	Phenomenex	
Optical table	4' x 8', pneumatic isolation	Newport (NRC)	

3.2.2 The Trapping Region

The advantages of an inverted geometry for the microscope objective and sample cell have been noted earlier in this chapter. However, the availability of a standard geometry Carl Zeiss microscope assembly [Fig. (10)] made its use preferable to the design and construction of an entirely new structure. In particular, the presence of a side video port and internal beam splitter gave a convenient way to introduce the trapping beam while allowing the trap region to be viewed with the standard eyepiece assembly or a video camera. The built-in half-silvered beam splitter was later replaced with a larger diameter dichroic reflector (described in Sec. 3.2.3).

Figure 10: Carl Zeiss Microscope Assembly

Several high N.A. objectives were used, primarily a Leitz Wetzler 100x, 1.30 N.A. A second identical objective was also available, as well as a Leitz Wetzler 100x 1.25 N.A. objective. The transmission of the 514.5 nm beam through the 1.30 N.A. objectives was approximately 89%. This could be expected to drop sharply for an infrared beam.

A convenient feature of the Zeiss microscope was a sample stage with micrometer-driven translation in x, y, and z. A programmable motion controller and two stepper motors (Compumotor) were installed to provide automated stage motion. The motors were coupled to the stage micrometers, giving a maximum resolution of about 2 nm per step, expected to be adequate for the calibration experiments described in Sec. 2.4 involving displacements on the order of hundreds of nanometers. A limit switch was designed and installed to override potential controller or programming errors.

In this thesis work calibration was attempted for only axial forces, so the stepper motors were not used. The motors were removed from the assembly to reduce the effect of their mass (1.2 kg each) on the stage vibrations, a significant source of low frequency noise in the Brownian motion frequency analysis. Future instrument development work will explore the degree to which such amplification of vibrations due to the motor mass will be a problem for radial force measurements and calibration.

Sample cells consisted of a standard glass microscope slide and a glass cover slip separated by a piece of Scotch transparent, double-stick tape. With the tape affixed to a slide, an oblong hole was cut out of the center of the tape with a razor blade and peeled off the glass, leaving a shallow well with depth equal to the thickness of the tape (about

0.1 mm). One or two drops of sample solution were then placed on the slide within the cut-out area, the fluid was spread to wet the entire well area, and a cover slip was placed over the sample, taking care to avoid air bubbles. The slide was then placed on the microscope stage, a drop of immersion oil placed on the cover slip, and the stage was raised until the tip of the objective was in contact with the oil.

Trapping for purposes of calibration was done with polystyrene and silica spherical beads suspended either in distilled water or in a solution⁶ of 11% NaCl with 0.2% Triton X as a buffer to minimize spheres sticking to the cell boundaries and to each other. This solution has a specific gravity of 1.1 g/cm³ and provides significant buoyancy to polystyrene spheres (specific gravity 1.05 g/cm³), while silica spheres (specific gravity 2.01 g/cm³) still sink fairly quickly to near the bottom of the cell.

3.2.3 The Beam Delivery Optics

The entire optical tweezers assembly was built on a standard four-foot by eight-foot Newport optical table that was physically isolated from the laboratory floor with pneumatic pistons. A diagram of the optical layout is shown in Fig. 7 (p. 34). The Nd:YAG and Ar⁺ lasers were placed side by side to facilitate switching from visible to infrared wavelengths, or for simultaneous use, in future applications. Using the full length of the table provided ample room for all the steering and focusing optics, as well as room for future modifications.

The assembly was designed with the intention of being easily modified for various applications. Design objectives included:

- Two independently steerable trapping beams
- The ability to calibrate both radial and axial trapping forces
- The ability to utilize two different wavelengths simultaneously
(*e.g.* to induce fluorescence with one beam and trap with the other)

As noted above, the Zeiss microscope assembly included an internal beam-splitter originally intended for use with a video port. The beam splitter was a half-silvered mirror. The original assembly is pictured in Fig. 11(A). As can be seen from the diagram, the cylindrical apertures on either side of the beam splitter have inside diameters of 7 mm, compared to the 8 mm objective entrance aperture. The upstream edge of the first aperture is at a distance of about 4.5 cm from the objective aperture. The principle of the trap steering design is that the beam should have constant diameter and intensity as it rotates about the objective aperture. The apertures in the original beam splitter geometry were not consistent with this design intent.

The beam splitter assembly was replaced by a new assembly [Fig. 11(B)] with a larger (12.5 mm diameter) dichroic reflector (CVI Laser Corp.) and no restricting apertures. To minimize the obstruction problem while working with the original assembly, the initial locations of L3 and L4 were chosen to make the beam splitter surface itself the conjugate plane to the gimbal mirror, rather than the objective aperture.

An additional advantage of the dichroic mirror was its wavelength-dependent reflectance. As indicated in the manufacturer's calibration curve [Fig. (12)], the mirror reflected 90% or more of light incident at a 45° angle for wavelengths between roughly 450 nm and 540 nm, depending on polarization. The 514.5 nm trapping beam was

Figure 11: Internal Beam Splitter and Apertures

- A) The original geometry of the internal beam splitter in the Zeiss microscope. The 7 mm apertures before and after the beam splitter limit the size and angle of the beam approaching the 8 mm objective aperture.
- B) With a larger reflector and open geometry the objective aperture is the limiting aperture of the system, as desired.

LWP-45-R(450-540)-TVIS

Figure 12: Dichroic Reflector Calibration Curve Supplied by CVI Laser Corp.

within this range, so that replacing the half-silvered mirror with the dichroic reflector nearly doubled the power delivered to the objective lens and trapping region. At the same time, a 632.8 nm He-Ne beam used for illuminating the trapping region from below was transmitted through the reflector at a rate of approximately 85% (S-polarization). The He-Ne light that was reflected back along the trapping beam was used for alignment, but also increased the background to the backscattered light used for measuring axial displacements of trapped objects. The dichroic reflector dramatically reduced this background light, while also reducing the background at the quadrant detector and video camera due to light from the backscattered trapping beam. For applications using an infrared beam as the trapping beam, a different dichroic reflector would be needed.

The steering optics were chosen following the Fällman and Axner model (Sec. 3.1.3), with two gimbal-mount motorized mirrors for the two trapping beams, each preceded by a set of afocal axial steering lenses (L1 and L2 in Figs. 6 and 7, pp. 33-34). After the beams are recombined, a single afocal pair of lenses (L3 and L4) was used to image the two gimbal mirrors (GM1 and GM2) at the objective aperture.

The position of the microscope assembly and of the gimbal mirror for the primary trap were chosen for convenient access and to have a large distance between them to accommodate the optical elements that would need to be placed between them. These include [Fig. (7)] the polarizing cube beam splitter which recombines the two trapping beams, the two lenses L3 and L4, a mirror pair to raise the beam level from near the table to the height of the side port on the microscope (not shown), a beam splitter to split off the backscattered light for axial force measurements (and to provide a beam power

monitor just before the microscope), and an aperture at the beam waist to limit background light into the backscatter detector.

With the locations of the GM and objective aperture (OA) established, the distance between them

$$d_{tot} = d_m + f_3 + f_4 + d_o \quad (30)$$

was used as a constraint in choosing lenses L3 and L4. (As noted above, the distance used in this calculation was modified when using the original half-silvered beam splitter, using the position of the beam splitter in place of the objective aperture. The positions of L3 and L4 were adjusted accordingly.)

Recalling Eq. (21), the relationship between the focal lengths and distances is

$$f_3 + f_4 = d_m \frac{f_4}{f_3} + d_o \frac{f_3}{f_4}. \quad (21)$$

Using the total distance as a constraint allows the determination d_m and d_o for a given set of focal lengths f_3 and f_4 . These calculations were done for various lens combinations using a Matlab program (Appendix A.1).

For a two-beam system, there is a minimum allowable distance d_m between the gimbal mirror and the first lens that depends on the axial steering components. As can be seen in Fig. 7 (p. 34), the distance d_m must be great enough to accommodate the lens set L1/L2 and gimbal mirror (GM2) for the second trap, which are parallel to the line between GM1 and L3.

Taking these logistical requirements into account, as well as the desired beam expansion of about 150%, and choosing from lenses available in the lab, lenses L3 and

L4 were chosen with focal lengths of 35 mm and 50 mm, respectively. The distances between optical elements and their focal lengths are listed in Table 2.

Table 2: Distances between Optical Elements

Element	Focal Length (cm)	Distance from previous (cm)	Distance from laser (cm)
Newport Attenuator		228	228
Polarizing cube beam splitter		49.5	277.5
L1 / (second trap)	2.5 / (5.0)	23.5 / (12)	301 / (289.5)
L2 / (second trap)	2.5 / (5.0)	5 / (10)	306 / (299.5)
Gimbal Mirror main / (second trap)		2.5 / (5)	308.5 / (304.5)
Polarizing cube beam splitter		27.5 / (31.5)	336 / (336)
L3	35	9	345
L4	50	85	430
Beam splitter (for backscatter detector / power monitor)		20	450
Adjustable aperture		9.5	459.5
Microscope entrance (3 cm dia.)		11.5	471
Dichroic reflector		2	473
Microscope objective aperture (8 mm dia.)		4	477

In order to minimize the distance between the polarizing cube beam splitters, L1 and L2 were chosen with short focal lengths. The focal lengths of L1 and L2 for the primary trap were each 2.5 cm. L2 was placed one focal length from the surface of the GM, and L1 was mounted on a translation stage with micrometer controlled motion in the

z-direction, 5 cm from L2. This is the afocal arrangement described in Sec. 3.1.3 and shown in Fig. 5(A) (p. 31). Because of the geometry of the corner mounted mirror used for the secondary trap, the minimum distance between L2 and the mirror, and hence the focal length chosen for L1 and L2, was 5 cm.

The Fällman and Axner afocal design is based on an infinity-corrected microscope system, which requires a collimated beam at the objective lens to produce a waist in the trapping plane. Since the Leitz Wetzlar objective used in the San Jose State Optical Tweezers assembly is designed for a 170 mm tube length, it was necessary to produce a waist in the trapping beam at approximately this distance from the OA. This was accomplished by reducing the distance between L1 and L2 by approximately 15%, producing a divergent output beam from a collimated input beam [as in Fig. 5(B)]. Again, because of the location of L2, the beam at the GM remained the same size, even as the divergence was changed to produce the desired waist before the objective.

The polarizing cube beam splitters (Newport) used to split the trapping beam were coated for visible light (400–700 nm). The total transmission of each cube was measured at 514.5 nm as approximately 90%. The cubes would be replaced with two identical pieces coated for near infrared light (900–1300 nm) when the Nd:YAG beam is used for trapping. In order to have light in both traps, the initial beam was elliptically polarized by introducing a quarter-wave plate immediately after the laser. Rotating the quarter-wave plate changed the ellipticity of the polarization, and thereby the relative intensities of the two traps. The beam splitters and quarter wave plate were removed for single-trap operation.

The main gimbal-mount mirror in this assembly was a New Focus model 8812, a true gimbal-mount assembly with a one-inch diameter aluminized mirror, driven with a multi-axis Picomotor (New Focus). The secondary trap used a New Focus 8809 corner mount with similar Picomotor drive. The 8809 is not strictly a gimbal mount, but a reasonably close approximation. While tipping the 8812 in the horizontal or vertical directions produced pure x or y motion of the trap, similar motion of the 8809 produced slightly arced trajectories for the secondary trap. The Picomotor can be driven either with the control pad, which has sliders for each axis of motion, or by analog voltages. Mirror motion can be automated, though it is limited to fairly slow translations because of the mass of the mount assembly. Calibration is difficult because of the lack of indexing in the Picomotor drive mechanism,⁴⁰ which limits the possible precision of the motor (and mirror) motion.

An optical multimeter (ILX Lightwave) was used to measure beam intensities at various points along the trapping beam. A cover slip was placed in the beam at a 45° angle approximately 140 cm from the laser to reflect a small percentage (8-10%) of the beam into the ILX Lightwave sensor as an intensity monitor. The same sensor head was moved to various points along the beam path for specific measurements to determine relative intensities, most often at the beam waist just before the microscope.

The overall transmission of the system was measured for a single trap, with the polarizing beam splitters removed. With the discharge current of the Ar⁺ laser at its maximum value of 30 A, the output at the laser was 1.7 to 1.8 watts. Approximately half of this power was transmitted through all the steering and focusing elements to the beam

splitter just before the microscope. Half of the remaining beam was transmitted through the beam splitter, so that about one quarter of the original beam intensity was present at the entrance to the microscope. The actual transmission coefficient at the microscope entrance was between 0.228 and 0.255 in various measurements, the variations due primarily to accumulating dust on optical surfaces and gradual misalignment due to vibration.

For single trap operation, the vertical polarization of the beam was left unchanged. The beam struck the dichroic mirror in the microscope as P-polarization. At 514.5 nm, the transmission for P-polarization is approximately 7% (Fig. 12, p. 47), so that 93% of the beam was reflected toward the microscope objective. The transmission through the aperture of the objective was measured as 90%, and the transmission of the objective lens was measured as 89% using the method described in Sec. 3.1.2.

With immersion oil ($n_{\text{oil}} = 1.52$) between the objective and the cover slip the light lost between the objective and the trap is less than one percent, so the total transmission from the entrance of the microscope to the trap can be estimated as about 74% (again, this would be dramatically lower for an infrared beam since objectives are generally coated for use with visible light).

Combining the measured transmission to the microscope with the estimated transmission to the trap gives an overall transmission of approximately 17 – 19%. This means that with the maximum power of 1.7 watts at the laser, the power at the trap would be approximately 285 – 320 mW. This is at least a factor of 10 greater than the minimum power needed to trap silica spheres in the 1 – 5 μm range. Beads were stably trapped

with powers as low as 25 mW at the microscope entrance, corresponding to about 18 mW at the trap. This is comparable to values reported by others.^{6, 7, 29}

In a two-beam system, the combination of the two polarizing cube beam splitters introduces an additional loss of about 10% of the total beam power. The remaining power is split between the two traps, so that traps of equal strength could provide about 130 – 145 mW each.

To vary the trapping beam intensity, a dual shearing attenuator (Newport) was inserted into the beam path at the position indicated in Fig. 7 (p. 34). The attenuator uses multiple reflections to vary the intensity of the transmitted beam with minimal effect on beam shape or quality. The output of the attenuator can be varied continuously from zero to 70% of the input intensity. Above 70% the only option is to allow 100% of the beam through the half-inch diameter aperture. The attenuator dial is rather coarse, but settings were repeatable to within about 5%.

3.2.4 Observation and Measurement

To illuminate the trapping region for viewing, the original incandescent bulb provided with the Zeiss microscope was initially employed. Later, the 2 mW He-Ne (Uniphase) beam that had been used as an alignment guide was also used for illumination. Two intermediate lenses between the objective and the eyepiece of the Zeiss assembly were removed so that the illuminating beam exited the top port of the microscope after passing through only the objective and the beam splitter. A reflector sent this beam into an 8 mm video camera (Samsung) with output to a monochromatic

monitor (Zenith Data Systems) for observation of objects in the trapping region. A 10 cm focal length convex lens was used to magnify the image for the video camera, which was set to its full 32x zoom. Color filters were placed before the camera to prevent saturation from laser light. For radial force calibration and measurements, which were not attempted in this work, the beam would be split, illuminating both the video camera and a quadrant photo detector, as in Fig. 8 (p.36) and Sec. 3.1.4.

To calibrate the axial trap stiffness, a silicon photodiode (Hamamatsu S1406-03-04) was employed to collect backscattered light in the manner described in Sec. 3.1.4 and pictured in Fig. 9 (p. 38). The S1406 has a built-in op-amp, and requires only positive and negative bias voltages with appropriate feedback resistor and filter capacitors. The circuit diagram is pictured in Fig. 13. The bias voltages were provided with two 9 volt batteries.

Voltage data from the photodetector were collected using a C++ data acquisition program (Appendix A.2) run on a PC with a 100 MHz 486 processor. Data were digitized with a DAS-8 12-bit ADC data acquisition board (Keithley Metrabyte). The sampling rate for the board was 24 kHz. For each data set the program collected data at a sampling rate of 2000 Hz for a period of 20 seconds, or a total of 40,000 measurements. Data files were analyzed using several programs written and run with Matlab 5.3.0, as described in Sec. 4.2.1.

Figure 13: Circuit Diagram for Backscatter Photodetector
 Detector used for axial displacement measurements. Photodiode and operational amplifier are packaged together as Hamamatsu S-1406.

CHAPTER 4

4.0 Results and Discussion

In this chapter the operation of the San Jose State University Optical Tweezers is described, including the qualitative results of successful trapping and manipulation of beads in two and three dimensions, a discussion of the data acquisition techniques employed and the quantitative results of preliminary calibration of the axial spring constant of the optical tweezers trap.

4.1 Qualitative Results: Trapping in Two and Three Dimensions

Numerous authors have reported success trapping polystyrene spheres with diameters between 1 μm and 10 μm with optical tweezers.^{4, 6, 10} Initial trials at San Jose State employed samples of 1.069 μm and 2.90 μm diameter polystyrene latex spheres (Duke Scientific) suspended in distilled water.

Successful two-dimensional trapping, sometimes referred to as "impaling," was quickly achieved. In this situation the sample cell was raised until the waist of the trapping beam was just above the cell floor. Beads floating in the region of the beam were drawn into the beam and pushed toward the waist where they became impaled against the cell floor. In this position a trapped sphere could be manipulated in the x-y plane by steering the trapping beam at the gimbal-mount mirror, providing confirmation of the steering optics design.

Video recordings of this trapping show beads being captured and held by the two trapping beams. Trapping was verified by translating the sample stage, producing a Stokes drag force as the suspension medium was swept past the trapped spheres. While other objects floating in the medium were swept along with the stage, trapping was demonstrated when the trapped beads remained stationary or were translated within the trap by one bead diameter or less but not dislodged. The beam steering optics were demonstrated by the motion of the trapped spheres in regular patterns (lines, circles) in response to steering at the gimbal mirrors. A sequence of frames from one such video is shown in Fig. 14. Two trapped beads appear in the upper left portion of the image. One bead is held motionless throughout the sequence, while the other is moved in a zigzag pattern by steering the trapping beam. A rectangular guide has been superimposed on the image to make the bead motion easier to judge. In contrast to the trapped beads, the collection of beads on the right part of the image changes position randomly from frame to frame as it undergoes Brownian motion.

Trapping in three dimensions proved more difficult, at least with polystyrene beads. If the cell were lowered in an attempt to lift a trapped sphere from the cell floor, the sphere would slip out of the trap. Similarly, when the trap was positioned so that the waist was several sphere diameters or more above the cell floor, beads were drawn into the beam but were pushed through and out of the trapping region. The difficulty trapping in three dimensions suggests that there was no equilibrium point where the gradient force balanced the scattering force.

Figure 14: Polystyrene Beads in Dual Optical Tweezers Traps

Video images show two 1 μ m-diameter polystyrene spheres impaled against the cell floor by dual trapping beams. The upper left bead is held stationary with one beam while the other bead is manipulated in the x-y plane. In frames A) through D) the bead follows as the trap is moved in a zigzag pattern (The rectangular guide is drawn in the same position in each frame). Note the cluster of beads on the right side of the frame moving freely under Brownian motion.

A possible reason for an inadequate axial gradient force is underfilling of the objective aperture. It is important to have adequate intensity at the outer part of the lens because the rays from the outer edges of the objective lens strike the trapped bead at the greatest angle from normal and contribute the most toward the gradient force.^{6, 30} The transmission through the objective aperture was measured as 90%, somewhat higher than the recommended $1/e^2$ value of 85%, suggesting that the beam diameter might be too small. A beam expander was introduced to increase the beam diameter by 10%, giving the desired 85% transmission, however there was no apparent improvement in trapping of polystyrene beads.

In contrast to these difficulties, both yeast cells and 1 μm to 5 μm silica spheres were routinely trapped and manipulated in three dimensions. The yeast samples were made by dissolving a few grains of Fleischmann's baker's yeast, purchased at a grocery store, in 30 – 40 ml of distilled water. Individual yeast cells appeared on the video monitor as slightly elongated objects approximately 4 – 5 μm across. When trapped, the long axis of the yeast cell tended to align with the beam axis, so that a circular cross-section was visible in the video monitor. In video recordings three-dimensional trapping is demonstrated when an object is captured near the cell floor and then lifted away from the floor and the surrounding objects. Because the trap lies in the focal plane of the objective, the trapped object remains in focus while the images of the surrounding objects blur and enlarge. The trapped object can be manipulated with the steering optics, or the cell can be translated while the trapped cell is held fixed. Figure 15 shows two frames from such a video tape of a yeast cell (approximately 4 μm across) in the optical tweezers

A)

B)

Figure 15: Yeast Cell Held in Optical Tweezers Trap

Video images show trapped cell lifted above cell floor with optical tweezers, demonstrating stable three dimensional trapping. The trapped cell is marked. The other objects in the images are yeast cells that lie A) 10-15 μm and B) 20-30 μm below the trapped cell.

trap. The trapped cell is marked with an arrow in the figure. The other objects in the figure are also yeast cells, but appear out of focus because they are lying behind (below) the trapped cell. In Fig. 15(A) the trapped cell has been lifted with the tweezers approximately 10 to 15 μm above the floor of the sample cell and most of the other yeast cells, which have drifted to the cell floor. In Fig. 15(B) the yeast cell has been lifted 20 to 30 μm above the bottom of the sample cell. In the video the trapped cell can be clearly seen to pass over other cells without interference. The ability to manipulate trapped objects in this way confirms that the forward scattering force due to the trapping beam is balanced by the axial gradient force, producing stable three-dimensional trapping.

The ability to trap silica and yeast in three dimensions but only to impale polystyrene spheres could have several causes. Svoboda and Block,³⁰ describe their experience that silica spheres could be trapped at depths of up to 200 μm below the cover slip, while polystyrene beads could be stably trapped to depths of only 10 μm . The presumption is that the spherical aberrations, which affect the beam when the waist is deep below the cover slip, are a greater problem when trying to trap polystyrene. At San Jose State polystyrene spheres were suspended in distilled water, hence they sank well below 10 μm from the top of the cell. This may have been a key problem in trapping polystyrene, which might be remedied by using the buffered saline solution to keep polystyrene beads floating near the top of the cell. A systematic study of this problem has not yet been performed.

The reason that the polystyrene spheres are more difficult to trap, and more susceptible to instability due to spherical aberration, may be their high refractive index.

The polystyrene spheres used in this experiment had a published refractive index of 1.59, while the index for fused silica is generally near 1.45, and the index for the 1 μm silica spheres from Duke Scientific is listed as 1.40.

A simple calculation is suggestive. Using Fresnel's equations for reflectance and transmittance,²⁵ the power reflectance R at normal incidence for light passing from a material with refractive index n_1 into one with index n_2 is

$$R = \left(\frac{n_2 - n_1}{n_2 + n_1} \right)^2. \quad (31)$$

Setting $n_1 = 1.33$, the refractive index of water, Eq. (31) gives the reflectance at a water to polystyrene interface as 7.9×10^{-3} , more than three times the value of R for silica, 2.2×10^{-3} . Without a similar expression for the dependence of the gradient force on relative refractive index this calculation is intended only to be suggestive that the scattering force could be more difficult to overcome for high-index materials.

The implication of the reflectance calculation is supported by numerical calculations. In a 1998 paper, Nemoto and Togo²⁷ used numerical integration of a non-paraxial ray optics model to find forces acting on a refractive sphere near the waist of a Gaussian beam. They found that a sphere 10 μm in diameter with index of refraction between 1.40 and 1.45 can be trapped by a beam with a waist about 1 μm in diameter. In this model, however, if the refractive index is 1.5 or greater, the radiation force is always in the positive z -direction and there is no axial trapping.

Another study used an electromagnetic model based on the Lorenz-Mie theory⁶ for numerical calculations of trapping efficiency as a function of relative refractive index

$m = \frac{n_2}{n_1}$. It was found that trapping efficiency was maximized for values of m between 1.1 and 1.2, and was poor for $m \leq 1.05$ and for $m \geq 1.25$. The value of m for the polystyrene and silica spheres used here were 1.20 and 1.10, respectively. Both are within the preferred range, but if the index of the polystyrene had increased with age it might move into the range of lower trapping efficiency. The beads used in this experiment were packaged in 1983, making them more than four times older than the three to four year shelf life recommended by the manufacturer. The manufacturer could not predict how optical properties of the beads might be affected by such aging, but it is possible that they had become more reflective or absorptive with time.

Other researchers have noted that microscope objectives that have been manufactured to be identical in their imaging properties can have very different success in forming traps.⁴¹ There is no known way to predict trapping characteristics, and it is not uncommon to purchase several objectives and return or exchange those that do not trap well. Since the equipment available for this study was limited to three objectives which had been used previously in other applications, it is possible that they produced traps sufficient for trapping silica and yeast, but not suitable for higher index materials such as polystyrene.

4.2 Quantitative Results

For both two- and three-dimensional trapping as described above, the relative strength of the trap could be observed qualitatively by the degree to which trapped

objects exhibited Brownian motion. With low laser power the amplitude of motion of a trapped bead due to Brownian collisions as viewed with the video camera and monitor was nearly as great as that of nearby free-floating beads. The difference was that a trapped bead continued to oscillate randomly about a fixed point, while free-floating beads drifted in and out of the sample viewing area. As the beam intensity was increased, the amplitude of the motion of a trapped bead decreased. At high beam powers the bead appeared to be completely motionless to within the resolution of the video monitor. In order to quantify this effect and to calibrate the trap stiffness as a function of power, data were collected and analyzed as described below.

4.2.1 Data Acquisition and Analysis

In order to use the Brownian motion spectral analysis described in Sec. 2.4 to calibrate the trap stiffness for force measurements, position vs time data for a trapped sphere were transformed into frequency space and the resulting curve was fit to a Lorentzian [Eq. (17)] using the trap stiffness as a fitting constant.

Recalling Eqs. (10) and (11), the Fourier transform and its inverse can be defined as

$$X(f) = \int_{-\infty}^{\infty} x(t) e^{2\pi i f t} dt \quad (10)$$

$$\text{and} \quad x(t) = \int_{-\infty}^{\infty} X(f) e^{-2\pi i f t} df, \quad (11)$$

where $x(t)$ is the position as a function of time and $X(f)$ is its Fourier transform in

frequency space. The data used to calibrate trap stiffness are a discretely sampled series of position measurements taken at regular time intervals during the continuous motion of the bead. The above integrals must therefore be rewritten as sums in order to be used with discrete data. The discrete Fourier transform and its inverse for a set of N data points are defined^{42, 43} as

$$X_n \equiv \sum_{k=0}^{N-1} x_k e^{2\pi i k n / N} \quad (32)$$

and

$$x_k \equiv \frac{1}{N} \sum_{n=0}^{N-1} X_n e^{-2\pi i k n / N}, \quad (33)$$

where x_k represents the position of the bead as measured at the k^{th} time interval, and X_n is the coefficient of the n^{th} frequency in the Fourier series representation of the transform.

The frequencies are defined by

$$f_n \equiv \frac{n}{\tau N} \quad (34)$$

where τ is the time interval between samples, *i.e.*, the inverse of the sampling rate in hertz. Because of aliasing, an artifact of a finite sampling rate, the coefficients of only the first $\frac{N}{2}$ frequencies are independent, so that a data set of N points generates a Fourier transform of $\frac{N}{2}$ frequencies ranging from $\frac{1}{\tau N}$ to $\frac{1}{2\tau}$ (known as the Nyquist frequency)^{42, 43} at intervals of $\frac{1}{\tau N}$.

The discrete form of the power spectral density (PSD) at each frequency [Eq. (16)] is

$$P(f_n) = \frac{1}{N^2} |X_n|^2. \quad (35)$$

Recalling Eq. (17) and Fig. 2 (p. 18), the PSD for a trapped particle driven by Brownian collisions is expected to be a Lorentzian of the form

$$P(f) = \frac{|F(f)|^2}{(4\pi^2 f^2 - \alpha)^2 + 4\pi^2 f^2 \beta^2}. \quad (17)$$

Plotted on a log-log graph, the curve has a plateau at low frequencies which drops off above the critical frequency $f_c = \frac{\alpha}{2\pi\beta}$, where α is the trap stiffness constant and β is the Stokes damping constant.

The ratio $\frac{\alpha}{\beta}$ is equivalent to the ratio $\frac{k}{D}$, where k is the spring constant of the trap and D is the Stokes drag coefficient, determined from the modified Stokes equation [Eq. (7)]. Taking the viscosity of water as 1.0×10^{-3} N-s/m² for a 3 μ m diameter sphere gives the value $D = 2.83 \times 10^{-8}$ N-s/m. The correction term λ has been set to unity since the data reported here were collected with the trapped bead held far from the cell boundaries, so that $\frac{r}{h} \ll 1$ and $\lambda \approx 1$.

Trap stiffness is expected to increase linearly with power.^{6, 7, 30} This has been observed by other groups, with published values of the radial spring constant k_{xy} ranging between 25×10^{-6} and 250×10^{-6} N/m for trap power varying from 13 mW to 140 mW.⁷ The axial force is expected to be only 3–4% as strong as the radial force,⁶ and values for k_z have been reported in the range of 1×10^{-6} to 40×10^{-6} N/m for trap powers from 3 mW to 60 mW.²⁹ The latter figures include low power measurements which were made

with a doughnut cross-section beam instead of a Gaussian beam. This reduces the scattering force relative to the gradient force and allows stable trapping and higher spring constants at lower powers than for a TEM_{00} beam. Based on these published values the San Jose State Optical Tweezers would be expected to have axial trapping stiffness coefficients between approximately 1×10^{-6} and 10×10^{-6} N/m.

Given these approximate values for k and D , f_c for a $3 \mu\text{m}$ sphere is expected to lie approximately between 100 Hz and 1500 Hz for the radial force and 3 Hz to 100 Hz for the axial force. The range of frequencies in the PSD should be adequate to give a long, flat plateau below the shoulder frequency and a distinguishable drop above the shoulder frequency on a log-log plot. For the axial force calibration, this means low frequencies down to a fraction of a hertz, with an upper frequency near 1000 Hz. Because of the Nyquist cut-off frequency, the data sampling frequency must be at least twice the upper frequency desired in the Fourier series output, or 2000 Hz, while the lower limit requires sampling over a period of tens of seconds.

Axial calibration data were taken with a Hamamatsu photodetector and recorded by means of a C++ data acquisition program (Appendix A.2) as described in Sec. 3.2.4. The sampling rate was 2000 Hz and the total sampling time for each data run was 20 seconds, providing a total of 40,000 data points for each run.

The voltage output of the detector was taken to be linearly proportional to the incident power. Variations in the power are expected to be proportional to the axial displacement of the bead, as derived in Sec. 3.1.4. It was not necessary to use the proportionality constants for the calibration described in this thesis. The relative

amplitudes of motion provide adequate information to generate a normalized power spectral density that can be fit to Eq. (17) to determine the trap stiffness. The absolute axial displacement of the bead within the trap will be needed in order to use the tweezers to measure force or to map the topography of a surface. Absolute displacement can be determined from the variations in power at the detector through Eq. (29) (p. 40), using the overall magnification of the optics that project the backscattered image onto the detector.

The voltage data was analyzed with several original programs and functions written for and executed with Matlab 5.3.0 (Appendix A.1). Matlab has a built-in Fast Fourier Transform (FFT) function which uses the definition of Eq. (32) with a slight difference in indexing (the Matlab syntax does not allow indexing to start with zero, so the sums are from 1 to N instead of from 0 to N-1). A full 40,000 point FFT and 20,000 point PSD was computed for each set of data. Figure 16 shows typical voltage vs time data and the corresponding PSD.

The variance in $P(f_n)$ at each frequency can be reduced by averaging the $P(f_n)$ from several sets of data collected under similar conditions. Figure 17 shows such an average for five data sets, including the one shown in Fig. 16. The data used to produce Figures 16 and 17 were taken with the trapping beam power equal to approximately 82 mW at the microscope entrance (approximately 61 mW at the trap).

In addition to having less scatter, the averaged data more clearly show the expected Lorentzian shape (compare to Fig. 2, p. 18). To make a quantitative statement, however, it is necessary to fit the curve to Eq. (17), using the stiffness coefficient α as a

Figure 16: Detector Output and Power Spectral Density

- A) Backscatter detector voltage as a function of time. Variation in detector output is proportional to variation in axial position of trapped sphere (Sec. 3.1.4). Five-second excerpt from 20-second run.
- B) Power Spectral Density of single 20-second data run (82 mW at microscope entrance).

Figure 17: Averaged Power Spectral Density

PSD's from five data sets collected at the same power (82 mW at microscope entrance) are averaged to reduce variance at each frequency.

fitting constant. By fitting data collected at different laser powers, the system can be calibrated for force measurements.

A fitting routine was written in Matlab (kfit.m, Appendix A.1), using a built-in minimization function. In conjunction with two original functions, the program compares the PSD data to the theoretical curve, varying α to minimize the difference between the curve and the data. The difference is defined using a standard χ^2 function,⁴²

43

$$\chi^2 \equiv \sum_{i=1}^N \left(\frac{y_i - y(x_i; \alpha, \beta)}{\sigma_i} \right)^2, \quad (36)$$

where the y_i are the measured PSD values and the $y(x_i)$ are the expected values at the

corresponding frequencies as calculated from Eq. (17) using the current value for α and the known value for β .

The fitting program was tested on simulated data created with another original Matlab program (fftdata.m, Appendix A.1). This program took points from the theoretical Lorentzian [Eq. (17)] and used an inverse FFT function to generate a theoretical voltage vs time curve. Random noise was added to produce simulated data. The FFT and PSD were then calculated from the simulated data. The fitting program reproduced the original stiffness constant to within a few percent in trials with varying levels of added noise. Two examples with different noise levels are shown in Fig. 18.

4.2.2 Preliminary Calibration Results

Three sets of averaged PSD data with fitted curves are shown in Figs. 19(A), 19(B) and 19(C). Each data set is an average of four to five individual PSD's calculated from data taken at one of three power levels. The fitting constants and corresponding powers are tabulated in Table 3 and plotted in Fig. 20. Figure 20 also shows a linear fit generated with Matlab's built-in fitting routine. The fit confirms the expected linear relationship between laser power and trap stiffness.

Table 3: Laser Power and Fitting Constants for Axial Force Calibration

Newport Attenuator Dial Setting	Est. Power at Microscope Entrance (mW)	Est. Power at Trap (mW)	f_c (Hz)	k ($\times 10^{-6}$ N/m)
56	82	61	4.13	0.740
65	174	129	10.6	1.90
0	277	205	16.0	2.87

Figure 18: Simulated Data and Fitting Program Test

Simulated PSD data generated by adding random noise to inverse FFT of theoretical curve, fit with original Matlab program (Appendix A.1); original values $f_c = 15.0$ Hz and $k = 2.686 \times 10^{-6}$ N/m.

A) Moderate noise added; fit reproduces original values within less than 2%.

B) Higher noise level; fit reproduces original values within 5%.

Figure 19: Averaged PSD Data and Fitted Curves

A) 82 mW, B) 174 mW, C) 277mW at microscope entrance.

Figure 20: Axial Stiffness as a Function of Power.

Data collected from backscattered light at three power levels show expected linear relationship between laser power and trap stiffness.

The power estimates listed in the table are based on the setting of the Newport attenuator, not on individual measurements at the microscope. The attenuator settings were found to be repeatable within a few percent. The power at the microscope was measured once at the start and once at the end of the entire experiment to avoid interrupting the beam between measurements, which would cause the trapped sphere to fall from the trap. There was a wide variation in bead sizes (from about 1 to 5 μm) in the silica sample, and since β varies linearly with sphere radius it was important to keep a single bead in the trap for the entire experiment to insure that β was kept constant. In future experiments a detector will be placed in position to collect light from the incoming trapping beam reflected from a beam splitter in front of the microscope to allow constant

monitoring of beam power without interrupting the trapping beam. Allowing for the uncertainty due to the attenuator dial settings and long-term drift of the laser power, the uncertainty in the power measurements is estimated to be five to ten percent.

The results of Table 3 and Fig. 20 confirm the desired operation of the optical tweezers trap. The values of the spring constant vary from approximately 0.74×10^{-6} N/m with approximately 61 mW at the trap to approximately 2.9×10^{-6} N/m with approximately 205 mW at the trap. These can be compared to the value published by Ghislain *et al.*⁴⁴ for 2- to 6- μm polystyrene spheres in a 60 mW trap using 1064 nm wavelength light of 42×10^{-6} N/m. Friese *et al.*²⁹ measured the spring constant for a 2.84 μm polystyrene sphere in a 3 mW trap produced with 632.8 nm light as 1.95×10^{-6} N/m. These values cannot be directly compared to the results in Table 3 because the experiments involved different wavelengths, beads of different materials, and different distances from the cover slip, but they are of the same order of magnitude and suggest that these initial calibration values are reasonable. The linear relationship evident in Fig. 20 further supports the claim that the trap is operating as expected.

Calibration of the axial trapping force as a function of characteristics of the laser beam, beads, and suspension medium can be conducted by repeating the procedures and analysis described in this thesis while varying the characteristics of interest. Calibration of the radial force can be done with the same analysis tools, using data from a quadrant detector as described in Sec. 3.1.4.

CHAPTER 5

5.0 Conclusion

This thesis describes the design and demonstration of optical tweezers to be used for biophysics experiments. The design requirements of providing two independently steerable beams, each expanded to overfill the back aperture of the microscope objective lens used to form the trap were met. This was achieved with three afocal sets of lenses (one set for each beam and one set shared by the two beams) which allowed the angle and divergence of the beam to be changed, producing radial and axial translation of the trap position, without changing the size or position of the beam at the objective aperture.

Three-dimensional trapping and manipulation was demonstrated with silica spheres and with yeast cells. Two-dimensional trapping and manipulation was demonstrated with polystyrene spheres.

The optical tweezers were designed to exploit either the radial or axial trapping force, each used for different types of applications. The work described in this thesis focused on calibrating the axial trapping stiffness. Variations in the axial position as a function of time for a trapped sphere caused by Brownian collisions with molecules of the suspension medium were measured by collecting backscattered light reflected from the trapped sphere. The trap stiffness was calibrated by comparing the power spectral density of this motion to a theoretical model. Measurements were taken at three different power levels and the results confirmed the expected linear relationship between trap power and stiffness constant. The magnitude of the axial stiffness constant, varying from

approximately 0.74×10^{-6} to 2.8×10^{-6} N/m as the power at the trap varied from approximately 60 mW to 250 mW, is comparable to values published by other researchers.

Continued work can be done in several areas. The difficulty trapping polystyrene spheres can be immediately investigated by using a saline solution to float the spheres near the cover glass surface, potentially reducing trapping problems due to spherical aberrations. A newer sample of polystyrene spheres might also be tested to determine whether aging has affected the optical properties of the spheres currently available.

Uncertainties in trap power measurements can be reduced by monitoring the power without interrupting the beam by means of a beam splitter near the microscope entrance. The optics used to project the backscattered image of the trapped sphere onto the photodetector must be optimized for smaller particles before the axial stiffness can be calibrated for particles 1 μm or less in diameter.

Once these immediate questions have been investigated, the methods described in this thesis can be used to calibrate the axial trapping constant as a function of the size and material of trapped spheres, as well as the distance from the cover slip and the wavelength of the trapping beam. Following that, the radial trap stiffness can be calibrated for the same parameters by use of a quadrant detector. The frequency analysis technique established for axial calibration can be easily adapted to the radial calibration, and results can be confirmed with one of the Hooke's law methods, by translating the stage with stepper motors or translating the trap within the sample cell. The effects of

vibrations caused by the stepper motors and the amplification of vibrations due to their mass will need to be investigated before they can be used for calibration measurements.

Future development of the apparatus may include employing an inverted geometry of the sample and objective lens. Trapping efficiency could be increased by using a doughnut cross-section beam instead of a Gaussian beam, possibly generated with holographic filters. The resolution of radial position measurements could be increased with interferometric methods instead of the quadrant detector described here. It may also be interesting to investigate the effect of modulating the illumination beam, introducing a constant-frequency axial driving force to the trapped sphere. This may be particularly useful as a calibration tool.

Once calibrated, the optical tweezers can be used in the present format for a wide range of biophysical investigations. Single-molecule studies can be done using the Nd:YAG beam to form the trap while inducing fluorescence with the Ar^+ beam to allow visualization of molecules attached to spherical handles. A good test of the steering and force measurement capabilities of the system would be to measure the tensile strength of long molecules like DNA or myosin by attaching handles at both ends and trapping each handle with a separate beam. Similarly, the tensile strength and elasticity of membranes can be investigated by deforming them with one or two spheres held with the tweezers. This is a particularly interesting area of inquiry since communication through membranes plays a key role in many biological processes. The work described in this thesis has laid the ground for exciting investigations in any of these arenas.

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APPENDICES

A.1 Matlab programs and functions

PData.m: Matlab program used to calculate and average PSD's from multiple data sets:

```
% PData -- 2/28/01 program to average PSD's from several data sets

help PData;

fprintf('You can enter up to ten data sets to be averaged.\n');

sets = 0;
for k = 1:10
    A = input('Enter data set name (enter 0 to stop): ');
    if A == 0,
        break
    else
        data(:,k) = A(:,2);      % create matrix of k columns of data
        sets = sets+1;          % increment to number of data sets entered
    end
end
%f = input('Enter data collection frequency: ');
f=2000;

for m = 1:sets                % create matrix of PSD's
    [fourout Vnorm(m)] = fullfourier(data(:,m),f);
    P(:,m) = fourout(:,1);
end

freq = fourout(:,2);

P_rows = P';
P_ave = 1/sets*sum(P_rows);

P_ave = P_ave(2:20000);
freq = freq(2:20000);

Vave = 1/m*sum(Vnorm);

figure;
loglog(freq,P_ave);
axis([1e-1 1e3 1e-7 1e-1]);
title(['Average of ',num2str(sets),' data sets']);
xlabel('Frequency (Hz)');
ylabel('Normalized PSD');
```

fullfourier.m: Matlab function called by PSDData.m to calculate 40,000-pt. FFT and 20,000-pt. PSD from voltage *vs* time data set.

```
function [fourout, Vnorm] = fullfourier(data,f,n);

% function to take full N-point fft of V vs. t data set

V = V_ac(data);
Nvolt = length(V);

Vsqr = V.^2;
Vnorm = 1/Nvolt*sum(Vsqr);

tau = 1/f;
fstep = 1/(Nvolt*tau);
freq = linspace(fstep,Nvolt/2*fstep,Nvolt/2);

s = fft(V);
S = abs(s).^2;           % PSD (square of abs of FFT)

P = S(1:Nvolt/2);       % truncate at Nyquist freq.

Nrml = trapz(P);         % vector of Ndata integrals of FFTs

P_nrml = P/Nrml;        % normalize PSD's by dividing each by its integral

% create output column vectors of freq/PSD,
fourout(:,2) = freq';
fourout(:,1) = P_nrml';
```

V_ac.m: Matlab function called by fullfourier.m to subtract average voltage from data set to eliminate large low frequency spike from DC signal.

```
function V = V_ac(data);

%function takes V vs. time and subtracts
%average value to create "pure AC" signal

vt = data';
N = length(vt);
ave = 1/N*sum(vt);
V = vt - ave;
```

kfit.m: Matlab program used to determine trap stiffness constant $k = \alpha m$ from PSD data by fitting data to theoretical Lorentzian.

```
%kfit -- program to fit PSD of voltage data to Lorentzian,
%           using constant value for Stokes constant D,
%           fitting spring constant k

% input PSD data and frequency vector to be fit to Lorentzian
X = input('Enter data set to fit (PSD data only):' );
freq = input('Enter frequency vector:');
n = length(X);

Data = zeros(n,2);           % initialize data matrix

r = 1.5e-6;                  % sphere radius in meters
rho = 2.01e3;                % sphere density in kg/m^3
m = rho*4*pi*r^3/3;          % sphere mass
D = 1.9e-2*r;                % Stokes constant D
beta = D/m;

kstart = 1e-10;              % initial guess for k in N/m

alphastart = kstart/m;

% construct column matrix from frequency and voltage data
Data(:,1) = freq;
d = size(X);

if d(1) > 1                    % adapt for column or row vector data
    Data(:,2) = X;
else
    Data(:,2) = X';
end

% set max number of iterations and function evaluations for
minimization routine
lor_options = optimset('maxfunvals',1e6,'maxiter',1e6,'tolx',1e-
6,'tolfun',1e-9);

% fminsearch returns value of param which minimizes function 'chi2'
bestalpha = fminsearch('chi2alpha',alphastart,lor_options,beta,Data);

best = [bestalpha beta];

bestk = bestalpha*m;

fit = lorentz(freq,best);     % theoretical curve from best fit parameters
Nrm = trapz(fit);
fitplot = fit/Nrm;
```

```

f_c = best(1)/(2*pi*best(2));          % calculate shoulder freq from fit

fprintf('Fit gives k=%g, f_c=%g\n', bestk, f_c);

figure
loglog(freq,Data(:,2),freq,fitplot,'-');
axis([1e-1 1e3 1e-7 1e-1]);
legend('data','fit');
xlabel('Frequency (Hz)');
ylabel('Normalized PSD');
text(1.5e-1,3e-7,['Fit gives k = ',num2str(bestk,4), ...
    ' N/m and f_c = ', num2str(f_c,4),' Hz'])
title('PSD data and Lorentzian fit');

```

chi2alpha.m: Matlab function called by kfit.m calculates chi-squared error function for current values of constants α and β .

```

function err = chi2alpha(alpha, beta, data);

% function to determine chi-square of data set
% compared to Lorentzian with parameters alpha
% (spring stiffness) and beta (damping coefficient)

% separate data into vectors of frequency, PSD
f = data(:,1);
P = data(:,2);

param = [alpha beta];          % combine alpha, beta into vector

Y = lorentz(f,param);          % define theoretical function
term = (P-Y).^2;                % argument of chi-squared sum
err = sum(term);                % sum

```

lorentz.m: Matlab function called by chi2alpha.m calculates theoretical PSD for current values of constants α and β .

```
function Y = lorentz(f, param);

% function to calculate Lorentzian of PSD for
% parameters alpha (trap stiffness) and
%           beta (damping coefficient)
%           kT (thermal energy)

%separate parameters
alpha = param(1);
beta = param(2);

boltz = 1.38e-23;           % Boltzmann's constant
T = 285;                   % Temperature
C = 4*boltz*T*beta;        % Numerator of Lorentzian formula

omega = 2*pi*f;            % convert to angular frequency

y = C./((alpha - omega.^2).^2 + beta^2*omega.^2);
Nrml = trapz(y);

Y = 1/Nrml*y;
```

L3_L4two.m: Matlab program used to calculate positions of afocal steering lenses.

```
% L3_L4two
% calculates lens distances
% for delivery of laser into microscope port for steerable
% optical tweezers given specific focal lengths and overall distance
% from gimbal mirror to objective

clear; help L3_L4two;

dtot = input('Enter distance from gimbal mirror to objective (cm):');
f = input('Enter focal lengths for lenses [f3 f4]:');
f3 = f(1);
f4 = f(2);

d34 = f3 + f4;
d4 = (d34*(f3/f4+1) - dtot)/((f3/f4)^2 - 1);
dg3 = f3/f4*(d34 - f3*d4/f4);
out = [f3 f4 dg3 d34 d4 dtot];
fprintf('Dg3 = %g, D34 = %g, D40 = %g (Dtot = %g)\n',dg3,d34,d4,dtot)
```

fftdata.m: Matlab program starts with theoretical PSD, generates theoretical voltage vs time data, adds noise and calculates new PSD from simulated data.

```
%fftdata -- program to generate simulated data
% for Brownian motion-driven harmonic oscillator,
% starting with theoretical frequency spectrum, taking
% inverse fft, adding random noise and taking fft

% set constants

alpha = 1e8;           % stiffness (k/m)
beta = 1e6;            % damping (D/m)

boltz = 1.38e-23;      % Boltzmann's constant
T = 300;               % temperature
C = 4*boltz*T*beta;    % numerator of Lorentzian (PSD)

points = 40000;        % number of data points
n = 4096;              % number of fft points
f = 2000;              % sampling frequency
tau = 1/f;
gap = floor(points/n); % gap between selected points
deltat = gap*tau;       % time between sampled points
fstep = 1/(n*deltat);   % frequency increment
freq = linspace(fstep,n*fstep,n); % frequency vector
omega = 2*pi.*freq;     % angular frequency vector

X = C./((alpha-omega.^2).^2 + beta^2*omega.^2); % theoretical PSD
int = trapz(X);
Xplot = 1/int*X;

x = sqrt(C)./((alpha-omega.^2) - i*beta.*omega); % theoretical
x(omega)

V = ifft(x);           % inverse FFT for x(t)

Vreal = real(V);       % real part of V
Vimag = imag(V);       % imaginary part of V

Vave = 1/n*sum(V);
%v = V - Vave;

t = linspace(deltat,n*deltat,n); % create time vector for plotting
v(t)

% add random noise to v(t)
Vdata = addnoise(V, 15.0);

xdata = fft(Vdata);    % FFT for simulated x(omega)
xconj = conj(xdata);
Xdata = xdata.*xconj;  % simulated PSD
```

```

%Nrml = trapz(Xdata);
X_nrml = 1/int*Xdata;

figure(1);                                % plot theoretical PSD
loglog(omega(1:n/2),X(1:n/2));
%axis([.1 1e4 6e-32 1e-29]);

figure(2);                                % plot theoretical x(t)
plot(t,Vreal,t,Vimag,'-.');
%axis([0 10 -2e-17 2e-17]);
title('Voltage vs. time from IFFT');
legend('Real','Imaginary');

figure(3);                                % plot simulated x(t)
plot(t,real(Vdata));
%axis([0 10 -2e-17 2e-17]);

figure(4);                                % plot simulated PSD
loglog(omega(1:n/2),X_nrml(1:n/2),omega(1:n/2),Xplot(1:n/2));

figure(5);
loglog(omega(1:n/2),Xdata(1:n/2),omega(1:n/2),X(1:n/2));

Xout = X_nrml(2:n)';
Fout = freq(2:n)';

```

addnoise.m: Matlab function called by fftdata adds noise to theoretical voltage vs time data.

```

function data = addnoise(y,level);

%function to introduce random noise at given
%level to simulate data from calculated function

N = length(y);

%generate vector of N random numbers with Normal (Gaussian)
distribution
%about zero with standard deviation of one

R = randn(1,N);

mean = 1/N*sum(abs(y));                %take mean of data magnitudes

noise = level*mean*R;                  %noise term

data = y + noise;                      %add noise to data

```

A.2 C++ program

AD4.cpp: C++ program written by Randy Hoopai. Takes voltage data for 20 seconds at 2kHz through Keithly Metrabyte DAS-8 DAC board and writes to .dat file on hard disk.

```
#include <stdio.h>
#include <dos.h>
#include <fstream.h>
#include <time.h>
#include <string.h>

/*      AD4.cpp (11/1/00)                                */
/*      - 2 kHz sampling of 40,000 samples                */
/*      AD3.cpp (11/1/00)                                */
/*      - changed FOR loop to 600 for 3 kHz                */

/*      AD3.cpp (10/17/00)                                */
/*      - Accomodate negative data                        */
/*      AD2.cpp (10/16/00)                                */
/*      - Screen input of file name                      */
/*                                                        */

long int i, j, k;

int lreg = 0x0300;      /* ADlo reg          */
int hreg = 0x0301;      /* ADhi reg          */
int creg = 0x0302;      /* control & status  */

float fdata;           /* ADC data variable */

int temp = 0x0000;
int data = 0x0000;
int data55 = 0x55;
int dataAA = 0xAA;

char fname[13];         /* filename array */
char ftype[5] = ".DAT"; /* filetype string */

float volt = 0;         /* average voltage  */

ofstream fout;          /* file output      */

int main(void)
{
    data = inportb(lreg); /* flush ADlo      */
    data = inportb(hreg); /* flush ADhi      */

    outportb(creg, 0x00); /* set cntl reg    */
}
```



```

printf("\n \tDAS8 2 KHz Acquisition\n \n");

cout << " Enter filename (8 characters max): ";
cin >> fname;
strncat(fname, ftype, 4);
cout << "\n Data file: \t" << fname << "\n";

fout.open(fname);

for (i = 0; i <= 39999; i++)
{
    outportb(hreg, 0x00);          /* start conv          */
    data = inportb(creg);          /* get EOC            */

    while (data >= 128) { data = inportb(creg); } /* wait EOC */

    data = inportb(hreg);          /* get hiByte         */

    if (data >= 128)                /* test sign          */
    {                               /* positive routine */
        data = data - 128;          /* strip mostSigBit  */
        data = data * 256;          /* shiftLeft 8 bits  */
        temp = inportb(lreg);       /* get loByte        */
        data = data + temp;
        data = data / 16;           /* strip 4 leastSigBits */
        fdata = data * 0.00244;     /* convert to volts   */
    }
    else                            /* accomodate negative */
    {                               /* similar to positive */
        data = data * 256;          /* similar to positive */
        temp = inportb(lreg);
        data = data + temp;
        data = data / 16;
        fdata = data * 0.00244;
        fdata = fdata - 5;          /* negative data      */
    }

    fout << i << "\t" << fdata << "\n";

    volt = volt + fdata;            /* average buffer */

    for (j = 0; j <= 1070; j++) k=j+1; /* 2 KHz wait (486-100) */
}

fout.close();

/* printf("\n\t %d\t samples\n", i); */

volt = volt/i;

cout << "\n Average voltage: \t" << volt;

return 0;
}

```